Polyphenols: Mechanisms of Action in Human Health and Disease

Second Edition

Volume 1

Edited by

Ronald Ross Watson

Victor R. Preedy

Sherma Zibadi

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RONALD ROSS WATSON

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Preface

Polyphenols are a unique group of phytochemicals present in fruits, vegetable, herbs, and other plants. Their activity is based on functional groups capable of accepting a free radical's negative charge. Polyphenols are a very diverse and multifunctional group of active plant compounds with substantial health potential in many areas. There is a growing community of researchers interested in the medical benefits of polyphenols. In this second edition, 63% of the topics are new from the first edition, making this book very novel. The remaining 37% of the articles that have been revised have been substantially updated with new information and research published since the first edition was completed in 2013.

Section I begins with overviews of the biological activity of polyphenols and health. Polyphenols are constantly being tested and reviewed as supplements in foods and beverages. Baliga discusses prevention of pancreatitis with polyphenols.

In Section II Gollucke provides an overview of polyphenol supplements in foods and beverages. This is followed by a variety of new articles and topics: xanthuhumol benefits in beer, flavonoid inhibition of metalloproteases, and polyphenols in *Clerodendrum*, *Ecklonia*, and *Eryngium*. Additional chapters cover polyphenol benefits in cherries and citrus fruits. The developed world is experiencing an epidemic of chronic diseases, including diabetes due to obesity. Chapters on anthocyanins and diabetes, protection against oxidative stress, and protocatechuic acid were revised and updated.

Chapters in Section III discuss the role of genistein on type 1 diabetes, chocolate polyphenols and oxidative stress, and other therapeutic effects. Oxidation frequently causes disease pathologies, which are reduced by polyphenols, including neutrophils' oxidative burst. Fruit proanthocyanins are important bioactive polyphenols. Their chemical properties, bioavailability, and metabolomics are mechanisms. New chapters review resveratrol polyphenols' effect on attenuated pain, and polyphenol supplementation during pregnancy. Finally, manipulation of mitochondrial function by polyphenols is a new idea reviewed.

Section IV examines how polyphenols are sometimes inactivated or activated by metabolism in the gut and circulating blood and how polyphenols' absorption by tissues of nutrients and other compounds helps explain their actions. Specific polyphenol groups are reviewed for their metabolism to activity. The important topic of flavonoids and cell membrane transporters has been updated and expanded, along with the review of interactions of polyphenols with the intestinal absorption of bioactive polyphenols; the updated data included is particularly useful for analyzing ingredients in dietary supplements. A newly added chapter involves metabolism of polyphenols in the gastrointestinal tract to provide health benefits. Finally, two new chapters cover the latest information on the biochemistry and bioavailability of quercetin, as well as its health effects.

Section V moves on to the effects of polyphenols in clinical medicine. A wide range of clinical disorders is included, beginning with polyphenols in green tea altering arthritis, along with a chapter on hepatoprotective effects. An important chapter has been updated on clinical evidence and the role of predictive biomarkers, as well as caffeic acid in treating parasite infection. Finally, bone protection by hesperidin is described.

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SECTION I

OVERVIEW OF POLYPHENOLS AND HEALTH

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CHAPTER

1

Polyphenols in the Prevention of Acute Pancreatitis in Preclinical Systems of Study: A Revisit

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1 INTRODUCTION

Globally, the incidence of acute pancreatitis is on the rise and reports indicate that it has a relative frequency ranging from 5 to 80 cases per 100,000 population in the Western world [1]. Acute pancreatitis is biochemically an inflammatory condition affecting the exocrine pancreas and the severity can range from mild interstitial pancreatitis to severe pancreatitis with pancreatic necrosis; it may also lead to multiorgan failure [2]. Reports indicate that this upward trend in the incidence is due to changes in the prevalence of the main etiological factors (e.g., gallstones and alcohol consumption) and cofactors such as obesity and genetic susceptibility [3].

Traditionally, the severity of clinical presentation varies from a mild, self-limiting form to severe disease complicated by sepsis and multiorgan failure [4]. However, a new intermediate category of moderately severe acute pancreatitis has also been described with intermediate characteristics, including a high incidence of local complications but with a low mortality [2]. While about 80% of the patients recover without complications, 20% develop severe local and systemic damage [5]. Additionally, 25% of patients with acute pancreatitis have severe disease and a mortality rate of 30%–40%, making it an important clinical condition meriting an early clinical intervention [6]. Additionally, acute pancreatitis also confers a heavy financial burden and causes significant physiologic stress on the patient and their family [6].

Acute pancreatitis is best defined clinically by: a patient presenting with symptoms, such as epigastric pain, consistent with the disease; a serum amylase or serum lipase level greater than three times the upper limit of normal; and radiologic imaging consistent with the clinical diagnosis and biochemical results [7–10]. The mechanism/s responsible for severe acute pancreatitis is/are unknown. However, observations from studies conducted with laboratory animals have shown that the process starts with the activation of pancreatic enzymes within the acinar cells, the release of the activated enzymes into the interstitium, the autodigestion of the pancreas, and the release of activated pancreatic enzymes and other factors into the circulation, which then ultimately leads to the development of multiple organ dysfunction [4,11].

Currently there are no valid therapeutic agents/regimens that are universally acceptable for treating acute pancreatitis. The primary objective is to prevent the development of complications by providing supportive therapy to avoid secondary organ failure, in the form of early enteral feeding, intravenous fluid replacement, pharmacological agents like antisecretory agents, protease inhibitors, antioxidants, immunomodulators, nonsteroidal antiinflammatory medications, and prophylactic

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antibiotic treatment [12,13]. In lieu of these observations, efforts are being made to identify novel targets and therapeutic agents that are effective and nontoxic at their effective doses.

Data accrued from both clinical and experimental studies carried out in the past decade have conclusively shown that the various inflammatory cytokines (TNF-α, IL-6, IL-1) play an important role in the induction and severity of acute pancreatitis [14,15]. Additionally, reports also indicate that the nuclear factor-κB (NF-κB), a transcription factor and a key regulator of cytokine induction, plays an important role in the initiation and propagation of the pathogenesis and that targeting these molecules could be of potential therapeutic benefit [14,15]. Preclinical studies have shown that the plant polyphenols like curcumin, flavanoids, ellagic acid, and green tea polyphenols possess beneficial effects and these effects can be mediated by modulating the cytokines and the NF-κB. This review attempts to summarize the beneficial effects of these phytochemicals.

2 CURCUMIN

Curcumin (Fig. 1.1), the major constituent of the rhizome of *Curcuma longa* (turmeric), a spice and coloring agent widely used in Indian food, is one of the widely studied phytochemicals. It is a nontoxic agent with potent antioxidant, antiinflammatory, and cytoprotective effects. With respect to curcumin's effect in preventing pancreatitis, seminal studies by Gukovsky and coworkers [16] have shown that the treatment with curcumin was effective in preventing both ethanol (ethanol diet and low-dose CCK) and nonethanol (cerulean)–induced experimental pancreatitis in rats. In this study it was observed that curcumin significantly decreased the severity of the disease as measured by a number of parameters (histology, serum amylase, pancreatic trypsin, and neutrophil infiltration). Mechanistic studies showed that curcumin inhibited NF-κB and AP-1 activation, reduced induction of mRNAs for cytokines IL-6 and TNF-α, the chemokine KC, and inducible nitric oxide synthase in the pancreas [16]. Studies with cultured pancreatic acini have also shown that curcumin blocked CCK-induced NF-κB and AP-1 activation, validating the in vivo observations [16].

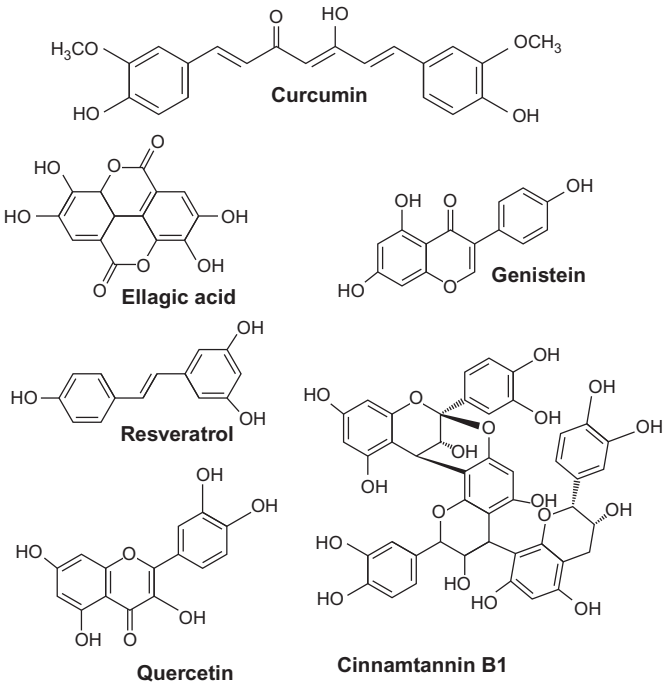

FIG. 1.1 Polyphenols effective against acute pancreatitis.

Contrary to these observations, subsequent studies have also shown that curcumin was not effective in protecting against sodium taurocholate–induced acute pancreatitis in rats [17,18]. However, when compared to the placebo-treatment acute pancreatitis group, administering curcumin reduced the serum amylase, lipase, malon dialdehyde, and nitric oxide, and was also effective in ameliorating free radical injury and prevalence of bacterial translocation [17]. Detailed studies showed that curcumin mediated these beneficial effects at least in part by reducing serum TNF-α and IL-6 levels [18]. Studies with mice have also shown that curcumin was effective in reducing cerulein-induced damage to the pancreatic tissue by inhibiting the release of inflammatory cytokine TNF-α and to mediate these effects possibly by upregulating PPARγ and concomitant downregulation of NF-κB [19]. Additionally, experimental studies with cultured rat pancreatic stellate cells have also shown that incubating with curcumin induced expression of HO-1 gene in a time- and dose-dependent manner and inhibited PDGF-mediated ERK1/2 phosphorylation and pancreatic stellate cell proliferation [20]. Activation of pancreatic stellate cells is a key process in the development of pancreatic fibrosis, a common feature of chronic pancreatitis and pancreatic cancer, and by upregulating HO-1, a stress-induced enzyme implicated in protection from a variety of injuries, curcumin reduced the pathogenic effects [20].

Mechanistic studies conducted by Zhong demonstrated the protective effect of curcumin in severe acute pancreatitis (SAP) in a rat model by lowering the levels of serum cytokines IL-10 and TNF-α, through the involvement of TLR-4/NF-κB signaling pathway [21–23]. Seminal studies by Shafik and coworkers [22] have shown that administering curcumin ameliorated severe necrotizing pancreatitis in rat-induced acute pancreatitis and reversed the histopathological changes. Detailed studies showed that curcumin administration mediated these effects by decreasing levels of serum amylase and lipase, levels of epithelial neutrophil-activating peptide, tissue content of protein carbonyls, levels of tumor necrosis

factor α and caspase-3 as well as myeloperoxidase activity; and by inducing apoptosis and inhibition of micro-thrombosis, inflammation, and oxidative stress by mediating its action on fibrinogen-like protein-2 (fgl-2) and some oxido-inflammatory and apoptotic markers [22]. The molecular mechanism of curcumin has its effect on the suppression of the JAK2/STAT3 pathway to reduce TNF-α and IL-6 levels in SAP-induced acute renal injury, thereby playing a prominent role in reversing renal failure in severe acute pancreatitis [23].

3 RESVERATROL

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) (Fig. 1.1), a phytoalexin produced by several plants including grapes, raspberries, blueberries, peanuts, and mulberries is a polyphenolic compound that possesses multiple pharmacological benefits [24,25]. Meng et al. [25,26] studied the protective effects of resveratrol on sodium taurocholate-induced pancreatitis in rats and observed that administering resveratrol reduced the levels of hemorrhage, exudates, and infiltration of inflammatory cells in pancreas and interstitial edema. Subsequent studies have validated these observations and have also shown resveratrol to be effective in reducing sodium taurocholate-induced pancreatitis [27–32]. Additionally, resveratrol imparted its beneficial effects in cholecystokinin-induced experimental pancreatitis, indicating that its beneficial effects extended to other experimental models [24].

Recent studies with cerulein-treated SAP rats have also shown that administration of dihydro-resveratrol, a prominent analog of trans-resveratrol, decreased SAP as evidenced by decreased α-amylase activities in the plasma, reduced levels of edema formation in the pancreatic parenchyma, generation of intracellular reactive oxidative products like malondialdehyde and protein carbonyls, and inhibition of NADPH oxidase and myeloperoxidase [33]. The protective effects of dihydro-resveratrol were associated with a down-regulation of NF-κB and phosphatidylinositol 3′-kinase-serine/threonine kinase signaling pathways [33].

In addition to preventing chemical-induced pancreatitis, resveratrol provided beneficial effects for other gastrointestinal organs. Studies have shown that resveratrol ameliorated hepatic injury (as assessed by histological and biochemical observations [ALT, AST and total bilirubin]) in rats with severe acute pancreatitis [32]. Detailed studies showed that this effect was mediated by reducing the levels of TNF-α and IL-6 in the serum and reducing the apoptosis in the liver by reducing the release of cytochrome C, up-regulating Bcl-2 and down-regulating Bax and caspase-3 [32]. Resveratrol is also shown to inhibit the apoptosis of the intestinal mucosa cells and maintain the integrity of the intestinal barrier to prevent bacterial and endotoxin translocation in SAP [32].

Mechanistic studies indicate that administration of resveratrol decreased serum amylase, levels of free radicals, and infiltration of neutrophils into the pancreatic tissue, to decrease MDA levels, reduce expression of ICAM-1 and VCAM-1, decrease intracellular calcium overload, increase the apoptotic indices of pancreatic acinar cells and the levels of FasL mRNA and protein, increase SOD levels, and reduce activation of NF-κB and the levels of TNF-α, IL-8, IL-1 and NO [24–32]. Studies have also shown that there was increase in apoptosis of pancreatic acinar cells, pancreatic myeloperoxidase activity, serum lactate dehydrogenase activity, amylase, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) levels, as well as a decrease in serum IL-10 level, pancreatic expression of heat shock factor 1 (HSF1), sirtuin 1 (SIRT1) and p53, but the ratio of acetylated HSF1 and p53 was markedly increased, which in turn reduced the morbidity due to SAP [34].

Acute pancreatitis is an inflammatory process originating in the pancreas; however, it often leads to systemic complications that affect distant organs. Acute respiratory distress syndrome (ARDS) is indeed the predominant cause of death in patients with SAP [28,35]. Administering resveratrol is shown to reduce the damage to the lungs [28,35] by decreasing leukocyte-endothelial interaction, reducing blood viscosity, improving the decrease of blood flow, stabilizing erythrocytes, and also is shown to reduce apoptosis by increasing the expression level of Bcl-2 and decreasing the expression of Bax, caspase-3, and cytochrome C [28,35]. Further, resveratrol is also shown to reduce acute pancreatitis-induced brain injury by up-regulating Bcl-2 and down-regulating Bax and caspase-3 [36]. Studies have also shown that dihydro-resveratrol was effective in mitigating cerulean and taurocholate-induced acute pancreatitis in rats by modulating the proinflammatory response, production of proinflammatory cytokines, activity of myeloperoxidase in pulmonary tissues, attenuation of suppression and activation of NF-κB signaling pathway, reducing pulmonary architectural distortion, hemorrhage, interstitial edema, and alveolar thickening [37,38]. Together all these observations clearly indicate the usefulness of resveratrol in preventing acute pancreatitis and associated multiorgan complications.

4 QUERCETIN

Quercetin (2-[3,4-dihydroxyphenyl]-3,5,7-trihydroxy-4H-chromen-4-one) (Fig. 1.1), is a flavonoid ubiquitously found in fruits, vegetables, leaves, and grains. Quercetin is shown to possess myriad pharmacological effects,

including free radical scavenging, antioxidant and antiinflammatory properties. Carvalho et al. [39] investigated the protective effects of quercetin on cerulein-induced acute pancreatitis in mice and observed it to be effective from both clinical (weight changes) and histopathological observations. When compared to the pancreatitis control, the cohorts administered with quercetin had reduced levels of histological changes such as pancreatic edema, inflammatory cell infiltration, acinar cell necrosis, and expression of TNF-α [39]. Pretreatment with quercetin attenuated the severity of cerulein-induced acute pancreatitis as evidenced by effective reductions in the pancreatic wet weight/body weight ratio [39]. Pretreatment with quercetin caused a partial or complete correction of blood flow disturbances in the pancreas and other organs of the digestive system [40]. It improved the functional state of these organs in the early stages of the disease and accelerated the full restoration of their functions [40].

Additionally, studies have also shown that administering quercetin to mice with pancreatitis caused reduction in the serum levels of amylase and lipase and the mRNA and protein expression of NF-κB, iNOS, COX-2 and phospho-JNK; reduced levels of IRE1α, sXBP1, C/EBPα and C/EBPβ in a dose-dependent manner [41,42]; reduced pancreatic myeloperoxidase activities, malondialdehyde and the proinflammatory cytokines TNF-α, IL-1b and IL-6; and increased the levels of antiinflammatory cytokine IL-10 [39]. Certain mechanistic studies showed quercetin played an important role in regenerative changes in the pancreas by increased levels of pancreatic proteins, nucleic acid content, and the histopathology report. In addition, MPE improved TGF-β1 and collagen levels, thereby preventing fibrosis and reducing severity of acute necrotizing pancreatitis [43]. Taken together, these results indicate that quercetin ameliorates the severity of cerulein-induced acute pancreatitis by acting as an antiinflammatory and antioxidant agent.

5 GENISTEIN

Genistein, also known as 5, 7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, and 4′, 5, 7-trihydroxyisoflavone (Fig. 1.1), is a phytoestrogen belonging to the group of isoflavons and is present in high concentration in soy. Recent reports indicate that genistein has a wide variety of pharmacological effects and its beneficial effects have been demonstrated in osteoporosis, cardiovascular diseases, menopause, and cancer [44]. With respect to its beneficial effects in acute pancreatitis, Shi et al. [45] have reported that genistein (1 mg/kg) was effective against the sodium taurodeoxycholate induced acute pancreatitis in rats. When compared to the acute pancreatitis controls, administering genistein significantly decreased the levels of MPO in the pancreas and lungs, and reduced the levels of IL-6 and IL-10 in plasma at 3 and 6 h after induction of acute pancreatitis [45].

6 ELLAGIC ACID

Ellagic acid (Fig. 1.1), a polyphenol found in fruits and vegetables including blackberries, raspberries, strawberries, cranberries, walnuts, pecans, pomegranates, wolfberry, and other plant foods, is one of the highly investigated phytochemicals. It possesses antioxidant, antimutagenic, and anticancer properties. With respect to the beneficial effects of ellagic acid in acute pancreatitis, studies have shown that feeding a diet incorporating ellagic acid to Wistar Bonn/Kobori rats (an experimental model of spontaneous chronic pancreatitis) for 10 weeks was effective in preventing pancreatitis [46]. When compared to the controls, the cohorts receiving ellagic acid had reduced levels of pancreatic inflammation and fibrosis. Additionally, there was an increase in pancreatic weight and decreases in myeloperoxidase activity (an index of neutrophil infiltration), collagen content, transforming growth factor-beta1 expression, and the number of alpha-smooth muscle actin-positive cells (activated pancreatic stellate cells) and ED-1-positive cells (macrophages/monocytes). in vitro studies have also shown that ellagic acid inhibited activation of pancreatic stellate cells, a major profibrogenic cell type in the pancreas [46]. Mechanistic studies showed that ellagic acid inhibited the production of reactive oxygen species in pancreatic stellate cells in response to transforming growth factor-beta1 or platelet-derived growth factor and reduced TNF-α, IL-1β and IL-6 serum levels [47]. Taken together, all these observations clearly indicate the protective effects of ellagic acid in chronic pancreatitis.

7 CINNAMTANNIN B-1

Cinnamtannin B-1 (Fig. 1.1), a naturally occurring A-type proanthocyanidin isolated from the bark of *Cinnamomum cassia*, is a pleiotropic molecule and modulates several biological processes, such as changes in cytosolic free Ca(2+) concentration, endogenous reactive oxygen species generation, protein tyrosine phosphorylation, and platelet aggregation [48,49]. Studies with mouse pancreatic acinar cells have shown that treatment with cinnamtannin B-1 (10 μM) reduced H_2O_2-induced oxidant effects; reduced intracellular Ca^{2+} overload; prevented loss of cell viability; and attenuated the inhibition of enzyme secretion in response to high concentrations of CCK-8 [48,49]. Together, all these observations indicate that the beneficial effects of cinnamtannin B-1 appear to

be mediated by decreasing ROS production and intracellular accumulation of digestive enzymes, all of which are a common pathological precursor to pancreatitis [48,49].

8 GREEN TEA POLYPHENOLS

Tea (*Camellia sinensis*), a plant native to Southeast Asia, has been cultivated and consumed by humans for thousands of years. Globally, tea is the second most widely consumed beverage after water and may be consumed as green tea (unfermented), oolong tea (partially fermented), and black tea (fully fermented). Innumerable scientific studies carried out in the past three decades have shown that green tea possesses myriad benefits owing to its properties that are attributed to its polyphenol content. The active compounds of green tea are the catechins [(−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG) and (−)-epigallocatechin-3-gallate (EGCG)] (Fig. 1.2), proanthocyanidins, flavonols (kaempferol, quercetin and myricitin in the form of glycosides), gallic acids and theanine [50–53].

Takabayashi et al. [50] investigated the protective effects of green tea catechins (Polyphenon) on DL-ethionine-induced acute pancreatitis in rats and observed it to be effective. Administering Polyphenon through drinking water to the rats with pancreatitis produced lesser levels of serum amylase and lipid peroxide as well as lesser tissue weight loss when compared to the cohorts being administered water [50]. Further the histopathological observations showed that the pancreas was almost correct; together all these observations indicated that green tea catechins have protective effects on the pathogenesis of experimental acute pancreatitis [50].

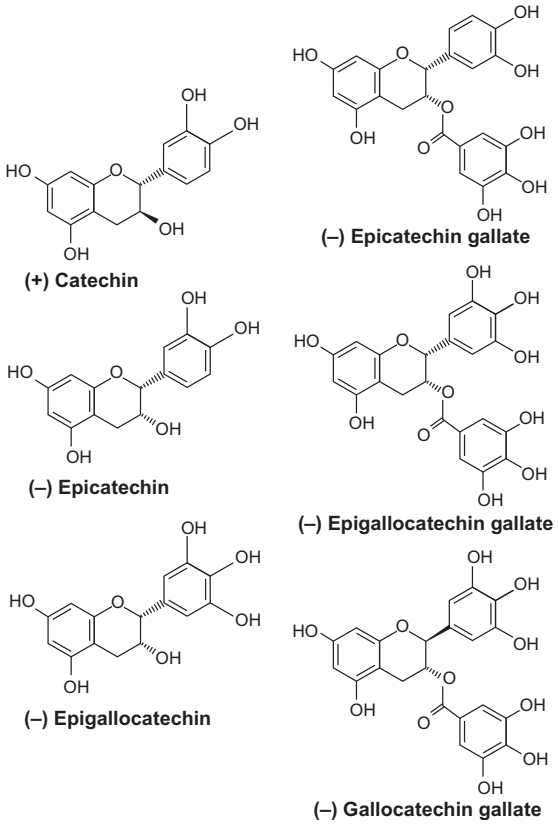

FIG. 1.2 Green tea chemicals effective against acute pancreatitis.

Subsequent studies have also shown that the protective effects of the green tea catechins also extended to another model of acute pancreatitis in rats (cerulein-induced) and that pretreatment with 0.1% green tea catechins in drinking water for 1 week before the induction of pancreatitis significantly decreased the wet weight of the pancreas, the serum level of amylase, and the tissue concentration of lipid peroxides [51]. Studies have also shown that administering green tea catechins to CD mice with acute pancreatitis reduced the levels of P selectin, intercellular adhesion molecule 1, TNF-α, TGF-β, VEGF, nitrotirosine, poly-(adenosine diphosphate ribose) synthetase and lipid peroxidation levels [52]. These observations were mediated by reducing the NF-κB/IkB (inhibitor kB) proteins, oxidative stress pathways and down-regulation of the apoptotic markers [52].

With respect to the phytochemicals, studies with cultured pancreatic stellate cells (PSCs) isolated from Wister rats have shown that pretreatment with EGCG (5 or 25 μM) abolished ethanol (50 mM)-induced lipid peroxidation of the cell membrane, loss of total superoxide dismutase (SOD) activity, and suppressed ethanol-induced gene expressions of Mn- and Cu/Zn-SOD [53]. Additionally, EGCG also suppressed ethanol-induced p38 mitogen-activated protein (MAP) kinase phosphorylation, alpha-smooth muscle actin production in PSCs and activated transforming growth factor-beta1 secretion into the medium [53]. Furthermore, EGCG inhibited ethanol-induced type-I procollagen production and collagen secretion, clearly indicating that EGCG could prevent pancreatic fibrosis by inhibiting pancreatic stellate cell activation through the antioxidative effect [53].

9 CONCLUSIONS AND FUTURE DIRECTIONS

Studies in the recent past have shown that polyphenols of green tea, curcumin, ellagic acid, resveratrol and the flavonoids quercetin and genistein are effective in preventing/ameliorating acute pancreatitis. The most promising aspect of these observations is that many of the studies conducted have been with experimental animals and validated their clinical applicability to humans. Polyphenols generally remain nontoxic, even at relatively high doses, and due to their abundance and low cost have immense potential. Future studies need to address

greater understanding of both the upper limit of consumption and their effectiveness at the various doses in humans. As polyphenols have a dietary origin, they should have wide acceptance as therapeutic agents, but only after gaps in the existing knowledge are bridged.

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Further Reading

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CHAPTER

2

Polyphenols as Supplements in Foods and Beverages: Recent Discoveries and Health Benefits, an Update

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1 POLYPHENOLS AND SUPPLEMENTATION

The abundance of bioactive polyphenols in fruits, teas, and their by-products such as pomace, skins and seeds has brought new possibilities to food researchers and industries to develop innovative food products. In 2017, Leonhart and colleagues were granted a patent for proposing a method of extraction of p-coumaroyl hexose and other polyphenols from strawberry pulp. The method consisted of extraction and purification using a hydrophobic adsorbent and a GRAS (Generally Recognized as Safe) solvent. The fractions comprising strawberry polyphenols and/or p-coumaroyl were available to be used in various applications, such as pharmaceutical, nutraceutical, and cosmetic. In the particular case of the granted patent, the extract was further formulated and tested as an antidiabetic supplement. The authors investigated the consumption of the product (containing 333 mg of polyphenols) in 116 prediabetic subjects for 6 weeks, observing a reverse in insulin resistance and improvement of glucose homeostasis [1].

An interesting process for obtaining cocoa products rich in polyphenols was described by Pons-Andreu and colleagues [2]. The method is based on producing liquid and powdered cocoa polyphenol concentrate from unfermented cocoa beans. Increasing polyphenol content in cocoa powder has a positive impact in antioxidant content and also in flavor. Total polyphenol content in unfermented, unroasted, defatted dried cocoa beans varies from 12% to 20% (in weight). The main polyphenols in cocoa beans under these conditions are catechins, dimers, and other oligomeric flavonoids. The extraction process involves blanching followed by drying of unfermented beans up to 15% moisture content, grinding, and finally polyphenol extraction with preferably polar solvents for human consumption (water, ethanol, or both). In contrast, Jacob and colleagues [3] proposed a method to obtain a fermented supplement from pomegranate, claiming that the aglycones resulting from fermentation of polyphenol-glycosides possess enhanced physiological properties. The authors proposed the use of the yeast *Saccharomyces boulardii* and at least one species of *lactobacilli* followed by a freeze-dry process to reduce water volume and obtain the final product. A more complex extraction procedure takes place to produce an algae extract rich in polyphenols to be used against inflammation processes. In the phylum of brown algae (Phaeophyceae), the phlorotannins are derived by polymerization from the same monomer: phloroglucinol (1,3,5-trihydroxybenzene). The extraction of polyphenols starts with the ground algae and is based on a solid/liquid extraction in the presence, or not, of added water. After centrifugation the solution undergoes membrane filtration or chromatographic purification of the desired polyphenols. Then, the extract is eluted in alcohol and spray-dried to its final commercial powder. This pharmaceutical product, according to the patent, can be used as such or integrated in a food matrix [4]. An unusual source of polyphenols to be used as supplements is the beer waste product. Taidi and colleagues [5] proposed a method of recovering the polyphenols removed from

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beer during processing. Some specific polyphenols in beer are undesirable due to their association with proteins forming insoluble compounds, causing permanent cloudiness after cold storage. In the brewing industry, these polyphenols are removed from the beer by a PVPP (polyvinyl polypyrrolidone) resin. The authors suggested a method for recovering these bioactive compounds and then applying the end product in the cosmetic/food/nutraceutic industry. The inventors found catechin, epicatechin, tyrosol, and ferulic acid to be relevant polyphenols in this beer by-product extract.

In 2017, Kurt Richards [6] described a flavonoid composition formula aimed at improving the bioavailability of quercetin. The flavonoid obtained by the proposed method was used in a chewable tablet formulation containing blueberry extract (33.9%), omega-3 powder (15.1%), green tea extract (13.6%), vitamin C (12.6%), ascorbyl palmitate (9.0%), quercetin (7.9%), and caffeine (7.9%). The benefits proposed for the new product are improvement of athletic performance and also of parameters such as cardiovascular and bone health. Moreover, the invention may be used to prevent fatigue and reduce recovery time after exercise. Ibarra & Zagiary [7] developed a process in which olive polyphenol concentrate is obtained by mixing the waste product of pressing with a polar solvent. Polyphenols are further extracted from the mixture and then concentrated up to 10% (w/w) by the use of membranes. The main compounds found in this polyphenol extract are oleuropein, demethyuloleuropein, and ligstroside. Aware of the polyphenol presence in fruit-processing residues, these previously cited patents have presented advanced extraction methods for skins, seeds, etc. Methods using hot water extraction and an adsorb resin followed by concentration and purification of polyphenols seem to be a promising challenge.

2 NEW INSIGHTS ON POLYPHENOL METABOLISM AND ACTION

Only recently, the role of microbiota in polyphenol metabolism has become known. In 2013, Cardona and colleagues published a review of the few studies available at that time, investigating the subject [8]. According to the authors, only 5%–10% of the dietary polyphenols is absorbed in the small intestine. The remainder accumulates in the large intestine where it becomes part of the enzymatic activities of the gut microbiota. These reactions result in the release of low molecular weight phenolic metabolites, which are then absorbed. For that reason, not only are the dietary differences interfering with specific polyphenol amounts in the blood, but also the individual microbiota composition. Moreover, it has been observed that polyphenols may also be able to modify gut microbiota composition, selecting the bacterial groups present in that environment.

On that note, in 2015, Roopchand and colleagues [9] tested the hypothesis that the mechanisms of action of polyphenols intrinsically involve the gut microbiota. In order to test it, the authors fed a high-fat diet to mice, containing 1% grape polyphenols. The results showed that, apart from the physiological benefits (attenuation of inflammatory markers and of glucose intolerance), the grape polyphenol supplementation increased intestinal expression of genes involved in barrier function, protecting its integrity. Moreover, it was demonstrated that grape polyphenols considerably increased the growth of *Akkermansia muciniphila* and decreased the proportion of Firmicutes to Bacteroidetes. Prior studies have reported that this change in the microbiota was associated with protection against obesity and metabolic disease. With parallel findings in 2017, Novotny and colleagues [10] compared polyphenol levels in plasma of overweight/obese subjects with lean individuals before and after consumption of resveratrol, grape seed extract, and grape juice for 11 days. After consumption, plasma levels of catechin, epicatechin, and quercetin were higher in lean individuals but did not differ in the obese group, suggesting that obesity may affect polyphenol absorption or metabolism, maybe due to disparity in gut microbiota between lean and obese individuals.

The ability of polyphenols to modulate fecal microbiota was successfully proposed by Moreno-Indias and colleagues in 2016 [11]. After a 30-day consumption of red wine (272 mL/day), changes in gut microbiota were observed in metabolic syndrome patients. Red wine polyphenols increased the number of fecal bifidobacteria and *Lactobacillus*, which are intestinal barrier protectors. At the same time, the presence of less desirable groups, such as the LPS (bacterial lipopolysaccharide) producers was diminished. The authors concluded that the decrease of LPS in the bloodstream may be due to the improvement of the intestinal barrier of the host, produced by the change in the gut microbiota promoted by red wine consumption. The result was an improvement in the insulin sensitivity and obesity in metabolic syndrome patients.

In spite of the great efforts and the success in explaining the role of gut microbiota in metabolizing polyphenols (and of polyphenols in modulating the microbiota), there are gaps that need further elucidation. In 2017, Espín and colleagues [12] listed some of the future challenges of scientists in this field. They are: conduct more studies in humans (most of the reported results are in mice); investigate the stability of the newly discovered polyphenol metabolites; undertake clinical trials to verify health parameters after exposure to such metabolites; investigate the large interindividual variability in gut metabolism and attempt to correlate the specific polyphenol with both the gut microbiota responsible for its metabolism as well as the observed biological activities.

3 BENEFITS OF POLYPHENOL'S CONSUMPTION: EXPERIMENTAL DATA

The efforts in investigating and proposing new forms of polyphenol supplementation are justified by the encouraging results observed in experimental data in recent years. In spite of the debate with respect to human applicability, the results of animal experimental data confirm the benefits of polyphenol-rich products [13].

Resveratrol, for instance, has been considered a "miracle molecule" for fighting cancer. According to Delmas and colleagues [14], in their extensive review, resveratrol protects cells from DNA adducts formation induced by various chemical agents. Such DNA alterations are responsible for the initiation phase of tumors, when cells start growing autonomously. Suppression of the metabolic activation and/or increasing of the detoxification rate are some of the mechanisms attributed to resveratrol leading to its antiinitiation property. Also decreasing reactive oxygen species (ROS) production, and the consequent procarcinogen activation and oncogenes mutation, is attributed to resveratrol.

Also credited to resveratrol are the stimulation of DNA repair by increasing the p53 activity, the cell cycle progression blockage (in G1, S and G2/M phases), induction of apoptosis in malignant cells, inhibition of inducible nitric oxide synthase (iNOS) (with consequent blockage of the metastasis) and inhibition of angiogenesis, all of these making resveratrol a promising molecule for preventing and treating cancers [14]. Treatments with resveratrol were also employed in liver disorders, simulated in several experimental designs [15,16]. Bujanda and colleagues [15] demonstrated that liver lesions and animal mortality were reduced in alcohol-exposed mice. According to the authors, resveratrol might have diminished the release of proinflammatory cytokines (such as IL-1), protecting the liver from damage. Alcohol-induced fatty liver was ameliorated by resveratrol treatment in studies by Ajmo and colleagues [16], which found that this polyphenol was able to reduce lipid synthesis and increase the rates of fatty acid oxidation, preventing alcoholic liver steatosis. The increment in rates of fatty acid oxidation seems to be modulated by increased mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ) coactivator α (PGC-1α) target genes, which encode the fatty acid oxidative enzymes [16]. The role of resveratrol was also investigated by Chan and colleagues in an animal model of cholestatic liver injury. As in the studies by Ajmo and colleagues [16], the survival of mice after resveratrol treatment was higher. Also, inflammatory markers (TNF-α and IL-6) were reduced, hepatic fibrosis also decreased, and the number of $Ki67^+$ hepatocytes increased, indicating that resveratrol stimulated hepatocyte proliferation [17].

Also, when applied to hyperlipidemic rats, resveratrol proved to be effective in restoring normal physiological conditions. As demonstrated by Zhu and colleagues [18], oral administration of resveratrol to cholesterol-fed rats resulted in lowered serum lipid, hepatic cholesterol, and triglycerides, besides an increment of the antioxidant capacity expressed by significantly increased levels of superoxide dismutase (SOD), catalase, and glutathione peroxidase. Also, hepatic thiobarbituric acid reactive substances (TBARS) were lowered, possibly by inhibition of the oxidation of LDL, maintaining antioxidant efficacy and denoting the antihyperlipidemic effects of resveratrol. However, some results are still controversial. A study with 66 middle-aged women with metabolic syndrome showed that a 16-week treatment with resveratrol did not improve inflammatory status, glucose homeostasis, blood pressure, or hepatic lipid content. On the contrary, resveratrol significantly increased total cholesterol, LDL cholesterol, and fructosamine levels compared with placebo [19]. That remains to be further investigated.

Quercetin, considered the major flavonoid found in the human diet, has also been vastly studied. Relevant results have been found by Tieppo and colleagues [20] when working with hepatopulmonary syndrome (HPS), a complication of liver cirrhosis. In an animal model of hepatic cirrhosis (through bile duct ligation), quercetin treatment showed decreased oxidative stress, by restoration of plasma TBARS, SOD and nitric oxide (NO), besides a lower severity of the consequent HPS and less pronounced evolution of hepatic injuries [20]. A slowing-down in the cirrhotic process was also observed by Hamed and colleagues [21] using quercetin in a model of thioacetamide-induced cirrhosis. The amelioration in the hepatic biochemical, morphological, and functional aspects was attributed to a potentiation of the antioxidant defense, breaking the "vicious circle" between oxidative stress and oxidative damage.

Proapoptotic properties have also been ascribed to quercetin by Bulzomi and colleagues [22]. This polyphenol was demonstrated to mimic the apoptotic effect 17β-estradiol (E2) in two transformed cell lineages, leading to the activation of the p38 MAP-kinase which, in turn, is responsible for caspase-3 activation and apoptosis. Such a role of quercetin, acting at the estrogen receptor (ER), ensures an anticarcinogenic potential for this molecule. Also, when applied to diabetic mice (by induction with streptozotocin), quercetin was shown to result in lowering of blood glucose and improving plasma insulin levels [23]. The authors suggested that quercetin might improve liver and pancreas functions by suppressing the streptozotocin-induced expression of the cell-cycle inhibitor CDKN1A, restoring the cell proliferative capacity. Corroborating with these results, it was observed that the administration of quercetin, epigallocatechin gallate and apple polyphenol concentrate improved disorders of lipid metabolism and pro-oxidant-antioxidant homeostasis in rats subjected to a high-calorie diet enriched with fructose [24].

The interference of quercetin with the cell cycle regulation in cancer cells was also very well documented in the extensive review by Gibellini and colleagues [25]. According to those authors, quercetin has many molecular targets within the cycle, including cyclin-dependent kinases (CDKs) and cyclins, making this polyphenol able to block the cell cycle progression at various transition points, depending on the cancer cell lineage investigated. Quercetin also acts in multiple ways to influence the p53 activity, leading cancer cells to apoptosis and also contributing to improve cell fighting against ROS, since p53 has been found to regulate a series of genes related to oxidative stress mitigation [25]. A prooxidant property was also proposed for quercetin by Vargas and Burd [26]. When at high concentrations (greater than 40 μM), quercetin is cytotoxic, leading cells to apoptosis. Such a property puts this molecule in consideration to be used as an adjuvant to current chemotherapies.

Curcumin has well-known benefits in a series of pathological conditions. According to Chiu and colleagues [27] diabetic nephropathy is a consequence of high glucose levels (and concomitant increased level of ROS), leading to an increasing of the extracellular matrix proteins which, in turns, is responsible for the thickening of the glomerular basement membrane. When applied to diabetic rats, curcumin prevented renal lesions and mesangial matrix expansion. The authors attributed to curcumin, besides a neutralization of oxidative stress, an inhibition of p300, a histone acetiltransferase that up-regulates specific genes in association with nuclear factor κB (NFκB) [27]. Like the other polyphenols cited previously, curcumin has a pleotropic effect, which includes proapoptotic properties, gene regulation, and cell cycle control (proliferation arrest), which indicates that curcumin is now used in phase II and III clinical trials for a variety of cancer types [28]. According to Aggarwall and colleagues [29], curcumin can suppress tumor initiation, promotion, and metastasis, being also a potent antiinflammatory agent.

Tea is one of the most consumed beverages in the world. Green tea polyphenols have been found to decrease oxidative stress, which has been implicated in the pathogenesis of some neurodegenerative diseases [30]. The authors showed that GT polyphenols protect the neural cells against the oxidative stress-induced nitric oxide (NO) toxicity, attenuating NO-induced apoptotic cell death by modulating the proapoptotic gene expression. Green tea (GT) polyphenols have also been found to maintain the bone architecture in females with and without ovariectomy [31]. It has been described that estrogen is a reactive oxygen species scavenger and that its decreasing with advanced age accelerates bone loss by increasing the oxidative stress and reducing thiol antioxidant defenses in osteoclasts [31]. These authors have found that GT consumption was able to maintain bone microarchitecture by increasing bone formation and suppressing bone erosion. Also, GT polyphenols have been found to have a beneficial role in neurodegenerative diseases [32]. Besides the antioxidant properties, GT seems to modulate various protein-kinase signaling pathways, mainly involving protein-kinase C (PKC), whose activation generates cytoprotection by apoptosis prevention, so preventing or delaying the neuronal loss in the neurodegenerative diseases [32].

Several studies have shown the benefits of catechins. A recent investigation showed that Annurca apple polyphenol extract (APE) with 500 μM catechin equivalent increased levels of p53 and p21, down-regulated the expression of the cell cycle regulatory protein cyclin D1, and strongly inhibited the proliferation of MCF-7 cells by inducing G2/M cell cycle arrest and apoptosis in human breast cancer cells. These results suggest a potential use of catechins in the development of novel therapeutics towards breast cancer [33]. Catechins and gallocatechin obtained from ethyl acetate extracts of Paliurus spina-christi Mill (PS) significantly reduced HO-induced DNA damage in single cell gel electrophoresis techniques (comet assay). PS fruits are widely used for different medical purposes in Turkey [34]. It has also been shown that catechins cause an increase in production and activity of endothelial nitric oxide synthase (eNOS), causing vasodilatation with consequent decrease in blood pressure. Catechins also reduce platelet adhesion; lower the concentration of C-reactive protein and tumor necrosis factor alpha and interleukin-6. All of these outcomes suggest a preventive effect of catechins in atherosclerosis development [35]. Besides, green tea catechins have been able to decrease depressive symptoms in experimental animals, possibly in part through the inhibition of monoamine oxidase (MAO) [36].

4 ANTIMICROBIAL ACTIVITY OF POLYPHENOLS

Early studies observed remarkable antimicrobial activity of flavonols against several Gram-positive bacteria, such as *Staphylococcus aureus*, *Lactobacillus acidophilus*, and *Actinomyces naeslundii*, and Gram-negative bacteria, such as *Prevotella oralis*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* [37]. The combination of quercetin and caffeic acid was very active against *Staphylococus aureus*, while a combination of kaempferol and quercetin was effective against *Pseudomonas aeruginosa* [38].

Simpson et al. observed that rooibos infusions with natural contents of polyphenols showed antimicrobial activity against three species of bacteria: Gram-positive *Staphylococus epidermidis* and *Staphylococcus aureus* and Gram-negative *Escherichia coli* [39]. Polyphenol-rich

fractions from *Sida alba* L. (Malvaceae) showed significant effects against 10 bacteria strains (Gram-negative and Gram-positive), especially *Enterococcus faecalis* [40]. Grapeseed oil has shown antimicrobial activity, mainly detected by in vitro studies. These effects have been related to grapeseed oil constituents, mainly tocopherol, linolenic acid, resveratrol, quercetin, procyanidins, carotenoids, and phytosterols [41]. Other investigations demonstrated that tannic acid, epigallocatechin gallate, rutin, and eugenol have high antibacterial activity against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Moreover, epigallocatechin gallate, tannic acid, quercetin, and epicatechin significantly inhibited the level of β-lactamase activity [42]. Extracts from *Sideroxylon obtusifolium*, a native plant from Brazil, with predominance of condensed tannins, showed antimicrobial potential on the growth of *Streptoccocus mutans* and moderate inhibitory activity on the growth of *Candida albicans* [43].

Early in the chapter, we discussed the fact that polyphenols are able to modulate the gut microbiota composition. It has been observed that one week of a diet including a daily small amount of cereal, vegetables, fruit, and herbal tea increase microbial diversity [44]. In a 3-day study, a blend of fruit/vegetable juice induced significant changes in the intestinal microbiota. The proportional abundance of the phylum Firmicutes was significantly decreased, while Bacteroidetes was significantly increased. Moreover, the results demonstrated a significant decrease in weight and body mass index of the individuals. Indeed, a relative proportional abundance of Firmicutes has been associated with increase of body weight and Bacteroidetes with low body weight [45]. Most and colleagues [46] showed that 12 weeks of epigallocatechin-3-gallate and resveratrol supplementation significantly decreased Bacteroidetes in obese or overweight individuals. Alqurashi and colleagues [47] observed that a mixed-culture fermentation with faecal inoculate, a simulated digested açai (*Euterpe oleracea*) pulp, rich in polyphenols was able to reduce the numbers of both Bacteroides-Prevotella spp and the clostridium-histolyticum groups.

5 NOXIOUS ACTIVITIES INDUCED BY POLYPHENOLS: AN INTRIGUING ISSUE

Few studies have discussed the injurious effects closely related to the toxic potential of polyphenols. In 2010, Keith and colleagues [48] published a review of the possible toxicity of high-dose intakes of polyphenols through supplementation. The consumption of a natural compound such as polyphenols, found in numerous foods, is commonly considered safe. The doses used in supplements, however, are often much higher than a normal daily intake. Halliwell [49] summarized the prooxidant effects observed in in vitro studies. In the presence of iron, high pH and depending on concentration, polyphenols can begin autooxidation. Other mechanisms may also be involved in the toxic effects of polyphenols in high-dose supplementation, such as the soyfoods and breast cancer association, for example. There has been considerable investigation of the potential for soyfoods to reduce risk of breast cancer due to the presence of isoflavones, compounds that bind to estrogen receptors, exhibiting weak estrogen-like effects. Recently, however, concerns have been raised suggesting the isoflavones could stimulate the growth of estrogen-dependent breast tumors [50]. The contradiction is supported by other evidence. In an in vivo rodent model, ovariectomized athymic mice fed genistein and genistin both displayed enhanced growth of mammary tumors [51,52]. Again, the dosage seems to play a fundamental role. Hepatoxicity from high dose intake of tea polyphenols supplementation has been described in human studies [53]. Other possible toxic effects have been reported [54–56]. Nevertheless, to date there is not enough evidence to support that a daily intake comparable to Asian soyfood habits is detrimental to the health.

The harmful effects of some polyphenols can be dubious. Studying the luminal surface of the gastrointestinal tract, covered by its protective mucus gel layer, D'Agostino and colleagues [57] have demonstrated that epigallocatechin gallate was very toxic to the HT29 cells. However, the substance was less toxic to the HT29-MTX-E12 cells, suggesting that the mucus gel layer on the HT29-MTX-E12 cells is able to protect the cells against epigallocatechin gallate toxicity. In contrast, epicatechin had no effect on the viability of either the HT29 or HT29-MTX-E12 cells, suggesting that proteins within the mucus gel layer on the apical surface of gut epithelial cells may bind to the galloyl ring of epigallocatechin gallate. In the same way, some authors have postulated that the antioxidant capacity of the tested polyphenols quercetin and epigallocatechin gallate is due to the stabilizing effect on the cell membranes, thus contributing to cell protection in various pathologies and as adjuvant therapy in highly toxic treatment regimens [58]. Epigallocatechin gallate also inhibited beta-glucuronidase activity in native Hepa 1c1c7 mouse hepatoma cells, while it failed to affect the enzyme in alamethicin-permeabilized cells, where the endoplasmic membrane barrier was eliminated. Such findings indicate that tea flavanols inhibit deglucuronidation in the endoplasmic reticulum at the glucuronide transport stage [59]. Epigallocatechin gallate induced apoptosis in the carcinoma HSC-2 cells, but not in the normal HGF-2 fibroblasts [60]. This research supports those studies suggesting that green tea is an effective chemopreventive agent of oral carcinoma cells in vitro.

Mice exposed during 28 days at doses of 0, 30, 300, and 3000 mg/kg body weight/day of quercetin, which are equivalent to 5, 50, and 500 times, respectively, the estimated mean human intake of these polyphenols (25 mg/day) revealed no mortality during the experimental period [61]. No significant body weight gain in the male or female groups was also observed. Red blood cell number and hematocrit increased after polyphenols administration compared to control groups. Biochemical parameters were not affected. Histopathological examination revealed no alterations in clinical signs or organ weight at any dose [61].

Resveratrol at a concentration of 10 μM or more (up to 100 μM) led to a significant dose-dependent increase in the population of dead cells, shrunken living cells, annexin V-positive cells, and cells with hypodiploidal DNA. In the presence of benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK), a pan-inhibitor of caspases, the resveratrol-induced increase in the population of cells with hypodiploidal DNA was partially inhibited [62]. Overall, it is suggested that resveratrol at a concentration of 10 μM or more induces apoptosis in normal cells as well as cancer cells. Thus, at concentrations that are suitable for chemopreventive and chemotherapeutic actions, resveratrol may exert a cytotoxic effect on normal cells [62].

6 CONCLUDING REMARKS AND FUTURE CHALLENGES

Many epidemiological and experimental studies support the action of polyphenols or polyphenol-rich foods on health, but there are still many gaps in current knowledge. More adequately powered, randomized, placebo-controlled human studies as well as animal studies are needed on polyphenols. There is a large number of structurally different polyphenols that are relevant for health, and obtaining enough information to set a DRI for each of these will not be feasible in the foreseeable future [63]. As far as supplementation is concerned, the future challenge may be to develop new ingredients and/or products as similar as possible to the polyphenols found in foods. Supplementation, however, should be carried out responsibly with great attention to the doses used. The challenge in this field is to provide the "right on the target" dose for the different polyphenols and the most adequate form of supplementation (i.e., beverages, foods, capsules, etc.). Delivery of polyphenols directly to human tissues so that local concentrations are increased seems an interesting future approach. The new insights on the relationship between polyphenol bioavailability and gut microbiota represent a new field for food researchers with plenty of challenges.

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CHAPTER

3

Xanthohumol and the Medicinal Benefits of Beer

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Abbreviations

ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
BJCP	Beer Judge Certification Program
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
EPC	endothelial progenitor cells
ER	endoplasmic reticulum
HDL	high density lipoprotein
IPA	India pale ale
LDL	low density lipoprotein
MAP	mitogen-activated protein
PPAR	peroxisome proliferator-activated receptor
RUNX2	runt-related transcription factor 2

1 INTRODUCTION

Though not as familiar of a functional beverage as red wine or green tea, beer, especially craft beer, has been shown to contain significant amounts of polyphenols. As with other beverages, it is the polyphenols in constituent plant ingredients of beer that impart this phenolic content. In this chapter, we discuss the various aspects of beer brewed from base plant ingredients, the effect these ingredients have on beer styles, and the polyphenols contributed by such ingredients. We also discuss xanthohumol, a polyphenol uniquely associated with beer and the potential benefit thereof, along with research conducted on the health effects of beer consumption.

The basic ingredients of beer are malted barley, hops, yeast, and water [1]. The proportion of these ingredients is selected based on style of beer: darker beers such as porters and stouts will have higher amounts of malted barley, whereas lighter, bitterer beers such as India pale ale will have more hops [2]. Barley and hops both contain significant amounts of phytochemicals and polyphenols that have been shown to contribute to health. Other ingredients (corn, rice, wheat, etc.) will be selected based on the style of beer being brewed. While some of the phytochemicals contained within the beer's ingredients may be destroyed or altered during the brewing process, studies have shown that significant amounts of polyphenols are contained in the final product, and that the phenolic content varies according to style [3,4]. Polyphenols may also be derived from various other ingredients added during brewing. Wheat is added to various styles such as weizens, hefeweizens, and witbiers. Added fruits will naturally contribute their constituent antioxidants to fruit-based beers, such as fruit lambics or fruit ales. Spices are also well-known for their phenolic content and are often added for flavor, most notably pumpkin beers. Coffee or chocolate stouts will also have the phytochemicals associated with those ingredients [2]. In this chapter we will elucidate the ingredients used in various beer styles, the polyphenols contained in these ingredients, notably xanthohumol from hops, and the medicinal benefits associated with beer drinking. Please note that the information presented here is based on broad generalizations; beer styles may vary in ingredients and designation according to the brewery.

2 HISTORY OF BEER AND BREWING

During the 1970s, light American lager was the most established beer style in the United States, with other styles discussed relegated to the burgeoning home-brewing movement. From these home-brewers, microbreweries (breweries producing less than 15,000 barrels annually) slowly emerged during the 1980s [5,6]. Though there was a significant jump in volume growth between 1991 and 1995, such microbreweries remained obscure and unknown until the mid-2000s. At this point, while

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still in the vast minority compared to major American beer sales, craft brewing began to rise to greater prominence [6]. Craft beers will have much more of the ingredients listed in this chapter, and will contain these ingredients in greater variety, leading to higher phenolic content compared to beer produced by major commercial breweries [3,7]. As such, it is understandable that research into beer as a phenolic and functional alcoholic beverage did not emerge until the mid-2000s and accelerated thereafter as phenolic-rich beer gained interest in the United States, though research into beneficial compounds in its constituent ingredients had been established before that.

3 STYLES

The Beer Judge Certification Program (BJCP) issues yearly guidelines of how beer may be generally classified into styles. These styles are based on ingredients used to brew the beer as well as alcohol volume, color (which will to some extent be determined by the ingredients; see Fig. 3.1), and resultant flavors. As the BJCP is quick to point out, these guidelines are by no means comprehensive or compulsory. Breweries may designate their beer as a particular style without conforming to the specifications laid out by the BJCP guidelines. As such, the beer styles listed here are very loosely and generally grouped into the general categories based on the most prominent ingredient typically found in this style. However, beer brewing involves a balancing of the base ingredients (hops and barley) to yield specific flavors and characteristics of the resulting brew. Thus, individual beers may deviate from these hop-forward or malt-forward designations based on choices made by the brewer regarding the balance of ingredients. The styles mentioned here are those that typically do have a prominent hop, malt, or wheat profile; styles not listed may be either less common or blended such that no particular ingredient is more prominent than the others. For more precise information on the typical composition of styles not listed here, please see the BJCP guidelines [2].

3.1 Hop-Forward Styles

India pale ale (IPA) is the best-known hoppy beer style. The name originates from the transportation of beer from British colonies to India [8]. The long voyage required that beer contain large amounts of hops (known to act as a preservative) in order to survive the journey. As such, this pale, highly hopped style bears the name of the country to which it was once regularly transported.

Any "Imperial" style will be higher gravity (i.e., will contain higher alcohol content). Imperial IPA (sometimes designated double IPA) thus is higher alcohol by volume (ABV) and typically higher in hops content [2].

Various other styles may have a strong hops flavor, including certain American versions of brown ale, red ale, or barleywine, especially in comparison to their English counterparts [2].

3.2 Malt (Barley)-Forward Styles

Most beers that are dark in color are barley-based. With a few exceptions, barley is typically malted in the brewing process, leading to the slightly sweet flavor referred to as "malty" [9]. Brown ales and red ales both have barley as the primary grain (as opposed to wheat, rice, or corn in other styles). Again, American versions of these styles may have stronger hops flavors balancing the malted barley flavor. Scottish ales are also barley-forward and have a caramel-like flavor due to the malt [2].

Porters and stouts are strongly associated with their barley component, as this ingredient leads to an almost black color characteristic of these styles. Porters contain roasted malt and thus should have a strong roasty character. Some dark beers, specifically black IPA and dark lagers, may have roasted malts to add color, but barley may not be the distinguishing ingredient for these. Often

FIG. 3.1 Different beer styles will have different appearances based on ingredients. Darker beers on the left have more barley; *red/pink* beer second from right has more fruit; beer on far right has more hops (photo by the author).

these styles strive to avoid the roasty flavor but retain the dark color from roasted malts. As such, these beers may not have the sweet or roasty flavor typical of other barley-based beers [2].

Barleywine is a highly alcoholic barley-based beer (hence the name). Due to the high alcohol content, this style is often sweeter in flavor than porters or stouts [2].

3.3 Wheat-Based Styles

Some beer styles contain wheat rather than or in addition to barley. The most obvious example of wheat-based styles is American wheat beer. Similarly, weizens and witbiers are wheat-based international styles translating to "wheat" and "wheat beer" in German and Dutch, respectively. The sour beer styles Berliner weisse, lambic, and gose all also incorporate wheat as the primary grain [2].

4 INGREDIENTS

The ingredients that contribute to phenolic content of beer are listed here. The most obvious are the base ingredients of hops and barley, but grains used in addition to or in place of barley may also contribute to the phenolic content. In craft beer in particular, added ingredients such as fruit, spices, coffee, and chocolate are often used and are known to have significant phenolic content.

4.1 Base Ingredients

4.1.1 Hops

In contrast to barley, hops are flowers rather than grains, related to cannabis [10]. They grow best in the United States in the northwestern region, particularly Oregon and Washington. Though hops have been used for a variety of purposes in traditional medicine, they were not always used in beer brewing. Hops contain antimicrobial compounds that preserve the sterility of beer, but beer consumption vastly predates hops cultivation. Before hops cultivation, the earliest recording of which was AD 760, beer tended to spoil much more quickly [11].

Hops are dioecious, with male and female flowers. The cones of the female flowers are used in brewing. Depending on the type of hops, other flavors may be imparted, such as citrus, grass, or pine. Hops grown in or originating from various parts of the world will have differing characteristics, and the specific strain will have further distinction in flavors. American hops tend to impart citrus (primarily orange and grapefruit) and pine/juniper flavors, whereas English hops tend to have more herbal, earthy, woody flavors. German hops will also have citrus (lemon) flavors, but may have more spicy characteristics [12]. The hops selection is based on the desired flavors and beer style [10].

The bitterness associated with hops is due to alpha acids, whereas the hops aroma and flavor are due to (non-phenolic) compounds in the essential oils. While alpha acids do contain cyclic rings and hydroxyl groups, the cyclic ring does not contain the necessary double bonds to be classified as a polyphenol. Despite the fact that many polyphenols are antimicrobial, it is these alpha acids that account for much of hops' preservative capabilities. Humulone and cohumulone are common alpha acids that impart bitterness to beer, especially cohumulone. More precisely, these compounds isomerize during the brewing process to yield iso-alpha acids that are truly the compounds responsible for the bitterness of the final beer product. The bitterness of a beer is indicated by International Bitterness Units, or IBUs, which indirectly indicate the amount of hops in a beer by indicating how bitter the beer is [13].

Though not exploited for their bitterness or flavoring characteristics as the compounds listed previously, hops polyphenols account for up to 30% of the phenols in beer. These polyphenols show significant antioxidant activity (rivaling green tea polyphenols) and other protective mechanisms [14]. As with many medicinal plants, the amount of polyphenols varies by strain. As the strain of hops selected for a beer is based on the hops characteristics desired (bitter, grassy, citrus-like, etc.), the amount and type of polyphenols in each strain vary according to the year and location in which the hops were grown [15]. Additionally, hops are boiled for varying lengths of time, which may also affect the amount of polyphenols extracted. All of this will yield variable amounts of hops-derived polyphenols.

4.1.2 Barley

With a few exceptions such as wheat, most beers are brewed with barley as the primary grain. The amount of barley-derived polyphenols in beer is due not only to the polyphenols in barley itself but also to the malting and roasting of the grain prior to brewing. Barley used for beer brewing is distinguished by two major characteristics: when it was grown and the configuration of its kernels.

Barley is designated as "winter" or "spring" barley based on what time of year the barley is actually in the ground; that is, winter barley is planted in autumn and harvested earlier in the year than spring barley. Winter barley is generally more efficient to grow, as it allows for earlier harvesting and higher yields. However, because barley cannot survive extremely low temperatures, certain climates necessitate growing spring barley. There has been little distinction found between these two types of barley [9].

Barley naturally grows with two configurations of kernels: either two rows of kernels on either side of the ear ("two-row"), or an extra four rows along the ear

("six-row") [9]. In this case, two-row barley tends to be preferable for brewing; indeed, six-row barley is rarely used for brewing outside of the United States, where it is used primarily for brewing mass-produced lagers [9,16]. There are a variety of reasons for this: the extra four rows on six-row barley have smaller kernels than on the main two rows, resulting in uneven grain size, which makes milling difficult. Additionally, these smaller kernels have lower starch content, resulting in lower alcohol production [9].

These characteristics lead to four main designations of barley for brewing purposes: winter two-row, winter six-row, spring two-row, and spring six-row. BJCP guidelines often specify whether two-row or six-row barley is typically used in beer styles, but do not indicate winter or spring distinctions [10].

As mentioned in the "Style" section (Section 3), barley and other grains are either malted or roasted during the beer-making process. Malting involves inducing barley seeds to germinate and generate nutrients and enzymes necessary to produce a new plant, then stopping that process and exploiting the nutrients and enzymes for brewing. During malting, barley is steeped and then dried in several cycles to induce seedlings to form. The barley enzymes then begin to break down the barley cell walls and the contents of the starch cells. Heat and air are then applied to stop the germination process while allowing the enzymes to continue their conversion of starches to sugars, which will later be fermented to alcohol. Once moisture has evaporated, the temperature is increased to cure the malt. The temperature at which this curing occurs determines which type of malt is produced; for example, the process of creating caramel or crystal malts involves allowing sugars in the barley to caramelize. Another type of malt is roasted malt, which is in fact roasted in order to create flavors reminiscent of coffee or chocolate [9,10,17]. It is important to distinguish roasted malt from roasted barley, or raw barley that is roasted and used in styles such as dry Irish stouts.

4.1.3 Other Grains

Other grains may be used in place of or in addition to barley. These include wheat and rye, both of which allow for the conversion of starches into sugars during the malting process. These grains may also contribute to the phenolic profile of the beer. Corn and rice have also been used in beer production, primarily in lagers, though often in combination with barley, as these grains do not produce sugars as easily during the malting process [17]. Alternative grain beer is also a family of beer styles assigned by the BJCP to beers that use grains other than barley, including buckwheat, millet, or sorghum [2]. Any polyphenols contained in these grains have the potential to contribute to the phenolic profile of beer.

4.2 Added Ingredients

The base ingredients listed previously will contribute polyphenols to the beer style they are used to brew, leading to variable amounts and types of polyphenols in beer styles. Adding to the complexity of the phenolic profile of beer is the addition of various phenolic-rich ingredients. The majority of research conducted on the topic of beer polyphenols has focused on styles incorporating primarily the base ingredients discussed previously; as such, there is not much information regarding the contribution of the following ingredients to beer. However, given the significant phenolic content of these ingredients, it is important to be aware of their inclusion in certain styles and the possible addition of phenols to these styles.

4.2.1 Fruit

There is no limit to the types or range of fruits that may be added to beer [18,19]. Peach, apricot, citrus fruits, and berries are all commonly added to various beers. Any of the various polyphenols from the constituent fruit may be present in the beer, though this depends on the amount of fruit added. It is important to be aware that some citrus or banana flavors may come from hops or yeast, respectively, rather than those fruits. That is, a beer with a strong citrus flavor may simply be brewed with especially citrus-like hops, unless the brewer specifically states citrus fruits have been added.

4.2.2 Spices

As with fruit, there are certain beers that have a spicy flavor without the addition of spices. German wheat beers, for example, are brewed with yeasts that produce banana and clove flavor, even though these beers do not contain either of these ingredients [2]. In fact, German law specifically forbids the use of any but the base ingredients of water, hops, barley, and yeast to prepare beer [20,21]. Furthermore, as mentioned in the "Hops" section (Section 4.1.1), certain hop strains yield spicy or herbal characteristics to the beer.

Nonetheless, many beer styles do actually incorporate spices. Indeed, the BJCP lists a broad category of "Spiced Beer," which allows for the inclusion of herbs and spices with the same limitless potential as fruit. In their guidelines, the BJCP actively encourages the use of creative combinations of spices when brewing spiced beer [2]. Chilies are sometimes added to give a literal spicy heat to the beer (as opposed to the spicy nature of cloves or cinnamon, which do not contain capsicum heat). Seasonal beers released during autumn and winter regularly include flavors traditionally associated with these seasons, such as cinnamon, cloves, nutmeg, ginger, and allspice. These are especially common in pumpkin beers, which typically strive to imitate the flavors of pumpkin pie. Given the various studies of the phenolic content

and health benefits of herbs and spices [22–24], it is logical to suspect that such styles will be higher in phenolic content than nonspiced beer, but this has not been studied specifically or conclusively.

4.2.3 Coffee and Chocolate

Both these ingredients are often (but not exclusively) included in dark beer, such as porters and stouts, and may contribute to the higher phenolic content seen with those styles. As with the other added ingredients, however, it is entirely possible to impart coffee and chocolate flavor to beer using roasted malt, caramel malt, or roasted barley [17]. In this case, however, the distinction is less crucial. Compounds such as ferulic acid are generated by the roasting process, whether it be malt, barley, or coffee being roasted [25,26]. Thus, phenols may be generated by similar processes in both these ingredients and in beer, regardless of the presence of these ingredients.

5 HEALTH BENEFITS OF BEER POLYPHENOLS: XANTHOHUMOL

It appears that approximately 70% to 80% of beer polyphenols originate in barley, with the remainder from hops [14]. Studies have shown that darker beers tend to be higher in phenolic content than lighter beers, likely due to the greater presence of malted barley and the generation of polyphenols such as ferulic acid during the malting process [3,4,25]. The hopping and fermentation process have also been shown to increase phenolic content as the brewing process progresses [27]. There is some overlap of polyphenols originating from hops and barley, but some very general distinctions may be made (Table 3.1).

Due to xanthohumol's unique presence in beer, any health benefits from this polyphenol may be distinctively associated with beer consumption. As such, the benefits studied in xanthohumol are discussed here. The benefits associated with the other polyphenols listed previously are not expounded upon here, as they have been listed elsewhere when related to foods and beverages in which they are also found. Regardless, it is important to keep in mind the variety of polyphenols found in beer when considering potential benefits of beer consumption.

TABLE 3.1 General Distinctions for Origin of Polyphenols in Beer [14,25,28]

Polyphenols Derived *Primarily* From Hops	Polyphenols Derived *Primarily* From Barley
Xanthohumol[a]	Ferulic acid[b]
Apigenin	Caffeic acid
Humulone	Catechins
Kampferol	Chlorogenic acid
Narigenin	Coumaric acid
Quercetin	Gallic acid
	Vanillic acid

[a] *Found exclusively in hops.*
[b] *Major polyphenol in barley.*

Xanthohumol is notable among beer polyphenols in that it is the only one that can be specifically and solely attributed to hops. That is, other hop-derived polyphenols may also be found in barley or other added ingredients, but xanthohumol is found almost exclusively in hops. This rarity of the compound among plants and the limited uses of hops for human consumption makes beer virtually the exclusive dietary source of xanthohumol and related compounds (isoxanthohumol, etc.) [29].

This prenylated flavonoid (specifically a chalcone) and compounds may be present in beer at concentrations as high as 4 mg/L, though reports differ on this and this number will vary based on the amount and type of hops [30,31]. Regardless of their concentration, the prenylation of these compounds increases their lipophilicity and, as such, their antioxidant activity [32]. Thus, even if these compounds represent the minority of beer polyphenols, they may account for a disproportionate degree of beer's antioxidant-related benefits.

Xanthohumol has been studied for potential benefit in preventing a variety of chronic diseases and medical conditions. These effects may be due in part to the free radical scavenging ability common to polyphenols and demonstrated in this compound specifically. Interestingly, xanthohumol's antioxidant effects may protect against ethanol-induced damage in various organs, thus potentially attenuating some adverse effects associated with excessive alcohol consumption (see Section 5.8 regarding human studies) [33]. Xanthohumol has also been shown to have benefits in various conditions, including those listed in the following subsections, via various mechanisms.

With many polyphenols, there may be concern that too much of the compound may cause adverse effects due to interfering with the body's natural processes. This may be a particular concern with xanthohumol's antiestrogen properties. However, studies have shown that high doses of xanthohumol did not affect normal metabolism or organ function in vivo [34].

5.1 Cancer

There are various mechanisms by which xanthohumol may prevent the development of cancer (see Fig. 3.2). As with many antioxidants, preserving the health of cell DNA is likely to prevent mutation and carcinogenesis. Other mechanisms have been found in various studies and reviews of the benefits of xanthohumol. Xanthohumol aids

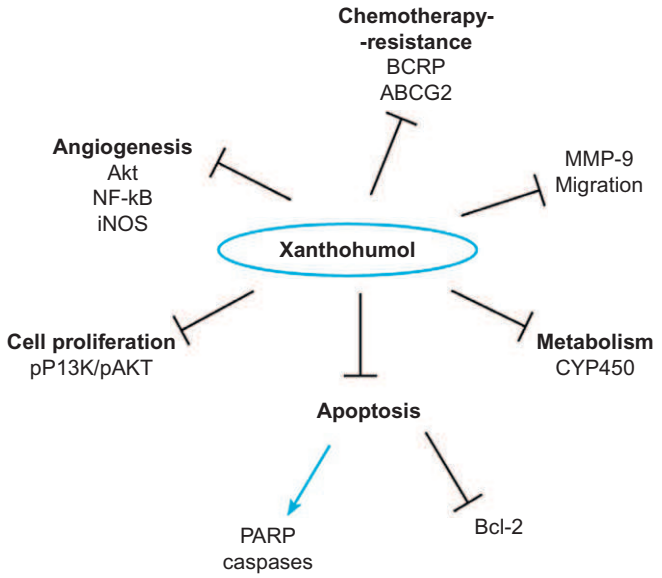

FIG. 3.2 Mechanisms of xanthohumol's chemopreventative properties. *From Rossi T, Gallo C, Bassani B, Canali S, Albini A, Bruno A. Drink your prevention: beverages with cancer preventative phytochemicals. Pol Arch Med Wewn 2014;124(12):713–722. Used with permission.*

in the elimination of carcinogens before they can cause damage by its differential activity towards the P450 enzyme system. In this system, the Phase I enzymes contribute to the activation of carcinogens, whereas Phase II enzymes are responsible for carcinogen excretion. Xanthohumol inhibits the 1A family of carcinogen-activating Phase I enzymes and induces the carcinogen-excreting Phase II enzymes, thus providing dual protection against carcinogen activity in the body [29,35].

Another mechanism by which xanthohumol may prevent or inhibit carcinogenesis is a somewhat counter-intuitive one; many antioxidants, including xanthohumol, exhibit paradoxical behavior in which they increase reactive oxygen species in certain cases. While reactive oxygen species are typically associated with cell damage and detrimental effects long-term, in the case of cancer prevention, generation of such compounds can be an asset. As cells contain a complex series of metabolic mechanisms utilizing reduction and oxidation (redox), disturbances to this balance can lead to cancer development. Typically antioxidants keep the redox balance in a normal state by removing reactive oxygen species, but in cancerous cells, which are already high in reactive oxygen species, increasing the concentration of these species can lead to apoptosis and prevent proliferation. This targeted cell death by xanthohumol appears to proceed via a mechanism not related to its antioxidant properties. Rather, the electrophilic ketone group of xanthohumol is the target of nucleophilic attack by proteins such as NF-κB. Xanthohumol may then modify a cysteine residue and suppress activity of this protein, ultimately inhibiting angiogenesis and cancer development [36].

Xanthohumol has also been shown to inhibit certain other cancer-inducing proteins. It inhibits β-catenin, a protein that accumulates in cells due to activation of the Wnt ligand. This accumulation is related to changes in gene target expression and cell proliferation and other cancer pathways [37]. Xanthohumol also inhibits TNF-α, a protein associated with NF-κB activation and inflammatory genes. TNF-α is a tumor promoter that has been implicated in all steps of tumorogenesis [37,38].

Xanthohumol also may inhibit topoisomerase 1, an enzyme overexpressed in many tumors and a target for drug therapy. By inhibiting this enzyme, xanthohumol appeared to induce cytoxicity in various cancer cell lines, namely lung, ovarian, colon, and melanoma cancers. It further decreased mRNA levels of drug efflux proteins responsible for drug resistance. Thus, xanthohumol may also inhibit drug resistance when given concomitantly with established cancer drugs [39].

Xanthohumol appears to inhibit the growth and metastasis of certain cancers. It was found that xanthohumol may prevent transcription and expression of cysteine X cysteine chemokine receptor 4, a receptor associated with tumor growth and metastasis [40].

Overall, it appears xanthohumol's cancer-preventative properties may be multifactorial. As described in following subsections, it has been shown to inhibit certain specific cancers, either by the mechanisms previously mentioned or by mechanisms specific to that cancer.

5.1.1 *Breast Cancer*

Xanthohumol has shown potential benefit against breast cancer, both in vitro and in vivo, and in estrogen-positive and estrogen-negative cancers. In various studies, xanthohumol has shown inhibition of DNA synthesis, cell cycle arrest, and induction of apoptosis in different breast cancer cell lines [34,35]. In vivo, xanthohumol inhibited the growth of estrogen-negative breast cancer tumor xenografts in mice and significantly decreased the size of established tumors [35].

Xanthohumol may also have a role in drug-resistant cancers. MCF-7 is an estrogen-responsive breast cancer line that has spawned a doxorubicin-resistant line. Liu and colleagues found xanthohumol decreased cell viability, induced apoptosis, and induced cell cycle arrest in the doxorubicin-resistant cell line. Xanthohumol appeared to accomplish this via decrease in anti-apoptotic Bcl-2 and procaspase 3 and increase in pro-apoptotic Bax. Xanthohumol also increased markers of apoptosis and DNA damage (cleaved PARP and γ-H2AX, respectively). Furthermore, xanthohumol decreased the fraction of cells that contributes to drug resistance and sensitized the cells to doxorubicin, indicating synergy between xanthohumol and the established doxorubicin treatment in breast cancer [41].

As described in the Section 5.8 regarding human studies, breast cancer incidence may increase with even light alcohol consumption. This may be due to an increase in estrogen and oxidative stress during alcohol consumption, particularly in postmenopausal women [42]. The beneficial effects associated with xanthohumol in beer may offset such effects in women who wish to achieve other medical benefits associated with moderate alcohol consumption without increasing their risk of breast cancer.

5.1.2 Ovarian Cancer

In ovarian cancer, xanthohumol shows cytotoxic and antiproliferative properties. Drenzek and colleagues found that, in addition to the cell cycle arrest and induction of apoptosis xanthohumol exhibits in this and other cancers, xanthohumol appears to prevent the transcription and protein expression of Notch 1, a protein overexpressed in ovarian tumors [43].

5.1.3 Prostate Cancer

Various studies have shown an effect of xanthohumol via various mechanisms [44–51]. Among the mechanisms suggested were those relating to the NF-κB and oxidative stress effects listed previously [45,48–50]. Xanthohumol appeared to induce apoptosis via NF-κB in both malignant cell lines and benign prostate hyperplasia (BPH) [45]. Another mechanism suggested was an increased effect of TNF-related apoptosis inducing ligand (TRAIL), a naturally occurring anticancer ligand that has no effect on healthy cells [47,51]. Furthermore, similar effects on the inhibition of tumor growth were seen in mice fed xanthohumol [50].

5.1.4 Pancreatic Cancer

As interest in beer consumption grows, xanthohumol has been studied in cancers with fewer treatment options. Pancreatic cancer is an example of a cancer that tends to be diagnosed at a point such that treatment options are very limited. Thus, it is encouraging that, though limited, recent studies have shown that xanthohumol may inhibit growth of pancreatic cancer cells and tumors and may induce apoptosis via the Notch-1 pathway (see "Ovarian Cancer," Section 5.1.2) [52,53].

5.1.5 Colon Cancer

Colon cancer is an excellent target for prevention via functional foods, as the pathogenesis is so closely linked to diet. Furthermore, various polyphenols may have significant benefit in colorectal cancers due to their pharmacokinetics: even if polyphenols are not significantly bioavailable and are excreted unchanged, they may affect colorectal cancers as they pass through the digestive tract [54,55]. Studies have shown prevention of the development of colon cancer in rats fed beer; other studies on xanthohumol suggest this may be due in part to xanthohumol [35]. When studied in colon cancer cell lines, xanthohumol increased apoptosis, differentiation, and cell cycle arrest, and decreased cell growth and proliferation, invasion, and angiogenesis [56]. Pan and colleagues found evidence this is likely due to activation of the caspase cascade and inhibition of anti-apoptotic Bcl-2 [57].

5.1.6 Leukemia

As with any cancer, various mechanisms have been proposed for the initiation and survival of leukemia. Xanthohumol has been suggested to induce apoptosis in leukemia cells via more than one mechanism and in more than one type of leukemia. Xanthohumol appears to induce apoptosis in acute myeloid leukemia (AML) in a manner related to its pro-oxidant and NF-κB suppression abilities. Bcr-abl is an oncoprotein related to proliferation and survival in myeloid leukemia cells expressing this protein. Via its NF-κB suppression, xanthohumol induced apoptosis in Bcr-abl+ chronic myeloid leukemia (CML) cells. Of particular interest, this apoptosis was maintained in drug-resistant cells. Xanthohumol also may act to prevent invasion of leukemia cells to tissues [58]. This is supported elsewhere; the NF-κB pathway was implicated in the ability of xanthohumol to induce apoptosis in vitro in acute lymphocytic leukemia (ALL), again showing apoptosis in drug-sensitive and drug-resistant cell lines. In this study, xanthohumol also increased survival in mice with an ALL xenograft by preventing cancer invasion to the brain [59]. Another mechanism of apoptosis may be the inhibition of CXCR4 (see previous), a protein that may allow for survival of chronic lymphocytic leukemia (CLL) in vivo [60]. This mechanism may be linked to the p38 MAP kinase pathway implicated in yet another mechanism of leukemia survival. Endoplasmic reticula (ER) are increased in chronic lymphocytic leukemia cells and ER stress has been shown to induce cell death in such cases. This is of particular interest, as the ER may be associated with drug resistance in these cancers. Studies have shown that xanthohumol may induce apoptosis via effects on endoplasmic reticula, a mechanism that may be related to p38 MAP kinase signaling [61,62]. Regardless of the mechanism, xanthohumol has been shown activity against various leukemia types via multiple mechanisms [58–63].

5.2 Cardiovascular Disease

Many chronic conditions are linked to inflammation, among them cardiovascular disease. As such, increasing dietary antioxidants is often recommended for prevention of inflammatory conditions. Along with xanthohumol's strong antioxidant capabilities, it may have

specific effects on inflammation and other conditions implicated in the development of cardiovascular disease.

The inhibitory effects of xanthohumol on inflammation and cardiovascular disease may be as multifaceted as its effects on cancer. Xanthohumol inhibits the inflammatory cyclooxygenase enzyme, expression of inducible nitric oxide synthase, and proinflammatory cytokine interleukin-1β [29,38,64]. Additionally, certain pathways associated with xanthohumol's cancer prevention effects are antiinflammatory, including its effects on TNF-α and NF-κB [38,64].

In addition to its antiinflammatory effects, xanthohumol may affect obesity and lipid profiles. Xanthohumol appears to inhibit the formation of new adipocytes and to induce apoptosis in those already formed via effects on various proteins, including peroxisome proliferative-activated receptor γ (PPAR γ), a protein that is also the target of diabetes treatment. Xanthohumol also may decrease triglyceride synthesis and apolipoprotein B levels, as well as increasing HDL levels by inhibiting cholesteryl ester transfer protein associated with this lipoprotein. Additionally, the antioxidant effects of xanthohumol again have a role here in their ability to inhibit LDL oxidation and thus prevent atherosclerosis. These obesity and lipid effects have also been demonstrated in vivo, with rats fed a high-fat diet supplemented with xanthohumol demonstrating decreased body and liver weight and triacylglycerol levels. Xanthohumol also increased HDL levels and inhibited atherosclerotic plaque formation in mice [65].

These mechanisms are summarized in a comprehensive 2015 review of xanthohumol's pharmacologic properties. More recently, studies have shown xanthohumol to prevent platelet aggregation in vitro and in vivo. This inhibition did not appear to increase risk of bleeding, an advantage over certain established therapies. Furthermore, xanthohumol prevented the formation of carotid arterial and inferior vena cava thrombosis in vivo [66,67].

Another recent study suggested beneficial effects on arrhythmias. In this study, xanthohumol prevented the release of calcium from leaky ryanodine receptors, thereby preventing membrane depolarization and arrhythmias [68]. Again, the research outlined previously suggests multiple mechanisms of benefit in cardiovascular disease by xanthohumol.

5.3 Diabetes Mellitus

As briefly mentioned in the cardiovascular disease section (Section 5.9.1), some studies have suggested xanthohumol may show benefit in diabetes. In addition to its effects on PPAR γ, xanthohumol also inhibits another target of diabetic therapy in vitro: α glucosidase, the enzyme responsible for breaking down starches into glucose. This and other mechanisms may account for blood glucose-lowering effects seen in various in vivo models. Xanthohumol may act to increase levels of adiponectin (a protein that may act to improve insulin sensitivity) and improve diabetic wound healing [65]. Recent studies support the potential benefits; Type 1 diabetic rats fed with xanthohumol-enhanced stout showed decreased fibrosis, suppression of glucose transporter upregulation, and reversal of glycogen depletion [69]. In another study, Type 2 diabetic rats fed with xanthohumol and 8-prenylnarigenen, another polyphenol strongly associated with hops, showed decreased weight gain and blood glucose levels as well as increased insulin sensitivity. Thus, xanthohumol may prevent diabetes or improve diabetic outcomes via effects on various enzymes and mechanisms [70].

5.4 Bone Health

Various studies have suggested xanthohumol may have an effect on bone health by inhibiting formation of osteoclasts (i.e., preventing bone resorption) or inducing formation of osteoblasts (i.e., increasing bone growth) [71]. While many cancer benefits are related to xanthohumol's antiestrogenic effects, xanthohumol seemed to stimulate osteoblast formation related to estrogen receptors α and β. The ability of xanthohumol to induce osteoblast formation without apparently increasing (and perhaps decreasing) breast cancer would be superior to that of hormone replacement therapy, which is associated with decreased risk of osteoporosis but increased risk of breast and endometrial cancer [72]. Another mechanism of osteoblast stimulation may be related to its MAP kinase effects; this enzyme is associated with RUNX2, a "master gene" of osteoblast differentiation. Xanthohumol was shown to increase the expression of this gene via MAP kinase effects [73].

In addition to estrogen and MAP kinase effects, the benefits associated with cardiovascular disease and cancer may also relate to the bone health benefits of xanthohumol. In addition to RUNX2, PPARγ and Wnt (related to obesity and cancer, respectively) are associated with the differentiation of mesenchymal stem cells to osteoblasts or to adipocytes. Xanthohumol's ability to regulate these mechanisms directs the differentiation pathway towards osteocyte formation [71].

A mechanism of osteocyte formation more recently studied is related to xanthohumol's NF-κB effects. A cytokine known as receptor activator of NF-κB ligand (RANKL) is associated with generation of osteoclasts. Xanthohumol was found to inhibit the RANKL pathway of osteoclastogenesis both in vitro and in vivo [74,75].

5.5 Cognition/Brain Health

Chronic neuroinflammation is associated with conditions such as Parkinson's disease, Alzheimer's disease,

and multiple sclerosis. Additionally, inflammatory diseases such as hyperlipidemia are a risk factor for stroke and other brain injury. As such, xanthohumol has recently been studied for its potential ability to decrease oxidative damage and improve outcomes after brain injury. Xanthohumol has been shown to act as an antiinflammatory agent in mouse microglial cells, decreasing inflammatory mediators and increasing the endogenous antioxidant glutathione [76]. In a rat model of cerebral ischemia (such as that associated with stroke), xanthohumol decreased ischemia and infarct volume and improved neurobehavioral effects. Ischemia normally increases hypoxia inducible factor, tumor necrosis factor, inducible nitric oxide synthase, and caspase; all these factors were decreased with xanthohumol treatment. Xanthohumol also decreased platelet aggregation, indicating that xanthohumol's effects on inflammation, apoptosis, and platelets may all contribute to neuroprotection following ischemia [77]. Another study showed similar effects in mice models of aging and senescence. Xanthohumol at 1 and 5 mg/kg/day decreased inflammatory and apoptotic markers, and the higher dose improved synaptic markers that were decreased in older mice [78].

Another mechanism of neuroprotection may be xanthohumol's effects on excessive glutamate. While glutamate is necessary for normal neural function, excessive levels are associated with neurodegenerative conditions, stroke, and other cognitive issues. Xanthohumol appears to decrease glutamate levels in the rat hippocampus via its effects on calcium channels [79].

5.6 Skin Conditions

Other potential health benefits of xanthohumol include antiinfective abilities, showing antibacterial, antiviral, antimalarial, and antifungal properties [80]. These and other properties have led it to be studied in dermatologic conditions. Its antiinfective nature suggests it may inhibit contact dermatitis, skin infections, and acne (due to inhibition of acne-causing bacteria) [32,81]. Other dermatologic benefits include wound healing capabilities (due to potential ability to prevent infection and inhibit oxidation and angiogenesis), decreasing elastase and thereby increasing elastin, increasing collagen, and increasing fibrillin in dermal fibroblasts [32].

5.7 Beer and Human Health

It has been well-established that moderate alcohol consumption may contribute to health benefits. Previously, much of this research was conducted on wine, particularly red wine associated with the Mediterranean diet. Much of the hypothesized benefit from wine related to the polyphenols contained within this beverage. However, as understanding of the polyphenolic content of beer increases, studies are increasingly focused on its health effects as a functional alcoholic beverage.

In 2016, a consensus document was published on the effects of beer consumption on health and disease. In this document, moderate, regular beer drinking was found to be as beneficial as wine and superior to liquor in preventing cardiovascular disease. Beer was found to have the same cancer-related risks as other alcohol types [82]. As such, beer can be considered a well-researched functional beverage. As discussed in the following, much, but not all, of this benefit can be attributed to the phenolic content of beer.

5.8 Human Studies

Many human studies have found a J- or U-shaped curve associated with alcohol consumption and various diseases, such that subjects consuming small and large amounts of alcohol appear to have a higher risk of developing certain diseases or conditions, with moderate alcohol consumers having lower risks. Various mechanisms to explain this have been proposed, largely related to alcohol metabolism in the body. Metabolism of moderate amounts of alcohol appears to have protective effects that are outweighed by detrimental effects that occur upon consumption of high amounts of alcohol. Additionally, the phenolic content is being considered regarding alcohol consumption: wine has long been considered a functional beverage due to its phenolic content, but beer is increasingly being considered in this same category of high-phenolic health-preserving beverages. As such, it may be that consuming phenolic beverages such as beer and wine may provide benefits above and beyond those of ethanol, as indicated by certain studies that have found increased benefit of beer or wine over distilled beverages (i.e., liquors) [82]. Specific mechanisms by which beer consumption might impart health benefits have been proposed by various studies and are discussed in the following sections.

5.9 Cancer

At one time, it was purported that beer may actually cause cancer due to nitrosamine content; however, the modification of the brewing process over the years has decreased such content, and it now appears beer is no more likely to cause cancer than any other alcoholic beverage [42]. Indeed, though the 2016 consensus document found no consistent benefit of beer consumption and cancer prevention, there was also no consistent evidence that beer consumption represented any greater risk of the development of cancer than any other alcoholic beverage. Indeed, it appeared that the alcoholic beverage that was most strongly linked to cancer development in many studies was the beverage most widely consumed in the country or countries where the study took place. That

is, in countries where beer is the most widely consumed alcoholic beverage (such as Asian and Northern European countries), beer consumption was associated with cancer incidence. In countries where wine was most widely consumed (e.g., Southern European countries), wine was associated with cancer development. These findings led the authors of the consensus document to advise that, unless an individual was at risk for an alcohol-related cancer, low to moderate beer drinking was associated with no greater risk of the development of cancer. Even for individuals at risk for alcohol-related cancers, alcohol rather than beer is the culprit. Overall, however, further studies are needed in order to more firmly elucidate the risk between beer consumption and cancer risk [42].

While research into individual polyphenols suggests benefits against certain specific cancers, it is difficult to analyze the population-based effect of the whole beverage on individual cancers. However, in analyzing oral cancer incidence data from various countries, the researchers of a 2005 study found no increased risk in countries in which beer and wine were the predominantly consumed alcohol, with the United States having the largest beer consumption among countries studied. In contrast, there was a statistically significant increase in oral cancers in countries in which liquors and other spirits were the chiefly consumed alcohol. It was hypothesized that this may be due to cultural drinking patterns associated with these beverages: beer and wine may be consumed in a moderate-drinking pattern, such as that associated with the Mediterranean diet, whereas spirits may be consumed in more of a binge-drinking pattern in the countries studied. The significant polyphenol levels in beer and wine compared to liquor may also contribute to this disparity [83].

5.9.1 Cardiovascular Disease

The implications of type of alcoholic beverage and/or drinking behaviors are especially prominent in terms of cardiovascular disease. Binge drinking and drinking at times other than typical mealtimes are both associated with increased risk of coronary heart disease; these behaviors are often associated with consumption of liquor rather than beer or wine. However, it is possible fewer people routinely consume beer or wine compared to those who regular consume liquor, which may result in a smaller sample size for analysis of drinking habits and health [84]. Moderate drinking, on the other hand, appears to be protective against coronary heart disease, stroke, and congestive heart failure. These effects seem to persist in patients with comorbidities such as diabetes and hypertension [42].

Review articles of the effects of beer consumption on cardiovascular disease strengthen the U-shaped curve associated with alcohol consumption [42,84]. In this case, it appears such ethanol consumption results in increased high-density lipoprotein, decreased low-density lipoprotein, and antithrombotic and antiinflammatory effects. A review of the literature since the early 2000s showed vascular events decreased with beer consumption up to about four standard drinks per day (55 g alcohol/day), with the greatest protection associated with consumption of around two to three drinks per day (43 g alcohol/day). In terms of vascular risk (as opposed to events actually occurring), the greatest benefit was associated with consumption of between one and two standard drinks per day (25 g alcohol/day). No cardiovascular protection was seen with liquor consumption; this could be due to either the binge-drinking behavior associated with liquor consumption, the lack of polyphenols in liquor, or to some combination thereof [84].

A few clinical studies (as opposed to retrospective, survey, or literature review studies) have been conducted on the effects of beer and beer polyphenols on cardiovascular health . In one study, human subjects consumed beer, nonalcoholic beer (with the same polyphenolic concentration as beer), or liquor (gin) for 4 weeks in a crossover study of men at high risk for cardiovascular disease. This design allowed for the comparison of the effects of polyphenols alone (nonalcoholic beer), alcohol alone (liquor), and polyphenols with alcohol (beer). The patients were provided with the beverages to consume, so the phenolic concentration of both the beer and nonalcoholic beer administered to each patient was consistent. The results showed that beer and nonalcoholic beer increased levels of endothelial progenitor cells (EPC) and EPC-mobilizing factors in these patients. EPCs are recruited upon endothelial damage and act to repair such damage. Exercise and certain drugs often administered to cardiovascular patients have been shown to increase EPCs, indicating this may be a mechanism associated with the cardioprotection from such interventions. The fact that beer and nonalcoholic beer both increased the levels of these cells indicates that beer polyphenols are responsible for this particular aspect of cardioprotection; had alcohol been responsible for this effect, it would have been expected that liquor consumption would also have shown an increase in EPCs. In contrast, there was a nonsignificant decrease in EPCs following liquor consumption. As patients at high risk for cardiovascular disease are more likely to experience endothelial injury, it is possible that beer polyphenols are of particular benefit in this patient population [85].

The researchers of the study described in the preceding paragraph also examined the effects of such beverage consumption on atherosclerotic markers. In this case, nonalcoholic beer consumption led to a statistically significant decrease in systolic blood pressure, indicating that the blood pressure–lowering effects of beer polyphenols may be counteracted by alcohol.

Nonalcoholic beer also improved markers of atherosclerotic risk, specifically lowering homocysteine and raising folic acid levels. As these effects were not seen with beer or liquor, alcohol may be countering the effects of polyphenols in these mechanisms of cardioprotection. In contrast, beer and liquor consumption increased levels of HDL, associated lipoproteins, and adiponectin, a protein associated with prevention of atherosclerosis and insulin resistance. This indicates that beer polyphenols do not appear to have an effect on HDL levels and that alcohol is likely to account for these particular cardiovascular benefits. However, markers of inflammation were decreased after consumption of both beer and nonalcoholic beer, consistent with the antiinflammatory effects of polyphenols. Overall, this study indicates that beer may offer cardioprotection via antiinflammatory polyphenols and HDL improvement via alcohol content, but that other polyphenolic benefits may be counteracted by alcohol [86].

The 2016 consensus states that low to moderate beer drinking is associated with protection against cardiovascular disease, but that binge drinking or heavy drinking (i.e., drinking more than four to five drinks in a single session or drinking more than the recommended daily amount, respectively) is strongly discouraged. Furthermore, despite the J- or U-shaped curve associated with alcohol and cardioprotection, it is not recommended that nondrinkers begin drinking, due to the propensity of some nondrinkers to consume more than the amounts recommended for disease prevention. Note that these recommendations do not extend to stroke prevention, as the current available data showed conflicting evidence [82].

5.9.2 Bone Health

Dietary intervention is consistently cited as an important factor in the prevention of osteoporosis. This includes not only the well-established link between calcium consumption and bone health [87], but the consumption of various polyphenols and antioxidants [88,89]. Additionally, moderate ethanol consumption has been shown to prevent osteoporosis and bone fractures [90], indicating that phenolic alcoholic beverages may be beneficial when consumed in moderation. The following studies strengthen the link between beer polyphenols and prevention of osteoporosis.

A literature review of studies of the effects of alcohol consumption and bone health found there may be benefit in this regard associated with moderate alcohol consumption, particularly beer consumption. As with any functional beverage, the whole product often contains nonphenolic ingredients that may partially account for beneficial properties. In this case, it is hypothesized that alcohol itself may be partially responsible for this benefit, perhaps by acutely inhibiting bone resorption or increasing estrogen activity and thus preventing estrogen-associated bone loss. However, these alcohol-based benefits may only be possible at concentrations sufficiently high as to cause detrimental effects that might outweigh any other benefits. As such, phenolic benefits may be of greater relevance in this disease state. Catechins, quercetin, and kampferol were all suggested to improve bone health based on in vitro, in vivo, and human studies. Again, however, xanthohumol and prenylnarigenin appear to be the primary phenols responsible for this benefit, due both to prenylnarigenin's estrogenic effects and the ability of both compounds to inhibit bone resorption. Interestingly, however, this study also suggested a mechanism for such benefit beyond alcohol or phenolic content: beer is an excellent dietary source of silicon, due to a high content of barley. Contrary to phenolic content, the content of silicon does not appear to vary according to style. As silicon is associated with increased bone density, this is yet another mechanism by which beer may prove beneficial to human health [91].

5.10 Summary of Human Health Effects

It appears beer may stand alongside wine as a high phenolic beverage; similar to wine, the combination of beneficial effects of alcohol and polyphenols may act synergistically to increase the benefits of moderate drinking by providing greater protection from polyphenolic content. The 2016 consensus document found no greater risk of adverse effects such as liver disease or alcohol-related accidents with beer compared to other alcoholic beverages. Overall, beer is a phenolic beverage that may impart similar benefits to those of wine and may be of greater benefit in certain conditions due to the xanthohumol content, though this has not been firmly established.

6 SUMMARY

Beer is a commonly consumed beverage that, with recent interest in craft brewing, has garnered new interest in the polyphenolic content. These polyphenols have been implicated in many of the health benefits associated with beer consumption, though alcohol and other beer components also contribute to such benefits. Of particular interest are the benefits of xanthohumol, a polyphenol found exclusively in hops and thus uniquely associated with beer. As xanthohumol has shown benefit in myriad conditions, this polyphenol and others contained in beer are likely to contribute to beer's role as a functional beverage for preserving health.

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Glossary

Chalcone
Nonphenolic aromatic compounds with ketone and enone groups.

Cohumulone
Nonphenolic alpha acid that, along with other compounds (see humulone), contributes to the bitterness in hops.

Craft Brewery
Brewery producing fewer than 6 million barrels of beer annually, with less than 25% ownership by alcohol industry, and producing majority of beers via traditional or innovative brewing.

Hops (*Humulus lupulus*)
Flowering plant used in brewing to impart bitter flavors; may also yield pine or citrus flavors.

Humulone
Nonphenolic alpha acid that, along with other compounds (see cohumulone), contributes to the bitterness in hops.

Microbrewery
Brewery producing less than 15,000 barrels annually.

Prenylation
Addition of lipophilic group to compound.

Prenylnaringenin
Prenylated flavonoid found in hops; strongest phytoestrogen identified.

Xanthohumol
Prenylated flavonoids found exclusively in hops; with the exception of hops teas or supplements, beer is the primary dietary source.

CHAPTER

4

Polyphenolic Flavonoids and Metalloprotease Inhibition: Applications to Health and Disease

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Abbreviations

ACE	angiotensin-converting enzyme
ECM	extracellular matrix
EGCG	epigallocatechin-3-gallate
HUVEC	human umbilical vein cells
KKS	kallikrein-kinin system
MMPs	matrix metalloproteinases
OA	osteoarthritis
RAS	renin-angiotensin system
TIMPs	tissue inhibitors of metalloproteinase
TPA	12-*O*-tetradecanoylphorbol-13-acetate
UV	ultraviolet
VEGF	vascular endothelial growth factor

1 INTRODUCTION

Flavonoids are hydroxylated polyphenolic compounds ubiquitously distributed in plants, where they perform important functions including attracting pollinating insects, combating environmental stresses, such as microbial infection, and regulating cell growth [1]. Apart from various vegetables and fruits, flavonoids are found in nuts, seeds, grains, spices, beverages like wine, tea and beer, chocolate, and other food sources and are consumed regularly with the human diet [2].

Over 9000 different naturally occurring flavonoids have been discovered [3] and the list is still growing. The basic structure of dietary flavonoids is composed of two phenyl rings (A and B rings) joined via a heterocyclic pyran ring structure (C ring) (Fig. 4.1). Based on their structural differences, flavonoids have been classified into six major subclasses, namely flavan-3-ols, anthocyanidins, flavonols, flavones, flavanones, and isoflavones [4]. The chemical nature and biological activity of flavonoids depend on their structural class, number and substitution pattern of the hydroxyl groups, other substitutions and conjugations, and degree of polymerization [5]. Flavonoids have long been recognized to possess antioxidant activity. In addition, hepatoprotective, antibacterial, anti-inflammatory, anticancer, and antiviral activities have been reported for flavonoids [1]. The antioxidant and free radical scavenging properties of flavonoids as typical natural polyphenols linked with their ability to act as potent metal chelators and to interact with many enzymes, including metalloproteases, are the main causes of their beneficial effects on human health [6].

Metalloproteases (metallopeptidases) are a class of proteolytic enzymes that contain a catalytic metal ion at their active site, which aids in the hydrolysis of peptide bonds leading to protein and peptide degradation [7]. Metalloproteases participate in numerous physiological processes such as cell differentiation, migration and proliferation, cellular adhesion, fertilization, and neurogenesis, and so their deregulation leads to diseases ranging from cancer, inflammation, and cardiovascular disease, to arthritis and neurological insults. To date, a great variety of synthetic and natural inhibitors with physiological and pathophysiological significance for the activity regulation of human metalloproteases are known. In fact, polyphenolic flavonoids as natural inhibitors are widely used for scientific and pharmaceutical studies [7–9].

This paper presents an overview of the investigations on the feasibility and application of flavonoids as natural inhibitors for well-known human metalloproteases: matrix metalloproteinases (MMPs) and angiotensin-converting enzyme (ACE).

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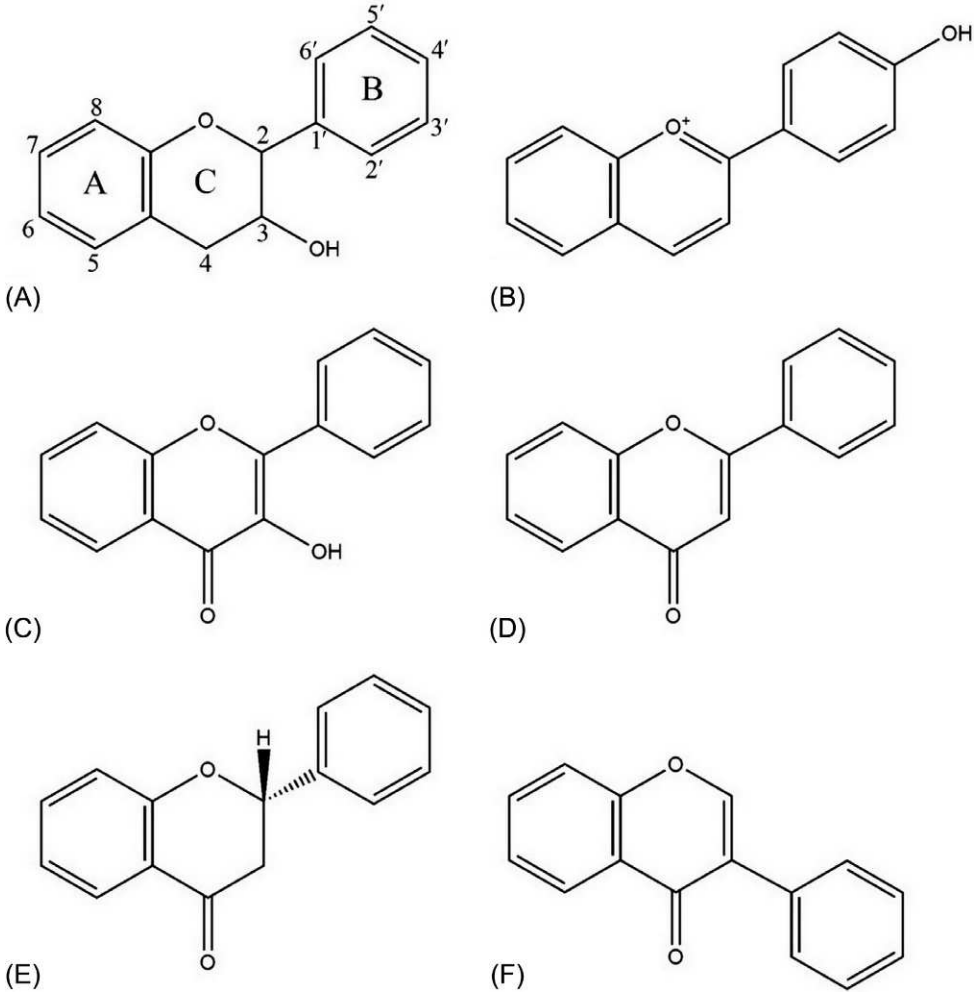

FIG. 4.1 The basic chemical structure of six mayor subclasses of flavonoids: (A) flavan-3-ol, (B) anthocyanidin, (C) flavonol, (D) flavone, (E) flavanone, and (F) isoflavone.

2 MATRIX METALLOPROTEINASES

Matrix metalloproteinases, collectively called matrixins, are zinc and calcium dependent proteases that participate in degradation of the extracellular matrix (ECM) and hence are closely linked with embryonic development, morphogenesis, tissue remodeling, tumor invasion, angiogenesis, and metastasis of cancer [10]. They are synthesized as secreted or transmembrane proenzymes and processed to an active form by the removal of an amino-terminal propeptide. The propeptide is thought to keep the enzyme in latent form by the interaction of a cysteine residue in this peptide with the zinc moiety in the enzyme active site. Disruption of this interaction triggers the cysteine switch mechanism and results in activation of the enzyme [11].

On the basis of structure and in terms of substrate specificity, MMPs were classified into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other nonclassified MMPs. Since some MMPs have been found to have multiple targets and some members cannot be classified into existing groups, this classification system has been replaced by numerical designations MMP-1 to MMP-28 [12]. Currently there are 24 known MMPs identified in vertebrates, including 23 in humans [13].

Under normal physiological conditions, MMPs are minimally expressed and their activities are precisely regulated at the level of transcription, activation of the precursor zymogens, interaction with specific ECM components, and inhibition by endogenous inhibitors like tissue inhibitors of metalloproteinase (TIMPs) [14]. Previous studies have shown that the MMP levels often increases in various pathological conditions such as cancer [15], atherosclerosis [16], lung disease [17], skin ulceration [18], arthritis [19], and Alzheimer's disease [20]. In addition to TIMPs, which constitute the major polypeptide inhibitors of this enzyme group, MMP inhibition

was also achieved by synthetic pharmacological agents such as diphosphonates, tetracycline derivatives, and hydroxamic peptidomimetics. These compounds have been shown to be effective in the treatment of bone diseases and dental periodontitis [21,22]. However, the negative results obtained in human clinical trials analyzing synthetic MMP inhibitors for anticancer activity stirred up the impellent need for compounds that could be more effective in cancer treatment and this search found its answer in the field of natural compounds [23].

2.1 Inhibition of Matrix Metalloproteases by Flavonoids

The ability to use flavonoids as MMP inhibitors in regulating severe pathological conditions has been studied over the past two decades and most of the flavonoids have proved to be effective in suppressing the activity of MMPs.

The effects of ultraviolet (UV) irradiation from sunlight on skin photoaging have been widely studied, and it has been shown that UV irradiation of human skin fibroblasts, either in vitro or on human skin in vivo stimulate the overexpression of genes of MMPs, including matrix metalloproteinase-1 (MMP-1, collagenase-1), which play an important role in the degradation of ECM components during skin photoaging [24]. In order to develop new antiphotoaging agents, Kim et al. [25] examined the inhibitory effect of extracts from the marine product *Zostera marina* on MMP-1 and found that luteolin suppressed the expression of MMP-1 in human skin fibroblasts (Hs68) cells, while Aslam et al. [26] demonstrated that MMP-1 accumulation in the fibroblast-conditioned medium was dramatically reduced in the presence of quercetin, luteolin, kaempferol, and naringenin, major constituents of pomegranate peel extract. Likewise, flavonoids, such as quercetin, kaempferol, apigenin and wogonin, were proved to be MMP-1 inhibitors, and also inhibited MMP-1 induction by suppressing activation of the transcription factor, activator protein-1 (AP-1) in 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treated human neonatal dermal fibroblast culture. Therefore, certain plant flavonoids may be beneficial to treat some inflammatory skin disorders and to protect skin from photo-aging [27]. Furthermore, flavonoids apigenin and luteolin suppress UV-A-induced MMP-1 expression via mitogen-activated protein kinase and AP-1-dependent signaling in human keratinocytes HaCaT cells [28] while methoxyflavonoid isosakuranetin inhibits UV-B-induced MMP-1 expression in HaCaT cells through the suppression of ERK1/2 kinase pathways [29]. To investigate the structure-activity relationship of flavonoid compounds on their inhibitory effects against the MMP-1 activity, Sim et al. [30] examined the effects of several flavonoids in vitro and in human dermal fibroblasts induced by UV-A and demonstrated that myricetin, quercetin, kaempferol, luteolin, apigenin and chrysin inhibited collagenase activities, in a dose-dependent manner, and MMP-1 expression. Also, they showed that the inhibitory effects of flavonoids on expression of MMP-1 in human dermal fibroblasts depends on the number and order of OH groups in the flavonoid structure.

In order to fit for the demands for MMP-1 inhibitor screening and the further structural and biochemical characterization of enzyme-inhibitor complex studies, Lu et al. [31] reported an improved method for high-level expression of soluble human MMP-1 catalytic domain (cd-MMP-1) in *Escherichia coli*. For this purpose 17 structure-related flavonoids were tested for their inhibitory effect on soluble cd-MMP-1 and six compounds (luteolin, fisetin, kaempferol, morin, myricetin, and quercetin) were active with an IC_{50} value less than 10 μM. Among them, fisetin, the most potent inhibitor, was identified as a mixed-type inhibitor of cd-MMP-1.

Osteoarthritis (OA) is a chronic progressive disease associated with complicated mechanisms that involve synovitis and articular cartilage destruction [32]. Proinflammatory cytokines, such as interleukin-1β (IL-1β), are suspected of causing damage to OA cartilage by inducing MMP expression in chondrocytes in an autocrine/paracrine manner [33]. Zheng et al. [34] for the first time showed that the flavonoid chrysin significantly inhibited the IL-1β-induced expression of MMP-1, MMP-3, and MMP-13 in human OA chondrocytes. Similarly, baicalin, a predominant flavonoid isolated from the dry root of *Scutellaria baicalensis Georgi*, notably prevented IL-1β-induced MMP-1, MMP-3, and MMP-13 expression at the mRNA and protein levels [32,35]. To identify the therapeutic potential for cartilage degradation and its action mechanisms, Lim et al. [36] examined the effects of apigenin and wogonin on MMP-13 induction and showed that these naturally occurring flavonoids down-regulate MMP-13 expression in interleukin IL-1β-treated human chondrocyte SW1353 cells. The results of these studies have shown that inhibiting the expression and activity of MMPs is an attractive strategy to counteract OA.

MMPs, in particular gelatinases MMP-2 and MMP-9, play an important role in cancer invasion and were found to be overexpressed in almost all types of cancers [16]. The association of green tea consumption with prevention of cancer development is based on several epidemiologic observations. The effect of the main flavanol present in green tea, epigallocatechin-3-gallate (EGCG), on two gelatinases most frequently overexpressed in cancer and angiogenesis (MMP-2 and MMP-9) and on tumor cell invasion and chemotaxis were examined [37]. The results from this study showed that EGCG inhibited in

a dose-dependent, precipitation-independent manner the activity of both basement membrane collagen- and gelatin-degrading metalloproteinases, MMP-2 and MMP-9, and it also increased the expression of TIMP-1 and TIMP-2, which block the activity of activated MMPs. Moreover, Cheng et al. [38] showed that EGCG forms a reversible complex with MMP-2, thus inhibiting enzymatic activity. Specifically, EGCG did not interfere with the binding of MMP-2 to type I collagen, but it significantly enhanced both pro- and activated-MMP-2 binding to TIMP-2.

To investigate interaction of flavonoids with the secretion of MMPs, Huang et al. [39] reported that quercetin suppressed the epidermal growth factor-induced production of MMP-2 and MMP-9 in human squamous carcinoma A431 cells. Furthermore, Lin et al. [40] showed that quercetin effectively suppressed MMP-9 gene expression induced by TPA via suppressing the protein kinase C/extracellular signal-regulated kinase and c-Jun/activator protein-1 cascades with consequent suppression of colony formation, tumor migration, and invasion by human breast carcinoma cells. By using docking and molecular dynamics simulations, Saragusti et al. [41] showed that quercetin interacted in the S1′ subsite of the MMP-9 active site. Moreover, the structure-activity relationship analysis demonstrated that flavonoid R 3′-OH and R 4′-OH substitutions were relevant to the inhibitory property against MMP-9 activity. Recently, Lan et al. [42] demonstrated that this flavonoid inhibited cell migration and invasion in human osteosarcoma cell line by regulating hypoxia-inducible factor, HIF-1α, vascular endothelial cell growth factor, VEGF, MMP-2 and MMP-9 expression in vitro and that quercetin ameliorated tumor metastasis in vivo in the osteosarcoma nude mouse model. Genistein, an isoflavonoid of the leguminosae family, also regulates cell invasion and the metastatic process by inhibiting MMP-2 and MMP-9 activity in prostate cell lines [43,44]. Among the natural compounds that help to protect from pathological states such as cancer, other flavonoids have been analyzed, including the citrus flavonoid nobiletin. This polymethoxy flavonoid inhibited an in vitro invasion of human fibrosarcoma HT-1080 cells in the Matrigel model and transcriptionally down-regulated the expression of MMP-1 and MMP-9 but up-regulated that of TIMP-1, suggesting that nobiletin prevents tumor invasion not only by suppressing the production of MMPs but also by augmenting TIMP-1 production in tumor cells [45]. Also, nobiletin has a distinct ability to strongly suppress MMP-7 expression and production, presumably by blocking AP-1 activity in HT-29 human colorectal cancer cells [46].

In order to find novel MMPs inhibitors from natural resources, Matchett et al. [47] demonstrated that flavonoid-enriched fractions from lowbush blueberries (*Vaccinium angustifolium*) can down-regulate the activities of MMP-2 and MMP-9 in DU145 human prostate cancer cells, suggesting that further understanding of the complex properties of flavonoids, in particular those from lowbush blueberry, may allow for the further development and refinement of the role of flavonoids in the prevention of carcinogenesis and metastasis. Furthermore, Zheng et al. [48] explored the effects of deguelin, a flavonoid isolated from several plant species, such as *Derris trifoliata* and *Mundulea sericea*, and demonstrated that this natural compound was capable of modulating migration and invasion in human pancreatic cancer cells by down-regulating the expression of MMP-2 and MMP-9.

The effects of baicalein in inhibiting MMP-2 and MMP-9 expression were studied in vitro, showing that baicalein inhibits colorectal cancer cell migration and invasion by reducing the expression of MMP-2 and MMP-9 via suppression of the protein kinase B and extracellular signal regulated kinases signaling pathway [49,50]. Likewise, baicalein significantly inhibits migration and invasion in B16F10 melanoma cells by suppressing MMP-2 and MMP-9 expression and activity. Thus, baicalein could be a potential candidate for the development of chemotherapeutic treatments for colorectal cancer and melanoma [51]. Diosmin, a glycosylated flavonoid from citrus species and olive leaves, exhibits cytotoxic potential by suppressing overexpression of MMP-2 and MMP-9 in A431 skin cancer cells, which indicates the anti-invasive potential of this polyphenolic compound [52]. The significant inhibitory effect on MMP-2 and MMP-9 in human fibrosarcoma cell line HT-1080 was also shown for glycosylated flavonoids from the halophyte *Salicornia herbacea*, isorhamnetin 3-*O*-b-D-glucoside, and quercetin 3-*O*-b-D-glucoside [53]. More recently, Crascì et al. [54] reported that flavones apigenin, luteolin, and their respective glucosides show different selectivity against MMP-1, MMP-3, MMP-8, MMP-9, and MMP-13. In particular, the aglycones apigenin and luteolin are very selective toward both the MMP-9 and MMP-13 in respect to the other MMPs. However, these flavones have a good ability to interact with metalloproteases and can also be lead compounds for further development.

3 ANGIOTENSIN-CONVERTING ENZYME

Angiotensin-converting enzyme (ACE) is a transmembrane zinc metallopeptidase that hydrolyzes peptides by the removal of dipeptide from the C-terminus. It is a central component of the renin-angiotensin system (RAS), which converts the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II [55]. ACE is present in different cell types, but its principal location is on the surface of endothelial and epithelial cells [56]. In humans, ACE is encountered in two distinct isoforms, the somatic and the germinal isoform. Somatic ACE

isoform is a large glycoprotein comprising two homologous domains, N and C, joined by an interdomain linker. Both domains are capable of cleaving angiotensin I and bradykinin [57]. This isoform is a key regulator of blood pressure, expressed in various tissues, mainly in lung, and cell types including cardiovascular system, liver, kidneys, intestine and adrenal gland [58]. Germinal ACE isoform is a lower-molecular-mass glycoform found exclusively in sperm cells and is thought to play a role in sperm maturation and the binding of sperm to the oviduct epithelium [59]. The most significant physiological substrates of ACE are angiotensin I, which is converted into angiotensin II by removal of the C-terminal His-Leu, and the potent vasodilator bradykinin, which is inactivated by ACE cleavage of the penultimate Pro-Phe bond. As a nonspecific metalloprotease, ACE was able to cleave neuropeptides such as neurotensin and substance P as well as luteinize hormone-releasing hormone (luliberin) [60]. As a bioactive component of RAS and the kallikrein-kinin system (KKS), ACE plays a significant role in blood pressure regulation and fluid and electrolyte balance [61]. The broad spectrum of ACE substrates, and its wide tissue distribution, indicate that this enzyme, in addition to an important role in cardiovascular homeostasis, may be involved in (patho)physiologic processes such as fertilization, atherosclerosis, and kidney and lung fibrosis. ACE levels are increased in many forms of vascular and cardiac hypertrophy, and the administration of ACE inhibitors has led to regression of hypertrophy. ACE inhibitors have achieved widespread use in the treatment of hypertension [56]. However, all of these synthetic drugs produced some side effects, such as angioedema and cough, when they are used for a long time [62]. Therefore, ACE inhibitors from natural sources, particularly dietary sources, are potentially beneficial.

3.1 Inhibition of Angiotensin-Converting Enzyme by Flavonoids

Abundant literature has reported that high fruit and vegetable consumption displays positive correlations in cardiovascular and cerebrovascular disease reduction, due to the antioxidant potential of phenolic compounds present in them [63–65]. A number of extracts obtained from Australian and Iranian medicinal plants rich in phytochemicals were found to be effective in ACE inhibition [66,67]. To investigate if the connection between tea and ACE might be an explanation of the pharmacological effects of tea on the cardiovascular system, Persson et al. [68] incubated cultured endothelial cells from human umbilical veins (HUVEC) with extracts of Japanese Sencha (green tea), Indian Assam Broken Orange Pekoe (black tea), and Rooibos tea, respectively. After incubation a significant and dose-dependent inhibition of ACE activity in HUVEC was seen with the green tea and the black tea, but no significant effect was seen with the Rooibos tea. Also, the authors have shown that the inhibitory effect on ACE activity was associated with the four major flavanols, epicatechin, epigallocatechin, epicatechingallate and epigallocatechingallate, isolated from tea. The ability of flavonoids to inhibit ACE through the generation of chelate complexes within the active center of ACE [69] may explain the action of green and black tea as ACE inhibitors. Flavan-3-ols and proanthocyanidins are of great interest in nutrition and medicine because of their potent antioxidant capacity and other protective efects on human health. In a search for potential ACE inhibitors from plants, Lacaille-Dubois et al. [70] prepared methanolic extracts, fractions, and pure substances from *Musanga cecropioides*, *Cecropia*, and *Crataegus* species and demonstrated that proanthocyanidins from these plants exhibited inhibitory activity against ACE. Furthermore, Actis-Goretta et al. [71] have determined that flavan-3-ols and procyanidins have an inhibitory effect on ACE activity, and the effect was dependent on the number of epicatechin units forming the procyanidin. To investigate the effect of bilberry (*Vaccinium myrtillus*) extracts on ACE activity, Persson et al. [72] treated the human endothelial cell culture with prepared extracts (rich in flavonoids i.e., cyanidin, delphinidin, and malvidin) and showed that the ACE activity had been significantly reduced in a dose-dependent manner.

Several anthocyanins (anthocyanidin glycosides) such as cyanidin-3-*O*-β-glucoside isolated from rose (*Rosa damascene*) and cyanidin-3-*O*-sambubiosides and delphinidin-3-*O*-sambubiosides isolated from hibiscus (*Hibiscus sabdariffa*) extracts have shown ACE inhibition in vitro [73,74]. Ethanolic extracts of *Senecio gibbosus subsp. gibbosus* and *S. vulgaris* exhibit ACE inhibitory activity [75]. Since *Senecio* species contain polyphenols such as quercetin, isoquercetrin, isorhamnetin-3-*O*-rutinoside, and quercetin-3-*O*-glucoside, the authors believed that flavonoids are responsible for the observed ACE inhibitory activity. Likewise, the flavonoids quercetin, quercetin-3-glucoside, quercetin-3-galactoside, and cyanidin-3-galactoside from apple skin extract exhibit a capacity to inhibit ACE with IC_{50} values in the 71 to 206 μM range [76] while quercetin 3-*O*-(6″-galloyl)-galactoside and kaempferol-3-*O*-(6″-p-coumaroyl)-glucoside from willow herb (*Epilobium angustifolium*) inhibit ACE in a dose-dependent manner [77]. The in vitro ACE inhibitory activity was also shown for apigenin, luteolin, kaempferol-3-*O*-α-arabinopyranoside, kaempferol-3-*O*-β-galactopyranoside, quercetin-3-*O*-α-arabinopyranoside, and luteolin-7-*O*-β-glucopyranoside isolated from *Ailanthus excelsa* with IC_{50} values in the 260 to 320 μM range [78]. In the cases when bioassay-guided fractionation of extract of *Sedum sarmentosum*

was performed, a new flavonoid, quercetin-3-*O*-α-(6‴-caffeoylglucosyl-β-1,2-rhamnoside), along with four known flavonoids, quercetin 3-*O*-α-(6‴-p-coumaroylglucosyl-β-1,2-rhamnoside), isorhamnetin-3-β-glucopyranoside, quercetin-3-β-glucopyranoside, and kaempferol-3-α-arabinopyranoside, was found to exhibit distinctive ACE inhibitory activity [79]. When evaluating the content of individual phenolics and ACE activity of the extracts of selected plants of the *Rutaceae* family, Alu'datt et al. [80] found that the highest ACE inhibitory activity of the extracted phenolics from lemon was associated with free phenolic extracts obtained at 30°C. Contrarily, Kwon et al. [81] reported that the strong ACE-inhibitory activity in water extracts of rosemary clonal line (Rosemary LA) and lemon balm (*Melissa officinalis*) did not correlate well with the total soluble phenolic content, antioxidant activity, or the concentration of individual phenolics in the extracts. Furthermore, Pinto et al. [82] reported that water extracts of red currents (*Ribes rubrum*) and black currents (*Ribes nigrum*) had ACE inhibitory activity but not the extracts of red and green gooseberries (*Ribes uva-crispa*). Although black currants had the highest quercetin derivatives content among all berries no correlation was found between ACE inhibition and quercetin content. The ACE inhibitory activity had been reported for several brown seaweed species such as *Ecklonia cava* and *Ecklonia stolonifera* [83,84]. More recently, Nagappan et al. [85] reported that crude extracts and fucoxanthin-rich fractions of *Sargassum siliquosum* and *Sargassum polycystum* are able to inhibit ACE activity. The results of a systematic screening of 21 flavonoids for the inhibition of ACE showed that luteolin, diosmin, quercetin and naringenin (inhibition approx. 45%) are the most effective compounds [86]. Finally, study of structure-activity relationships of the flavonoids and inhibitory activity of ACE showed that the combination of substructures in the framework of flavonoids that enhances the activity of ACE inhibitors consists of the following elements: a double bond between C2 and C3 on the C-ring, 4′-*O*-methoxylation on B-ring, 4-carbonyl group on C-ring, 3-hydroxylation on C-ring, and 3-*O*-glycosylation on C-ring [87]. These results suggested that flavonoids are an excellent source of antihypertensive compounds.

4 CONCLUSION

Flavonoids belong to polyphenolic compounds ubiquitously distributed in plants, many of which have been used in traditional herbal medicine for thousands of years. The data from ex vivo and in vitro experiments clearly indicate that flavonoids possess antioxidant, antibacterial, and antiviral activities. Moreover, the direct or indirect inhibitory action of flavonoids on metalloproteases such as MMPs, which participate in degradation of the extracellular matrix, and ACE, which plays a significant role in blood pressure regulation, allows these naturally occurring compounds to be considered as potential candidates for the prevention and treatment of skin disorders, osteoarthritis, cancer, and cardiovascular diseases.

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CHAPTER

5

Biological and Pharmacological Effects of Polyphenolic Compounds From *Ecklonia cava*

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1 INTRODUCTION

Oxidative stress is one of the causes of chronic diseases, such as cancer, heart disease, and inflammation. The relationship between inflammation and oxidative disorders supports the usefulness of antioxidant supplements in health and disease. Polyphenols work as antioxidants through various mechanisms, such as the direct scavenging of reactive oxygen species (ROS) and the inhibition of ROS generation. Antioxidants are found in vegetables, fruits, seaweeds, and cereals. Among these dietary antioxidants, polyphenols are the most abundant. Polyphenols from seaweed are a family of bioactive substances that humans have been consuming since ancient times. Recently, these compounds have attracted more attention for their various biological activities.

Ecklonia cava is an edible brown alga that widely inhabits the southern coast of Korea and Japan and is used in human food, animal feed, fertilizers, and folk medicine [1]. It contains many unique polyphenols called phlorotannins [2]. The specific phlorotannins found in *E. cava* are richer than those in other brown algae [3]. The main phlorotannins in *E. cava* are phloroglucinol (compound **1**), eckol (compound **2**), fucodiphloroetckol G (compound **3**), phlorofucofuroeckol A (compound **4**), 7-phloroeckol (compound **5**), dieckol (compound **6**) and 6,6′-bieckol (compound **7**), as illustrated in Fig. 5.1. They have various biological and pharmacological effects, including anticancer, antioxidation, antiinflammation, antidiabetes, neuroprotective, and hepatoprotective activities.

Recently, it is thought that polyphenols work with antioxidant-related actions or with antioxidant-independent actions. Following is a review of the biological and pharmacological effects of phlorotannins, unique polyphenols from *E. cava*, and their mechanisms of action.

2 BIOLOGICAL AND PHARMACOLOGICAL EFFECTS OF PHLOROTANNINS FROM *E.* CAVA

2.1 Antioxidant Properties

Free radicals are produced through intracellular metabolism of xenobiotics and UV radiation. During respiration, oxygen molecules react easily with these free radicals to form ROS. Various types of ROS such as the superoxide anion (O_2^-), the hydroxy radical (HO^-) and hydrogen peroxide (H_2O_2) oxidize intracellular components such as DNA, proteins, lipids, and cell membranes and, thereby, cause cell damage.

It is well known that oxidative stress such as ROS generation is closely involved in the progression of lifestyle-related diseases [4].

Li et al. first reported the systematic investigation of the chemical components of *E. cava* and the in vitro antioxidant properties of its phlorotannin derivatives [2]. They isolated 10 naturally occurring metabolites, including 7 phlorotannins (compounds **1**, **2**, **3**, **4**, **5**, **6** and **7** in Fig. 5.1) and three sterols (fucosterol, cholesterol, and ergosterol), from the methanol extract of *E. cava*. Then, they assessed the antioxidant activities of the seven phlorotannins, using total antioxidant activity in a linoleic acid model, the electron spin resonance (ESR) method for four free radicals (1,1-dipheny-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, superoxide anion radical, and peroxy radical) scavenging, and cellular reactive oxygen species inhibition assay. These results revealed the following similar reactivity order: compound **6** (or compound **7**)>compound **7** (or compound **6**)>compound **3**>compound **4**>compound **5**>compound **2**>compound **1**. This order suggests that the number

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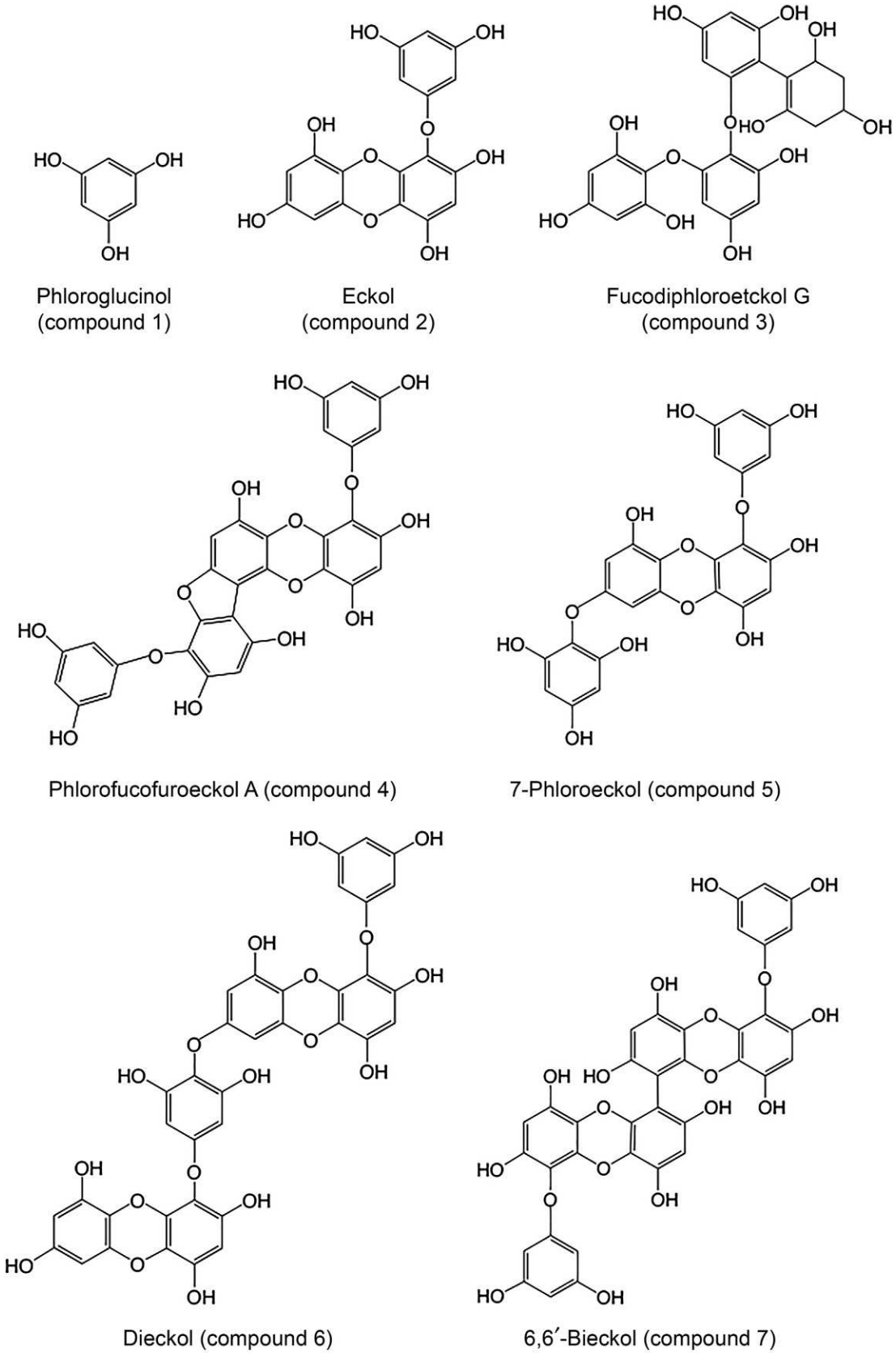


FIG. 5.1 Chemical structure of phlorotannins (compounds **1**–**7**).

of hydroxyl groups present in these compounds has an important role in their in vitro antioxidant activities.

Kim et al. have also shown that six of these phlorotannins (compounds **2**–**7**) had stronger antioxidant activities than polyphenols from green tea, including linoleic acid peroxidation inhibitory activity, DPPH radical scavenging activity, and tyrosinase inhibitory activity [5]. Furthermore, they reported that higher total phenolic content and antioxidant activity were found in grown *E. cava* than in young algae. These properties decreased during the reproductive period, demonstrating that summer is the best season for harvesting *E. cava* with high antioxidant activity [6].

Oxidative stress is associated with tissue damage and factors related to inflammation, aging, cancer, arteriosclerosis, and diabetes. Cells have developed a variety of antioxidant defense systems for cytoprotection against oxidative stress. The expression of antioxidant enzymes such as super oxide dismutase (SOD), catalase, and glutathione peroxidase plays an important role in the ROS removing system to protect cells. Kang et al. examined the cytoprotective effect of eckol (compound **2**) against oxidative stress-induced cell damage in Chinese hamster lung fibroblast cells and found that eckol scavenged hydrogen peroxide, an intracellular ROS, and thus prevented lipid peroxidation [7]. They also investigated the cytoprotective effect of phloroglucinol (compound **1**) against oxidative stress and found that phloroglucinol inhibited cell damage induced by serum starvation and radiation through scavenging ROS and increasing catalase activity. These results suggest that phloroglucinol protects Chinese hamster lung fibroblast cells against oxidative damage by enhancing the catalase protein expression [8].

Phlorofucofuroeckol A (PFF-A) (compound **4**), one of the phlorotannins found in *E. cava*, was also assessed for potential antilipid peroxidation activity in 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH)-stimulated monkey kidney cell lines and in zebrafish. The free radicals generated from AAPH were found to rapidly react with oxygen molecules to yield peroxyl radicals. These peroxyl radicals then attacked other lipid molecules to form lipid hydroperoxide and new lipid radicals repeatedly. Lee et al. demonstrated that PFF-A significantly inhibited ROS formation and cell death and exhibited a strong protective effect against lipid peroxidation [9].

These reports suggest that phlorotannins are effective against oxidative stress.

2.2 Antitumor Activities

ROS are generally considered toxic by-products of respiration. However, many recent studies suggest that ROS production is an integral component of intracellular signaling [10]. Particularly, aberrant ROS production has been found in many types of cancer cells [11], indicating that ROS is involved in tumor progression and metastasis.

Athukorala et al. evaluated the potential antiproliferative and antiradical activities of an enzymatic extract of *E. cava* together with crude polysaccharide and polyphenolic fractions in vivo and showed that both extracts selectively inhibit cell proliferation on all cancer cell lines [12]. The crude polyphenolic fraction was especially effective for inhibiting cell proliferation in murine colon cancer cell lines and enhancing DPPH radical scavenging activity [12]. The same study also reported that the crude polyphenolic fraction at 5 mg/mL scavenged 70% of DPPH radicals, which is much higher than those of BHA and BHT at the same concentration. Taken together, these findings suggest that the crude polyphenolic fraction of *E. cava* is a promising alternative to synthetic substances as a naturally occurring compound with high antiproliferative and antiradical activities.

Tumor metastasis is a complicated pathological process. In malignant tumors, the activities of matrix metalloproteinases (MMP) are deregulated, and their expressions are associated with poor prognosis. There are several reports on the suppression of migration and invasion of cancer cells by phlorotannins. Migration and inversion of cancer cells are key steps for the metastasis of tumor cells. Zhang et al. reported that dieckol (compound **6**) inhibited inversion of human fibrosarcoma cells with suppression of MMP-2 and MMP-9 via nuclear factor kappa B [13]. The MMP family is involved in the breakdown of the extracellular matrix in normal physiological processes and in the disease process such as cancer metastasis. Furthermore, Park and Jeon showed that dieckol also inhibited migration and invasion of HT1080 cells by scavenging intracellular ROS [14]. In HT1080 cells, ROS generation increased migration and invasion, which correlates with Rac1 activation and increased expression and phosphorylation of focal adhesion kinase (FAK). Therefore, they suggest that the Rac-ROS-linked cascade enhances migration and invasion of HT1080 cells by inducing expression of MMPs through activation of the FAK signaling pathway, whereas dieckol down-regulates FAK signaling by scavenging intracellular ROS [14]. Another paper of the same authors reported that dieckol down-regulated the Rac1-ROS-linked cascade and inhibited the invasive migration of B16 mouse melanoma cells [15]. Dieckol also exerted anti-breast cancer activity by regulating the expressions of metastasis-related genes, such as MMP-9, vascular endothelial growth factor, tissue inhibitor of metalloproteinase (TIMP) 1 and TIMP-2 [16]. For ovarian cancer cells, dieckol suppressed cell growth by inducing caspase-dependent apoptosis through increased ROS formation and down-regulation of AKT and p38 signaling [17].

Anticancer properties of phlorotannins other than dieckol have also been reported. Kong et al. evaluated the ability to inhibit the proliferation of human breast cancer cells with dioxinodehydroeckol (compound **8** in Fig. 5.2), and 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin (compound **9** in Fig. 5.2) was evaluated by measuring cell death via induction of apoptosis [18]. They reported that compound **8** exerted a higher antiproliferative activity in MCF 7 human breast cancer cells than that of compound **9**. Compound **8** induced an increase in caspase (3 and 9) activity, DNA repair enzyme poly-(ADP-ribose) polymerase cleavage. In addition, it down-regulated the nuclear factor kappa B (NF-κB) family and their dependent activated genes. These results suggested that dioxinodehydroeckol (compound **8**) induced the apoptosis of MCF-7 human breast cancer cells via the NF-κB-dependent pathway. Furthermore, Eo et al. have shown that phlorofucofuroeckol A (PFF-A) (compound **4**) exerts an anticancer effect by up-regulating the activating transcription factor 3 (ATF3) against human colorectal cancer cells. Induction of ATF-3 often correlates with cellular damage [19]. Therefore, ATF-3 can be thought of as an important molecular target in the induction of apoptosis for cancer prevention.

These results suggest that phlorotannins are potential therapeutic agents for cancer treatment.

2.3 Antiobesity Activities

Obesity is one of the most common metabolic syndromes and has become a major threat to human health. Obesity causes several abnormalities in lipid metabolism, antioxidant defense, and inflammatory response and is a main risk factor for metabolic diseases, such as type 2 diabetes, cardiovascular disease, and chronic kidney disease. Recent studies have suggested that intracellular ROS production plays an important role in excessive lipid accumulation and adipocyte differentiation [20]. Several drugs have been developed for the treatment of obesity that regulate appetite, fat absorption, and fat oxidation. However, many of these drugs have been withdrawn from the market due to low efficacy and side effects. Natural compounds are excellent sources for developing new drugs because of their minimal side effects. Therefore, natural compounds are considered a promising therapeutic strategy for obesity.

There are several reports on the antiobesity effects of phlorotannin or a polyphenol mixture derived from *E. cava*. Jeon et al. [21] found that the polyphenol mixture effectively inhibited lipid accumulation in adipocytes, zebrafish, and mice by suppressing adipogenic factors. Furthermore, they showed that the polyphenol mixture of *E. cava* activated AMP-activated protein kinase (AMPK), an energy sensor. Choi et al. [22], who were with the same research group as Jeon et al., demonstrated that dieckol (compound **6**), a major phlorotannin in *E. cava*, regulated mRNA expression of early adipogenic genes in 3T3-L1 cells, resulting in the down-regulation of late adipogenic factors and a decrease in lipid accumulation. They also found that AMPK activation is involved in the inhibition of lipid synthesis in 3T3-L1 cells and the high-fat diet (HFD) mouse model.

Eo et al. [23] investigated the effect of an *E. cava* polyphenol extract on the regulation of lipid metabolism, inflammation, and the antioxidant defense system in HFD-induced obese mice. These mice were administered the polyphenol extract (500 mg/kg/day) by oral gavage 5 days/week for 12 weeks. They observed that the *E. cava* polyphenolic extract reduced body weight gain, adipose tissue mass, plasma lipid profiles, hepatic fat

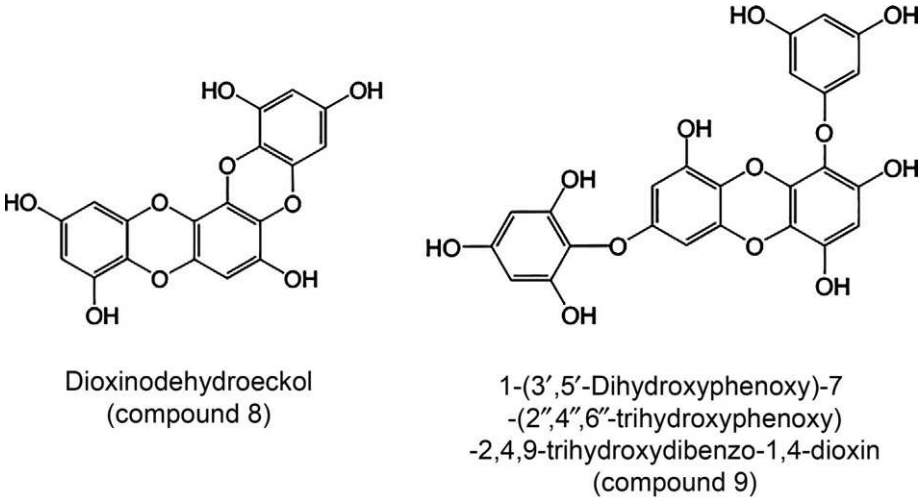


FIG. 5.2 Chemical structure of phlorotannins (compounds **8** and **9**).

deposition, insulin resistance, and the plasma leptin/adiponectin ratio derived from HFD-induced obesity. They also found that the extract induced the activation of AMPK and SIRT1, suggesting that the extract has potential antiobesity effects by regulating AMPK and SIRT1 in HFD-induced obesity. They also showed that the extract reduced the protein levels of tumor necrosis factor (TNF α), interleukin 1β (IL-1β) and (NF-κB), which are related to inflammatory response. Furthermore, they reported that antioxidative enzymes such as glutathione peroxidase and catalase were activated by the extract.

Eo et al. [24] also demonstrated the ameliorative effect of the *E. cava* polyphenol extract on renal inflammation, associated with a decrease in lipid accumulation and oxidative stress in in vivo obese mice. Furthermore, the extract activated the SIRT1/AMPK pathway and thereby reduced renal lipid accumulation in HFD-induced obese mice. This finding suggests that *E. cava* is a functional food for improving renal health in conditions of obesity.

Kwon et al. investigated the antiobesity activities of various phlorotannins by evaluating the inhibition of 3T3-L1 adipocyte differentiation and the expression of transcription factors [25]. They treated differentiated 3T3-L1 cells with five purified phlorotannins (6,6′-bieckol (compound **7**), 6,8′-bieckol (compound **10** in Fig. 5.3), 8,8′-bieckol (compound **11** in Fig. 5.3), dieckol (compound **6**) and phlorofucofuroeckol A (compound **4**)) for 8 days and found that these phlorotannins suppressed 3T3-L1 adipocyte differentiation in a dose-dependent manner, without toxicity. In addition, they observed that three transcription factors (peroxisome proliferator-activated receptor γ, CCATT/enhancer-binding protein α and sterol of regulatory element binding protein-1c (SREBP-1c)) were decreased at the mRNA and protein levels.

Park et al. [26] investigated the effect of the polyphenol-rich fraction of *E. cava* on nonalcoholic fatty liver disease in HFD-fed mice. C57BL6 mice were fed HFD (60% fat primarily from lard) for 6 weeks, and then the HFD group was administered 300 mg/kg of the *E. cava* extract. They found that body weight and fat mass decreased, and the mRNA expression of inflammatory cytokines hepatic lipogenesis-related genes such as SREBP-1c, fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC) was decreased in the extract-treated HFD mice, suggesting that *E. cava* extract may ameliorate hepatic steatosis.

A clinical trial on the hypolipidemic effects of *E. cava* extract in patients with hypercholesterolemia was reported by Choi et al. [27]. Eighty subjects with total cholesterol >200 mg/dL or LDL-cholesterol >110 mg/dL were randomly assigned to receive an *E. cava* extract ($n=40$) or a placebo ($n=40$) for 13 weeks. The results obtained suggest that the *E. cava* extract is effective in the prevention and treatment of atherosclerotic cardiovascular disease; however, more studies are needed to establish the long-term safety and effectiveness of this extract.

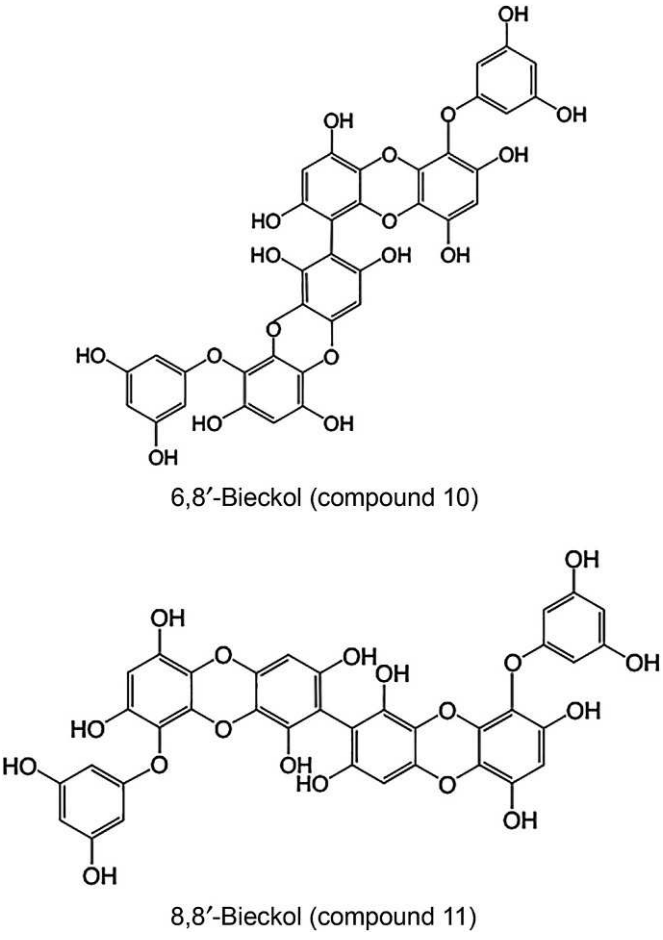


FIG. 5.3 Chemical structure of phlorotannins (compounds **10** and **11**).

2.4 Antidiabetic Activity

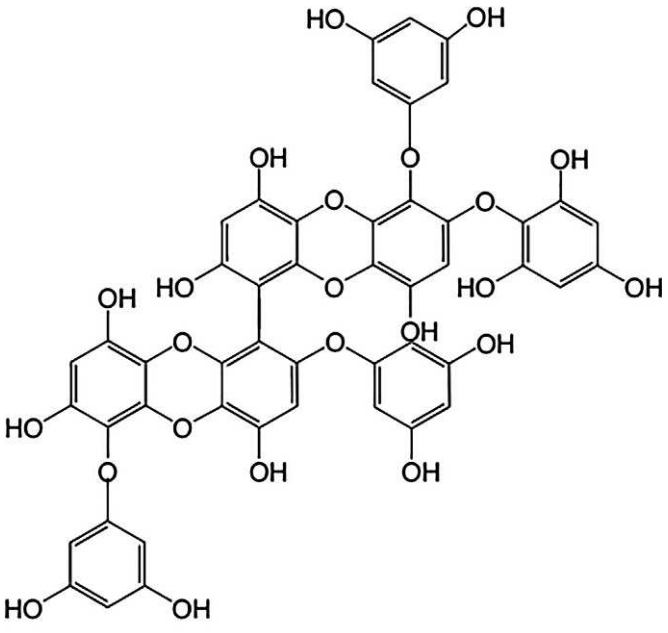
Diabetes mellitus is a chronic disease that develops with obesity and aging. It is known that the inflammatory process is involved in the initiation of diabetes type 1 and 2. Furthermore, some studies suggest that the relationship between ROS and proinflammatory markers is a direct interaction [28].

Drugs available for treating type 2 diabetes are associated with several serious adverse effects. Therefore, natural antioxidants with minimal side effects are screened as potential antidiabetic drug candidates.

One therapeutic method to decrease postprandial blood glucose is the reduction of glucose absorption by inhibiting carbohydrate-digesting enzymes such as a-glucosidase and a-amylase in the digestive organs [29]. Lee et al. evaluated the inhibitory effect of dieckol (compound **6**) against a-glucosidase and a-amylase and found that dieckol inhibited the activities of a-glucosidase ($IC_{50}=0.24$ mM) and a-amylase ($IC_{50}=0.66$ mM). These inhibitory activities were higher

than those of acarbose, an antidiabetic drug. Furthermore, they investigated the effect of dieckol on postprandial hyperglycemia in in vivo mouse models and found that dieckol significantly suppressed the increase of postprandial blood glucose levels in dieckol-treated mice compared to those in the streptozotocin-induced diabetic mice or in normal mice [30]. Lee et al. also investigated the antidiabetic effects of dieckol in a type 2 diabetic animal model, male C57BL/Ksj-db/db mice, and found that dieckol improved glucose and lipid metabolism and the activities of antioxidant enzymes [31]. Furthermore, they reported a double-blind, randomized, placebo-controlled clinical trial of supplementation with a dieckol-rich extract to 80 prediabetic men and women. Compared with the placebo group, the dieckol group showed a significant decrease in postprandial glucose, insulin and C-peptide levels after 12 weeks, suggesting that dieckol-rich extract contributes to lowering hyperglycemia and reducing insulin resistance [32]. Two other phlorotannins, phlorofucofuroeckol A (compound **4**) [33] and 2,7″-phloroglucinol-6,6′-biecckol (compound **12** in Fig. 5.4) [34], also alleviated postprandial hyperglycemia in diabetic mice.

A second target to decrease blood glucose is the regulation of hepatic glucose formation. The liver can produce glucose by breaking down glycogen and by *de novo* synthesis of glucose from lactate, pyruvate, glycerol, and alanine. Therefore, the regulation of hepatic glucose production can contribute to the reduction of blood glucose in diabetes [35]. Yoon et al. investigated the effect of phloroglucinol (compound **1**) on the control of blood glucose levels and the regulation of hepatic glucose production [36] and found that phloroglucinol significantly improved glucose tolerance in male C57BL/6j mice fed a high-fat diet and inhibited glucose production in mouse primary hepatocytes. In addition, the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), two enzymes involved in gluconeogenesis, were inhibited in liver tissue from phloroglucinol-treated mice and in phloroglucinol-treated HepG2 cells. On the other hand, phloroglucinol treatment increased AMPK activation. Furthermore, treatment with compound **C**, an AMPKa inhibitor, inhibited the activation of AMPK and the decrease of PEPCK and G6Pase expression caused by phloroglucinol treatment. These results indicate that phloroglucinol (compound **1**) may inhibit hepatic gluconeogenesis via modulating the AMPK signaling pathway, and thus lower blood glucose levels [36].



2,7″-Phloroglucinol-6,6′-biecckol
(compound 12)

FIG. 5.4 Chemical structure of phlorotannin (compound **12**).

A third therapeutic method for treating diabetes is to prevent the oxidative damage of pancreatic β cells. Pancreatic β cells are sensitive to oxidative stress and may play an important role in β cell death associated with diabetes. Mitochondria in β cells produce excessive amounts of ROS under a high glucose concentration as they utilize an alternative glucose-metabolizing pathway prone to the induction of oxidative stress [37]. Therefore, chronic exposure of β cells to high glucose concentration results in β cell dysfunction and apoptosis [38,39]. These results suggest that antioxidants can prevent the pathological damage induced by hyperglycemia-induced oxidative stress. A high glucose (30 mM) treatment induced the death of rat insulinoma cells. Lee et al. investigated the protective effect of dieckol (compound **6**) against high glucose-induced oxidative stress using rat insulinoma cells. However, dieckol protected pancreatic β cells from cell damage by reducing ROS-induced oxidative stress and apoptosis. These results suggest that dieckol may be a potential pharmaceutical agent to protect against hyperglycemia-induced oxidative stress that is associated with diabetes [40].

Lopes et al. have reviewed this potential in "Phlorotannins: Toward new pharmacological interventions for diabetes mellitus type 2" [41]. In the review, they described the effect of phlorotannins on various pharmacologically relevant mechanisms and pancreatic, hepatic, and intestinal enzymes, glucose transport and metabolism, glucose-induced toxicity and β-cell cytoprotection.

2.5 Neuroprotective Activities

The world population increasingly suffers from aging-dependent neurodegenerative diseases. Currently,

24 million people suffer from a neurodegenerative disease, and this number is expected to double every 20 years to 42 million by 2020 and 81 million by 2040 [42]. Alzheimer's disease (AD) is the most common age-dependent neurodegenerative disorder and causes a progressive decline in cognitive function. Therefore, it is highly desirable to find a method for preventing AD.

In AD, ROS-induced oxidative stress increases the production of amyloid β peptide [43] and then contributes to memory and cognitive function deficits [44]. Yang et al. investigated whether phloroglucinol (compound **1**) displays neuroprotective activities in HT-22 hippocampal cell lines and in 5XFAD (Tg6799) mice, an AD animal model. They observed that phloroglucinol displays therapeutic potential for AD and may delay the onset or inhibit the progression of AD by attenuating cognitive function deficits through its antioxidant activity [45].

Parkinson's disease (PD) is a neurodegenerative disease characterized by the progressive loss of dopaminergic neurons in the substantia nigra. These neurons are particularly sensitive to oxidative stress, which may lead to protein aggregation and subsequent cell death, causing PD. Cha et al. investigated the neuroprotective effect of dieckol (compound **6**) on rotenone-induced oxidative stress in human dopaminergic neuronal cell lines (SH-SY5Y cells, an in vitro PD model). They found that dieckol reduced intracellular ROS, slowed rotenone-induced α-synuclein aggregation, and reduced rotenone-induced cell death [46].

Ischemic stroke is the most common type of stroke and is among the leading causes of long-term disability and mortality [47]. Neuronal cell death during the progression of acute or chronic ischemic stroke is caused by the production of excessive extracellular glutamate, which activates its receptors abnormally. Kim et al. used glutamate-stimulated PC12 cells for an in vivo ischemic stroke model and found that phlorofucofuroeckol (compound **4**) significantly inhibited glutamate-induced apoptosis of PC 12 cells. Furthermore, they observed the exposure of PC12 cells to glutamate-induced production of ROS and mitochondrial dysfunction, and the production and dysfunction were attenuated by phlorofucofuroeckol dose-dependently [48].

These reports suggest that phlorotannins may be useful for protecting against or treating various neurodegenerative diseases.

3 PROTECTIVE EFFECT AGAINST ETHANOL-INDUCED LIVER INJURY

Chronic alcohol abuse is a significant cause of cirrhosis and liver failure. In particular, the excessive intake of alcohol causes serious liver damage that leads to steatosis, steatohepatitis, fibrosis, and cirrhosis. Eventually, cirrhosis may progress to hepatic decompensation and hepatocellular carcinoma [49]. Oxidative stresses, especially ROS, play an important role in this process.

The liver is the main organ responsible for metabolizing alcohol. Alcohol is primarily metabolized in the liver in two steps. The first step is the oxidation of acetaldehyde by the following three enzymes: alcohol dehydrogenase (ADH) in the cytosol, cytochrome P450 2E1 (CYP2E1) in the microsome, and catalase in the peroxisome. ADH is used to degrade limited quantities of alcohol, while alcohol-induced CYP2E1 occurs only after excessive alcohol intake [50] (Fig. 5.5). The second step is the oxidation of acetaldehyde to acetate. Acetaldehyde is oxidized primarily by aldehyde dehydrogenase (ALDH), which is a mitochondrial, NAD^+-dependent enzyme [51]. In alcohol metabolism, it is well known that CYP2E1 overexpression is induced by chronic alcohol intake, resulting in ROS overgeneration [52], impairment of mitochondrial function [53], and inhibition of fatty acid oxidation [54]. Furthermore, it has been reported that the use of the CYP2E1 knockout model resulted in decreased ethanol-induced malondialdehyde (MDA) accumulation and glutathione depletion [55]. These results suggest that CYP2E1 plays a critical role in increased oxidative stress. Therefore, it is widely thought that the inhibition of CYP2E1 overexpression contributes to the decrease in various impairments in hepatocytes.

Ecklonia cava polyphenols (ECP) have various biological and pharmacological effects. However, there is no study on the effect of these polyphenols on ethanol-induced liver injury. Therefore, we examined the effect of ECP on the development of liver fibrosis in (1) an in vitro alcohol-injury model in hepatocytes, (2) an in vitro alcohol-injury model in hepatic stellate cells (HSCs), and (3) an in vivo ethanol-CCl_4-induced cirrhosis model [56]. In addition, we studied mechanisms of the

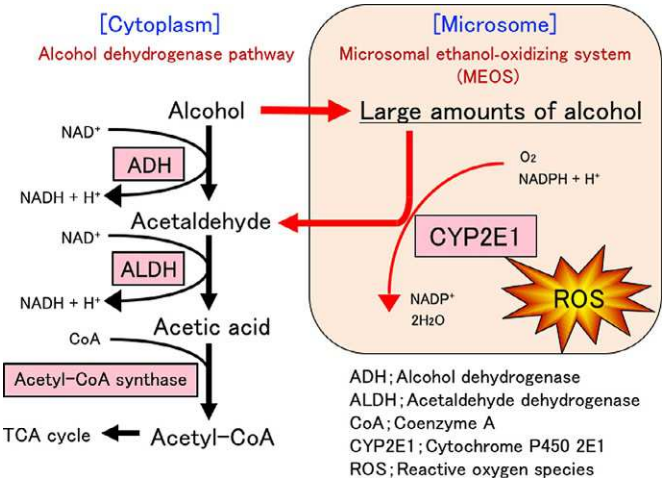


FIG. 5.5 Metabolic pathway of alcohol.

protective effect of ECP in ethanol-treated hepatocytes [57]. Here, we show the effect of *E. cava* polyphenols on ethanol-induced liver injury.

3.1 An in vitro Alcohol-Injury Model in Hepatocytes

First, we examined the effect of ECP in an in vitro alcohol-injury model in hepatocytes. Hepatocytes were isolated by collagenase perfusion of livers, following their removal from 10-week-old male Wistar rats. After preincubation, the cells were cultured in 10% fetal bovine serum containing Williams' medium E with 100 mM of ethanol with or without various concentrations of ECP for 0–24 h. Then, we measured the cell viability of hepatocytes with 100 mM of ethanol since serum ethanol concentration in heavy drinkers ranges from 100 to 200 mM. A dose of 6.25–25 μg/mL ECP significantly prevented cell death in the hepatocytes treated with 100 mM ethanol. Thus, experiments that followed were conducted with 6.25 or 12.5 μg/mL of ECP.

Ethanol-induced liver injury is characterized by the increased formation of ROS [58]. Therefore, we measured intracellular ROS levels in hepatocytes incubated for 6 and 9 h with 100 mM of ethanol, with or without 6.25 μg/mL ECP. With the cotreatment of ECP, ROS generation in the ethanol-treated hepatocytes was suppressed. To further verify the scavenging of ROS by ECP, we examined the effect of ECP on ethanol-induced increases in intracellular lipid peroxidation using the thiobarbituric acid reactive substances (TBARS) assay [59]. Cells with ECP treatment maintained intracellular MDA levels below those of the control cells. Induction of CYP2E1 in ethanol-treated hepatocytes is an important contributor to ethanol-induced oxidative stress [58]. Therefore, we examined the effect of ECP on the activity of CYP2E1. The ethanol-treated hepatocytes for 9 h significantly increased CYP2E1 activity to the levels of the control cells. However, ECP treatment suppressed the ethanol-induced increase in CYP2E1 activity to the levels of the control cells.

To know the potential protective mechanism of ECP against ethanol-induced hepatotoxicity, we examined the activities of the two major alcohol metabolizing enzymes, ADH and ALDH. ECP treatment significantly enhanced ADH activity in these cells over a period of 2, 4, and 9 h. Furthermore, ALDH activity was also significantly enhanced after 4 h incubation with ECP.

Potter et al. reported that cyclic AMP may be a common pathway for the effects of epinephrine and glucagon in enhancing ADH activity [60]. It has been reported that CYP2E1 activity is controlled by hormones posttranslationally, resulting in the phosphorylation of Ser^{129} by cyclic AMP-dependent protein kinase and degradation of the enzyme by the microsomal Mg^{2+}-ATP-dependent proteolytic system [61,62].

These reports suggest that ECP regulates the activities of ADH and CYP2E1 via cyclic AMP/protein kinase A signaling. Therefore, we examined the effect of H-89, an inhibitor of protein kinase A, and found that the treatment of H-89 increased CYP2E1 activity in ethanol- and ECP-treated hepatocytes. On the other hand, treatment with H-89 inhibited ADH activity induced by treatment with ethanol and ECP.

To ascertain the involvement of cyclic AMP on the regulation of CYP2E1 and ADH activities, we examined cyclic AMP concentrations in cells incubated for 6 h with or without ethanol and ECP. ECP treatment significantly increased cyclic AMP concentrations compared with control cells [57].

To examine whether ECP protects ethanol-induced liver injury via regulating alcohol metabolic enzymes in a cyclic AMP-dependent manner, we tested the effects of H-89 on ROS production in hepatocytes after ethanol and/or ECP incubation. ECP caused a decrease in intracellular ROS levels, but H-89 treatment increased intracellular ROS levels to those of ethanol-alone-treated hepatocytes [57].

These results suggest that ECP is a candidate for preventing ethanol-induced liver injury via regulating alcohol metabolic enzymes in a cyclic AMP-dependent manner (Fig. 5.6).

3.2 An in vitro Alcohol-Fibrosis Model in HSCs

HSCs were isolated by density gradient centrifugation in Nycodenz AG (Axis-Shield, Poc. Oslo, Norway) after digestion of the liver with pronase and collagenase [63]. Cells were cultured in uncoated plastic dishes with DMEM containing 10% fetal calf serum. HSCs have the

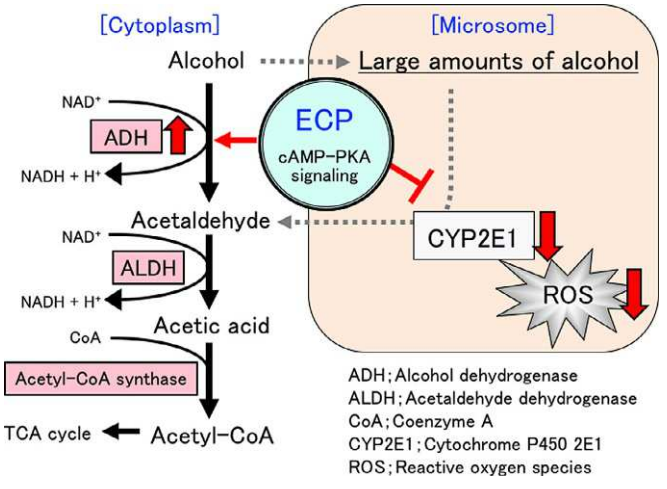


FIG. 5.6 Effect of *Ecklonia cava* polyphenols (ECP) on alcohol metabolism.

most important role in the regulation of liver extracellular matrix homeostasis and play a key role in the development of liver fibrosis. Therefore, we used cultured HSCs as a liver fibrosis model.

Increased ROS levels mediate the adverse effect of ethanol ingestion in liver injury and fibrogenesis. ROS levels are increased by ethanol metabolism, which is catalyzed by the hepatic microsomal cytochrome CYP2E1. Neito et al. demonstrated that CYP2E1-derived ROS stimulate the synthesis of type I collagen by HSCs in a paracrine manner [64]. They also reported that antioxidants and a CYP2E1 inhibitor prevent this increased collagen synthesis.

We examined the expression of type I collagen and α-smooth muscle actin (α-SMA), markers of HSCs activation, in ethanol-treated HSCs by immunohistochemistry. Our findings showed increased staining intensities for type I collagen. However, ECP treatment suppressed the ethanol-induced increase in type I collagen and α-SMA expressions to near control levels. Furthermore, we measured intracellular ROS levels to investigate the relationship between ethanol-treated HSC activation and ROS formation. HSCs were incubated for various amounts of time with 100 mM ethanol with or without ECP. ECP treatment maintained intracellular ROS levels below those of control cells [56].

These results suggest that ECP prevents liver fibrosis induced by ethanol via the suppression of ROS production.

3.3 An in vivo Ethanol-CCL_4-Induced Cirrhosis Model

Animal models of liver fibrosis are important for researching the underlying mechanisms of treatment for this disease. Currently, some models for the administration of alcohol to animals have been developed, including the Lieber-De Carli liquid diet [65] and the Tsukamoto-French gastric model [66]. In the Lieber-De Carli liquid diet, the ethanol replaces the carbohydrates of a normal diet. Tsukamoto and French developed an in vivo animal model in which enteral ethanol is continuously administered via intragastric infusion. A third model is the ethanol-carbon tetrachloride (CCl_4)–induced fibrosis model. In this model, experimental fibrosis was induced in rats within 4 weeks of administering low-dose CCl_4 (0.1 or 0.2 mL/kg of body weight diluted with olive oil to 25%) and a 5% ethanol solution. The hepatic histological changes in the ethanol-CCl_4-induced fibrosis model were similar to those found in human alcoholic cirrhosis [67]

The effect of ECP treatment on plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and liver fibrosis in ethanol plus CCl_4 (0.1 mL/kg of body weight) treated rats was examined. The plasma AST and ALT activities in the ethanol-CCl_4-treated group increased by 5.7- and 11.1-fold, respectively, compared with those of the control group. However, these enzymes were significantly less active in the ECP-treated groups than in the ethanol-CCl_4-treated group. Furthermore, histological analysis was performed by hematoxylin and eosin staining, as well as Elastic van Gieson staining, to assess liver damage. No significant abnormalities were observed in the control rats or the CCl_4-treated rats. However, collagen accumulations in the liver and hepatic necrosis were observed in the ethanol-CCl_4-treated rats. On the other hand, ECP treatment fully protected rats against ethanol-CCl_4-induced liver fibrosis and necrosis [56].

These results suggest that ECP could prevent ethanol-induced liver injury.

4 AMPK IN THE PROTECTIVE EFFECTS OF PHLOROTANNINS

There are several studies on the relationship between AMPK and the protective effects of phlorotannins. However, this relationship is still unclear. Here, the involvement of the AMPK pathway on the biological and pharmacological effects of phenolic compounds from *E. cava* is reviewed.

AMPK acts as an intracellular energy sensor that is activated by energy deficiency resulting from metabolic stresses that interfere with ATP production or accelerate ATP consumption [68]. AMPK activation occurs through phosphorylation by upstream kinases. The activation of AMPK is induced by numerous drugs, xenobiotics, and nutraceuticals [69]. Once activated, AMPK phosphorylates and inhibits key enzymes within energy-consuming biosynthetic pathways [70] (Fig. 5.7).

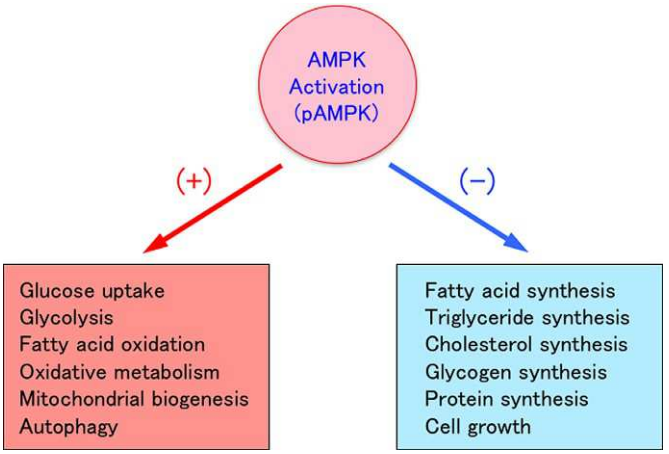


FIG. 5.7 Functions of activated AMPK.

Kim et al. reported that the AMPK pathway was involved in the cytoprotective effect of eckol (compound **2**) against H_2O_2-induced mitochondrial dysfunction in Chang liver cells [71]. They showed that eckol prevents the induction of mitochondrial ROS by H_2O_2 and the ROS-mediated overloading of mitochondrial Ca^{2+} in Chang liver cells. Eckol increased the expression of manganese superoxide dismutase, a mitochondrial antioxidant enzyme with a cytoprotective effect against oxidative stress, by phosphorylating AMPK and forkhead box O (FoxO). FoxO proteins are transcription factors that are involved in the detoxification of ROS, DNA repair, apoptosis, and cell cycle arrest. The FoxO3a evoked by AMPK mediates transcriptional up-regulation of the ROS scavenging enzyme superoxide dismutase 2. These results showed that eckol (compound **2**) protects cells from mitochondrial oxidative stress by activating AMPK/FoxO3a-mediated induction of manganese superoxide dismutase.

As previously described, Eo et al. reported that ECP extract reportedly ameliorates hepatic protein levels associated with lipogenesis, inflammation, the antioxidant defense system, and activation of AMPK and SIRT1 [23]. Furthermore, Kohjima et al. reported that activated AMPK negatively regulates sterol regulatory element binding protein (SREBP), a major transcription factor of fatty acid synthesis [72]. Horton also showed that an overexpression of SREBP1c, one of the SREBP isoforms, caused an increase in the expression of lipogenic enzymes, including ACC1 and FAS [73]. AMPK negatively regulates the activities of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) and ACC, which are rate-limiting enzymes of cholesterol and malonyl-CoA synthesis, respectively. Malonyl-CoA is a material of fatty acid synthesis and an allosteric inhibitor of carnitine palmitoyltransferase-1, which takes up long-chain fatty acyl-CoA into mitochondria. Therefore, inactivation of ACC leads to reducing lipogenesis and increasing fatty acid β-oxidation [74]. These results suggest that phloroglucinol (compound **1**) inhibits lipogenesis and cholesterol synthesis via modulating the AMPK signaling pathway.

As previously described, treatment with compound **C**, a specific AMPK inhibitor, suppressed ECP-induced phosphorylation of AMPK and acetyl-CoA carboxylase, suggesting that ECP inhibits lipid accumulation by activating AMPK signaling [21]. As previously shown, treatment with compound **C** inhibited the activation of AMPK and the decrease in the expressions of PEPCK and G6Pase, key enzymes involved in gluconeogenesis, caused by phloroglucinol (compound **1**) treatment [36].

There is growing awareness that activation of AMPK may oppose tumor progression in various cancers [75]. In addition, several reports support a neuroprotective role of AMPK [76]. However, there is currently no study that examines the relationship between activation of AMPK and phlorotannins in the context of antitumor and neuroprotective effects. Further investigations are necessary for understanding the molecular events associated with phlorotannin-dependent functions (Fig. 5.8).

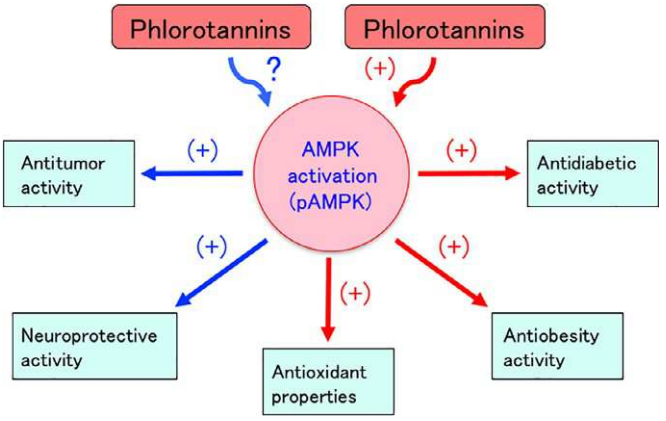


FIG. 5.8 Effect of phlorotannins on the functions of activated AMPK.

5 CONCLUSION

Ecklonia cava is an edible brown alga that widely inhabits the southern coast of Korea and Japan and contains many unique polyphenols called phlorotannins. The in vitro antioxidant activity of phlorotannins is dependent upon the number of hydroxyl groups present in each compound. The antioxidative activities of all phlorotannins, except for phloroglucinol, were stronger than the polyphenols found in green tea.

This review examined the biological and pharmacological effects of phlorotannins and their mechanisms of action. Many studies in vitro and in vivo show that phlorotannins have various biological and pharmacological effects, including antioxidation, antitumor, antiobesity, antidiabetic, neuroprotective, and hepatoprotective activities. These results strongly suggest that phlorotannins may be beneficial in the prevention of lifestyle-related diseases. However, further study in this area should examine the clinical effect of phlorotannins to more fully understand their potential.

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CHAPTER

6

Clerodendrum volubile: Phenolics and Applications to Health

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1 INTRODUCTION

The health benefits of phenolics are well documented [1,2]. Phenolics are secondary metabolites in plants responsible for most of their health-promoting properties [3]. They are utilized in plants as defense mechanisms against pathogens and protection against ultraviolent rays [4]. They also contribute to the color, acidity, taste, fragrance, and oxidative permanence of plants [5].

Chemically, phenolics possess an aromatic ring with one or more hydroxyl groups, including functional derivatives such as methyl ethers, esters, and glycosides [6]. They are classified into phenolic acids, hydroxycinnamic acids, flavonoids, and lignans, which all arise from the amino acids, phenylalanine or tyrosine. The amino acids are deaminated to cinnamic acids, before entering the phenylpropanoid pathway [7]. One or more hydroxyl groups are then introduced into the phenyl rings to form the common carbon skeleton, the C6—C3 phenylpropanoid unit (Pereira et al. [7]). This is well reflected in the C6 aromatic ring of hydroxybenzoic acids, C6—C3 structure of hydroxycinnamic acids, C6—C3—C6 structure of flavonoids, and C6—C4—C6 structure of lignans [8].

2 PHENOLICS AND HEALTH

Several studies have reported the inverse correlation between phenolics intake and chronic human diseases such as cardiovascular diseases, diabetes, cancer, obesity, and inflammation [5,9,10]. This is dependent on their absorption and metabolism by the gastrointestinal (GI) system into the circulatory system and transportation to the liver [11]. Their structures play a major influential role in this regard by determining conjugation with other phenolics, degree of glycosilation/acylation, molecular size, and solubility [11–13].

These medicinal properties have been attributed to the potent antioxidative effect of phenolics, owing to their ability to (i) scavenge free radicals, (ii) chelate divalent cations, and (iii) modulate endogenous antioxidant enzymes [5,11]. These abilities are dependent on the hydroxylation of the aromatic rings. Phenolics have also been reported to bring about their medicinal effect by modulating intracellular signaling cascades involved in cellular metabolism [11,13].

2.1 *Clerodendrum volubile*

Phenolics are widely distributed in plants and are responsible for most of their medicinal properties [3]. The phenolic contents of some of these plants have been analyzed and their protective activities reported. Among such plants is *Clerodendrum volubile*, which belongs to the genus *Clerodendrum* L. under the Labiatae family.

The genus *Clerodendrum* L. is very large and diverse with over 580 identified species, comprising small trees, shrubs, and herbs widely distributed in the tropics and subtropics [14]. Most of these species are employed as folk medicine in Asian and African continents [14].

Clerodendrum volubile is indigenously known as obenetete by the Urhobos and Itsekiris, as well as eweta, dagba, or marugbo by the Yorubas all in Southern Nigeria [15,16]. It is often regarded as a magic leaf owing to its use in folkloric medicine for the treatment and management of several diseases such as diabetes mellitus, cancers, arthritis, ulcers, and neurological disorders [17]. Despite these folkloric claims there are, however, limited

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scientific data on its biological activities and phenolic constituents.

This chapter discusses the studied biological activities and phenolic constituents of *C. volubile*.

3 PHYTOCHEMISTRY OF *CLERODENDRUM VOLUBILE*

3.1 Preliminary Phytochemical Screening

Preliminary phytochemical screening of the leaves of *C. volubile* revealed the presence of flavonoids, tannins, and phenols as summarized in Table 6.1.

3.1.1 Flavonoids

Flavonoids are hydroxylated phenolics with a benzo-γ-pyrone structure, and one of the most abundant groups of plant phenolics [23]. Their pharmacological roles have been well established and are dependent on the structural class, hydroxylation degree, conjugations, substitution, and polymerization [23], thus illustrating a structural dependent activity [23,24]. This is evident in their ability to donate electrons [25], chelate iron catalysis [26], reduce Fe^{2+} [27], attenuate oxidation [28], and activate antioxidant enzymes [29].

Ogunwa et al. [16] revealed the presence of flavonoids in the aqueous, ethanol, and methanol extracts of *C. volubile* leaves, with the ethanol extract having the highest concentration. Akinpelu et al. [21], Ogunwa et al. [16], and Senjobi et al. [20] also reported similar observations in ethanol and methanol extracts. These studies correspond with a previous study by Erukainure et al. [18] on the phytochemical contents of the leaves.

3.1.2 Tannin

Tannins are water-soluble polyphenolic biomolecules responsible for the astringency of most plants [30,31]. They mostly consist of a large number of hydroxyl and other functional groups, a characteristic that makes them capable of binding with macromolecules, especially proteins [30]. This binding ability has been reported to have a negative impact on animal nutrition, as it decreases feed intake, feed efficiency, net metabolizable energy, and protein digestibility [31,32].

Tannins are classified as hydrolyzable and condensed tannins. Hydrolyzable tannins consist of a polyhydric alcohol and hydroxyl groups that are partially or wholly esterified by gallic acid or hexahydroxydiphenic acid [30], while condensed tannins consist of a group of polyhydroxy-flavan-3-ol oligomers and polymers that are linked by carbon-carbon bonds between flavanol subunits [33].

Erukainure et al. [18] reported the presence of tannin in the aqueous extract of *C. volubile* leaves. This was further corroborated in a study by Ogunwa et al. [16]. Ogunwa et al. [16] also compared the concentration in the aqueous and methanol extracts, and concluded the methanol extract had a higher concentration, which corresponded to a previous report by Jaiyesimi and Adekoya [19]. Senjobi et al. [20], however, reported the highest concentration in diethyl ether, when they compared the tannin contents in aqueous, methanol, and diethyl ether extracts. The presence of tannin in the ethanol extract reported by Akinpelu et al. [21], however, contradicts that of Ogunwa et al. [16] who reported the absence of tannin in the extract.

Although tannins may be considered as antinutrients, their presence in *C. volubile* leaves also poses therapeutic benefits. Tannins have been reported for their antioxidant, antihemorrhagic, antiseptic, antiinflammatory, anthelmintic, anticancer, antimicrobial, and antiviral activities [34,35].

TABLE 6.1 Summary of Preliminary Studies on Phenolic Constituents of *Clerodendrum volubile*

Phytochemicals	Extracts	Plant part	References
Flavonoids	Methanol; Aqueous; Ethanol; Diethyl ether	Leaf	[16,18–22]
Tannins	Methanol; Aqueous; Ethanol; Diethyl ether	Leaf	[16,18–22]
Phenol	Methanol; Aqueous; Ethanol; Diethyl ether	Leaf	[16,18,20–22]

3.2 High-Performance Liquid Chromatography Fingerprinting

Studies on the high-performance liquid chromatography (HPLC) analysis of various extracts of *C. volubile* leaves have revealed the presence of phenolic compounds, as shown in Fig. 6.1 and Table 6.2.

3.2.1 Hydroxycinnamic Acids

Hydroxycinnamic acids (HCAs) are natural phenylpropenoic acid compounds, which occur as esters, glycosides, and/or conjugates of proteins [39,40]. A few also exist as natural free acids [39]. They are major intermediates in the biosynthetic pathways of polyphenols [41]. They have been recognized as an important source of antioxidants and play an influential role in the stability, flavor, color, and nutritional bioavailability of foods rich in the compounds [39,42].

HCAs are derived from phenylalanine and tyrosine, comprising a nine carbon (C6—C3) skeleton with a side chain double bond (with *cis* or a *trans* configuration),

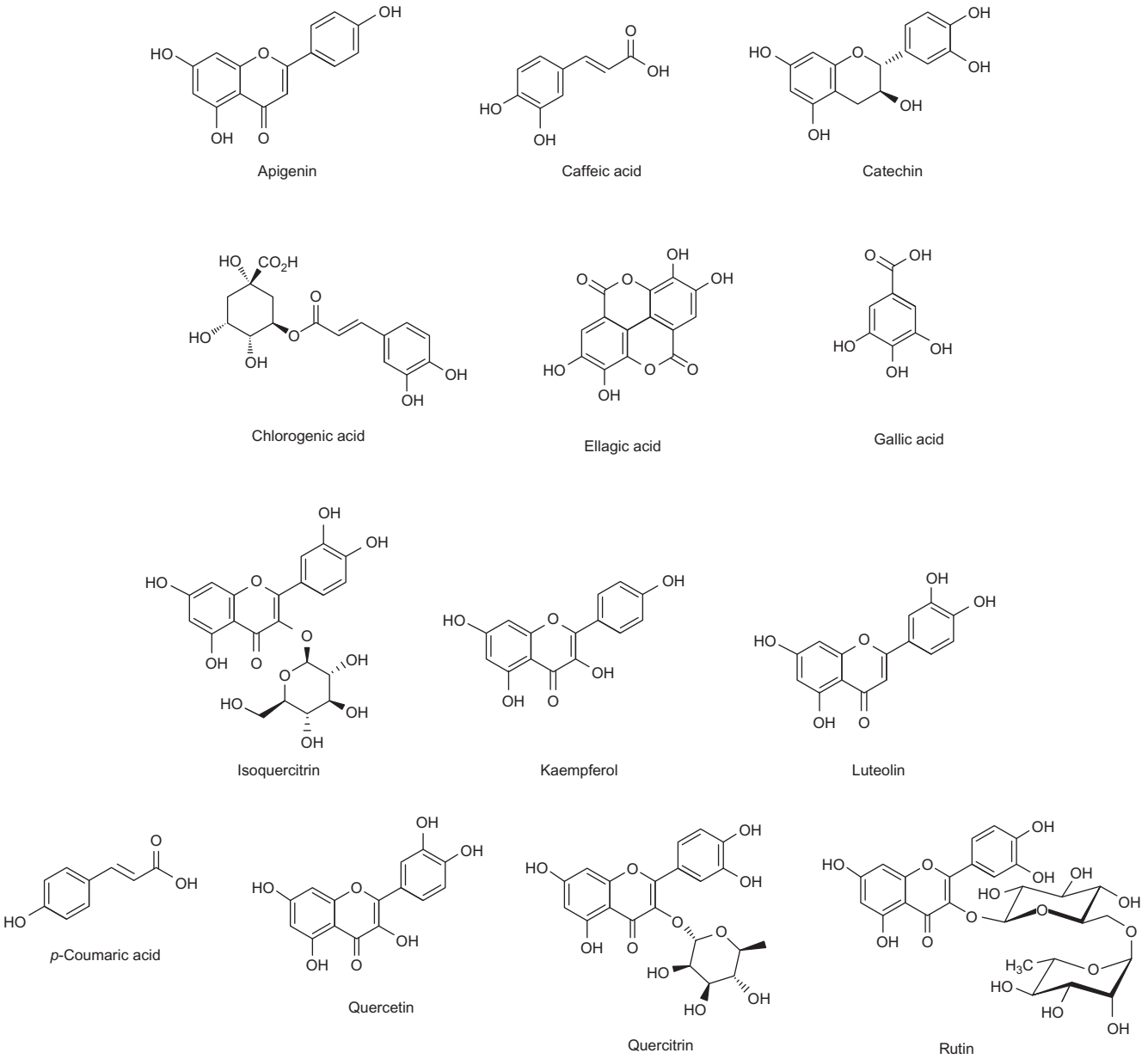


FIG. 6.1 HPLC-identified phenolics in *C. volubile* leaves.

representing a phenylpropanoid structure [41,43]. The hydroxyl functional groups on the benzene ring and the unsaturated bond of its ethylenic side chain are important sites for reactions with reactive oxygen species (ROS), portraying a structure-activity relationship [43,44]. Thus, their biological activity depends on the pattern of substitution of the aromatic moiety [43].

The most common HCAs are cinnamic acid, caffeic acid, sinapic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, and ferulic acid. Adefegha and Oboh [36] reported the presence of caffeic and chlorogenic acids in a phenolic rich extract of *C. volubile* leaves. This was confirmed by Oboh et al. [37], who also reported the presence of *p*-coumaric acid in the phenolic rich extract. Molehin et al. [38] also reported the presence of caffeic acid in the ethyl acetate, ethanol, and methanol extracts of the leaves.

3.2.2 *Flavonoids*

The presence of flavonoids reported in the preliminary screening of *C. volubile* leaves was further confirmed by HPLC analysis of the leaf extracts.

Adefegha and Oboh [36] and Oboh et al. [37] reported the presence of quercetin and its glycone rutin in the phenolic rich extract of the leaves. This was further confirmed by Molehin et al. [38] in the ethyl acetate, ethanol, and

TABLE 6.2 HPLC-Identified Phenolics in C. *volubile* Leaves

Phenolics	Compounds	Extracts	References
Hydroxycinnamic acids	Caffeic acid	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	[36–38]
	Chlorogenic acid	Phenolic-rich extract	[36,37]
	p-Coumaric acid	Phenolic-rich extract	[37]
Flavonoids	Rutin	Phenolic-rich extract; ethyl acetate, ethanol, and methanol extracts	[36–38]
	Isoquercitrin	Phenolic-rich extract	[36]
	Quercitrin	Phenolic-rich extract; ethyl acetate, ethanol, and methanol extracts	[37,38]
	Quercetin	Phenolic-rich extract; ethyl acetate, ethanol, and methanol extracts	[36–38]
	Kaempferol	Phenolic-rich extract; ethyl acetate, ethanol, and methanol extracts	[36–38]
	Apigenin	Phenolic-rich extract; ethyl acetate, ethanol, and methanol extracts	[37,38]
	Catechin	Phenolic-rich extract; ethyl acetate, ethanol, and methanol extracts	[37,38]
	Luteolin	Ethyl acetate, ethanol, and methanol extracts	[37]
Trihydroxybenzoic acids	Gallic acid	Phenolic-rich extract; ethyl acetate, ethanol, and methanol extracts	[37,38]
Ellagitannins	Ellagic acid	Phenolic-rich extract	[37]

methanol extracts. Quercetin is one of the most studied dietary flavonoids with diverse pharmacological applications, particularly antioxidant, anticancer, and neuroprotection [45]. It consists of five hydroxyl groups and two benzene rings that are linked by a heterocyclic pyrane ring, forming the backbone for other flavonoids such as naringenin, hesperidins, and tangeritin [23,46,47]. Although quercetin is an aglycon, it also exists in most plants as glycone [46]. Aside from rutin, Adefegha and Oboh [36] and Oboh et al. [37] reported the presence of isoquercitrin and quercitrin. These glycones bring about their biological activities by undergoing hydrolysis to release quercetin [48].

Other well-studied flavonoids identified in *C. volubile* leaves are kaempferol, apigenin, catechin, and luteolin [36–38]. Apigenin and luteolin belong to the flavonoid class of flavones, which are distinguished from other classes by the absence of a 3-hydroxyl group [23]. Kaempferol and catechin are flavonols whose benzene ring is condensed with an α-pyrone or a dihydro-derivative [23]. It is distinguished from flavones by the presence of a 3-hydroxyl group and C2—C3 double bond [23,49].

3.2.3 Trihydroxybenzoic Acids

Gallic acid is the most popular of trihydroxybenzoic acids. Also known as 3,4,5-trihydroxybenzoic acid, gallic acid has been reported in most plants. Adefegha and Oboh [36] and Oboh et al. [37] reported its presence in the phenolic rich extract of *C. volubile* leaves. Molehin et al. [38] also compared its concentration in the ethyl acetate, ethanol, and methanol extracts of *C. volubile* leaves, with the ethyl acetate extract having the least concentration.

Gallic acid is biosynthesized from 3-dehydroshikimate in a reaction catalyzed by shikimate dehydrogenase to give 3,5-didehydroshikimate, which then undergoes tautomerization to form gallate [50,51].

It consists of three hydroxyl groups and a carboxylic acid group attached to a benzene ring. The bonding of the hydroxyl groups in an ortho position results in a coplanar and bent configuration, which is favorable for antioxidative activities [52,53]

3.2.4 Ellagitannins

Ellagitannins are esters of hexahydroxydiphenoic acid and monosaccharide and are classified under hydrolyzable tannins [54]. They are recognized as the largest group of tannins, with over 500 compounds reported [55,56]. Their easy labilization in solution makes them prone to hydrolysis and polymerization reactions. On hydrolysis with acid and/or base, their hexahydroxydiphenoyl (HHDP) group undergoes a spontaneous rearrangement to the gallic acid dimeric derivative, ellagic acid [56].

The presence of ellagic acid has been reported in many plants. Oboh et al. [37] reported its presence in the phenolic rich extract of *C. volubile* leaves, thus confirming the previous reports on the presence of tannins in the leaves (Table 6.1) [18–22].

Ellagic acid consists of a hydrophilic domain made up of four phenolic groups and two lactones, and a lipophilic domain made up of four rings [57]. These domains play an influential role in its biological activities, particularly the hydrophilic, which can hydrogen bond and accept electrons [57,58], thus denoting a structure-activity relationship.

3.2.5 Bioavailability

Several studies have investigated the absorption and metabolism of these phenolics, thereby providing useful insights into their health benefits. Their bioavailability is summarized in Table 6.3.

3.3 Isolation and Structural Elucidation

In addition to HPLC fingerprinting, some phenolic compounds depicted in Fig. 6.2 and Table 6.4 have been isolated and structurally elucidated via different chromatography and spectroscopy techniques. The isolation of these compounds affirms the reported presence of phenol and flavonoids in the preliminary phytochemical investigation (Table 6.1) [18,20,22].

4 BIOLOGICAL ACTIVITIES OF *CLERODENDRUM VOLUBILE*

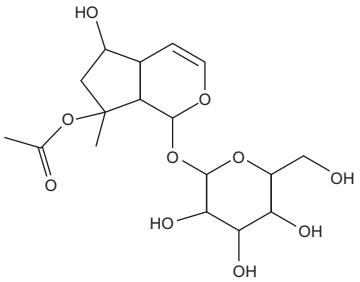
Although *C. volubile* has been employed in folkloric medicine for the treatment of several diseases and ailments, there are, however, fewer studies to back its medicinal properties. To the best of our knowledge, the studies done so far on the biological activities of *C. volubile* are summarized in Table 6.5.

4.1 Antioxidative Activities

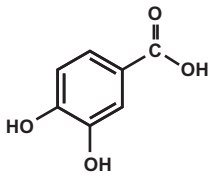
Oxidative stress has been implicated in the pathogenesis and progression of most diseases and their complications. It arises from an imbalance between free radical generation and the endogenous antioxidants, with the former being depleted. These free radicals are constituted from the reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are continuously produced by oxidative metabolism, immune activities, and cellular bioenergetics [75]. Lipid peroxides, nitric oxide (NO), superoxide anion ($O_2^{\bullet -}$), hydroxy radicals, ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), and singlet oxygen ($^1O_2$) constitute the most studied free radicals. Due to their unstable nature, free radicals react very fast with the cells' lipids and proteins, leading to physiological and biochemical changes characterized by increased cell differentiation, proliferation, growth, and death (apoptosis and/or necrosis). The body has

TABLE 6.3 Bioavailability of HPLC-Identified Phenolics in *C. volubile* Leaves

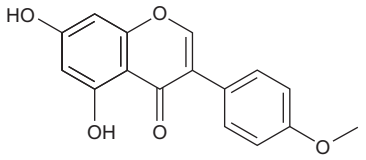
Phenolics	Bioavailability	Compounds	References
Hydroxycinnamic acids	Chlorogenic acid is hydrolyzed to caffeic and quinic acids in the stomach and intestine. Highest dose ingestion is associated with reduced bioavailability *p*-Coumaric acid has a higher bioavailability than chlorogenic and caffeic acids. In rats, it is absorbed in all sections of the gastrointestinal tract, with the jejunum having the highest absorption rate. In the liver, it is conjugated with glucuronide, sulfate and sulfoglucuronide	Caffeic acid Chlorogenic acid *p*-Coumaric acid	[59,60]
Flavonoids	They are absorbed from both the small intestine and colon, with absorption from the latter being the most efficient. They are conjugated with glucoronic acid or sulfate, after absorption from the small intestine. They may also undergo O-methylation. Those that are transported to the colon metabolized by the enzymes in intestinal microflora	Rutin Isoquercitrin Quercitrin Quercetin Kaempferol Apigenin Catechin Luteolin	[61,62]
Trihydroxybenzoic acids	They are readily absorbed from the gastrointestinal tract resulting in peak plasma concentration 1–2 h after ingestion. Mostly excreted unchanged in urine. Once absorbed, they undergo methylation, decarboxylation, and dehydroxylation	Gallic acid	[63]
Ellagitannins	They are absorbed from the stomach and/or the proximal small intestine. At the distal part of the small intestine and the colon, they are metabolized by microflora enzymes. They are then acted on by Phase II UGTs and/or methyltransferases before excretion in urine	Ellagic acid	[64,65]



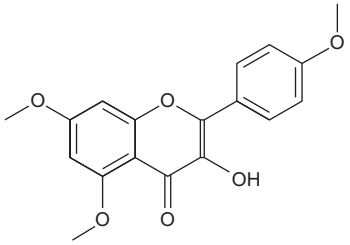
5-Hydroxy-7-methyl-1-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-7-yl (Ajugoside)



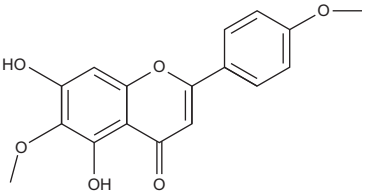
3,4–Dihydroxybenzoic acid (Protocatechuic acid)



4′-Methoxy-5,7-dihydroxy isoflavone (Biochanin)



5,7,4′-Trimethoxykaempferol



5,7-Dihydroxy-6,4′-dimethoxyflavone (Pectolinarigenin)

FIG. 6.2 Isolated phenolics from leaves of *C. volubile* leaves.

TABLE 6.4 Isolated Compounds From *C. volubile* Leaves

Isolated compounds	Common name	Fraction	Reference
5-Hydroxy-7-methyl-1-(3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yloxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-7-yl	Ajugoside	Butanol	[66]
3,4-Dihydroxybenzoic acid	Protocatechuic acid	Ethyl acetate	[67]
5,7-Dihydroxy-6,4′-dimethoxyflavone	Pectolinarigenin	Dichloromethane	[68]
4′-Methoxy-5,7-dihydroxy isoflavone	Biochanin		[69]
5,7,4′-Trimethoxykaempferol			[69]

developed an endogenous antioxidant system to defend itself against excessive free radicals, thereby mitigating oxidative stress [76]. This system consists of enzymatic and nonenzymatic antioxidants. Superoxide dismutase (SOD), catalase, xanthene oxidase, glutathione peroxidase, and glutathione reductase are among the most studied antioxidant enzymes, while glutathione, ascorbic acid, and tocopherol are the best-known nonenzymatic enzymes. They act by scavenging free radicals, thus preventing oxidative damage. In addition to the endogenous antioxidant system, the body defends itself against free radicals by utilizing phytochemicals obtained from food and other natural products.

Plant-based antioxidant activities are well documented, with phenolics playing a major role. The ability of *C. volubile* leaves to scavenge free radicals has been

TABLE 6.5 Studied Biological Activities of *C. volubile* Leaves

Biological activities	Experimental type	Target organ/cells	Mechanism	Plant part	Phenolic compounds	Nonphenolic compounds	References
Free radical scavenging/reducing power/Fe^{2+} chelating	In vitro		Scavenges DPPH, NO, OH^{-}, SO and ABTS; chelates and reduces Fe^{2+}	Leaves Flower	Chlorogenic acid Caffeic acid Rutin Isoquercitrin Quercitrin Quercetin Kaempferol Gallic acid Catechin Luteolin Apigenin Ellagic acid *p*-Coumaric acid	Triacetin 3,6-Dimethyl-3-octanol 2R-Acetoxymethyl-1,3,3-trimethtyl–4t– (3-methyl-2-buten-1-yl)-1c-cyclohexanol Stigmastan-3,5-diene	[15,16,36–38,70,71]
Antioxidative stress	Ex vivo	Brain; Liver	Increases SOD and catalase activities; decreases lipid peroxidation	Leaves	Ajugoside Chlorogenic acid Caffeic acid Rutin Isoquercitrin Quercitrin Quercetin Kaempferol Gallic acid Catechin Apigenin Ellagic acid *p*-Coumaric acid		[36,37,66]
	In vivo	Liver; Pancreas	Elevates GSH level; increases GPx, SOD, catalase activities; decreases lipid peroxidation	Leaves	Protocatechuic acid Biochanin 5,7,4′-Trimethoxykaempferol		[67,69,72]
Antiinflammatory	In silico		Molecularly interacts with COX-2 and TNF-α with negative predicted free binding energy	Flowers; Leaves	Protocatechuic acid	Triacetin 3,6-Dimethyl-3-octanol 2R-Acetoxymethyl-1,3,3-trimethtyl–4t– (3-methyl-2-buten-1-yl)-1c-cyclohexanol	[67,71]
	In vivo	Paws	Time dependently reduces linear circumference of paws				[19]
Immunomodulation	Ex vivo	Blood	Suppresses phagocytic oxidative burst in neutrophils and macrophages	Flowers; Leaves; Stem	Protocatechuic acid Pectolinarigenin	Triacetin 3,6-Dimethyl-3-octanol 2R-Acetoxymethyl-1,3,3-trimethtyl–4t– (3-methyl-2-buten-1-yl)-1c-cyclohexanol	[67,71,73]

Continued

TABLE 6.5 Studied Biological Activities of *C. volubile* Leaves—cont'd

Biological activities	Experimental type	Target organ/cells	Mechanism	Plant part	Phenolic compounds	Nonphenolic compounds	References
						Stigmastan-3,5-diene Fatty acids	
Hypolipidemic	In vivo	Serum	Reduces cholesterol, LDL-C, triglyceride and vLDL-C levels; elevates HDL-C level	Leaves	Protocatechuic acid Biochanin 5,7,4′-Trimethoxykaempferol		[21,68,69,72]
Analgesic	In vivo	Abdominal cavity	Time dependently reduces writhing count; and inhibits abdominal constriction	Leaves			[20]
Antihypertensive	In vitro		Inhibits ACE	Leaves	Biochanin 5,7,4′-Trimethoxykaempferol Chlorogenic acid Caffeic acid Rutin Isoquercitrin Quercitrin Quercetin		[36,69]
	In silico		Molecularly docks with ACE with a negative free binding energy		Biochanin 5,7,4′-Trimethoxykaempferol		[69]
Neuroprotection	Ex vivo	Brain	Inhibits AChE, BChE, and MAO	Leaves	Apigenin Caffeic acid Catechin Chlorogenic acid Ellagic acid Gallic acid Kaempferol *p*-Coumaric acid Quercetin Quercitrin Rutin		[37]
Anticancer	In vitro	Breast cancer cells (MCF-7)	Inhibits cell proliferation, arrests cell cycle progression, down-regulates MMP-9 expression, and attenuates oxidative stress	Leaves		Oleic acid Octadecanoic acid *n*-Hexadecanoic acid 6-methyl-2-heptanone	[74]

Antidiabetes	In vitro		Inhibits α-glucosidase, α-amylase	Leaves	Chlorogenic acid Caffeic acid Rutin Isoquercitrin Quercitrin Quercetin Kaempferol		[36,69]
	In silico		Molecularly docks with α-glucosidase with negative binding energy		Biochanin 5,7,4′-Trimethoxykaempferol Protocatechuic acid		[67,69]
	In vivo	Pancreas; Serum	Reduces blood glucose level; improves β-cell distribution and function, glucose tolerance; Increases serum insulin and pancreatic Ca^{2+} levels				
Toxicity	In vitro	CC-1 cells	Increases cell proliferation	Flower; Leaves	Pectolinarigenin	Triacetin 3,6-Dimethyl-3-octanol 2R-Acetoxymethyl-1,3,3-trimethtyl–4t– (3-methyl-2-buten-1-yl)-1c-cyclohexanol Stigmastan-3,5-diene	[68,71]
	In silico		Predicted toxicity class ranging from classes III–VI; Predicted inhibitors of cytochrome P (CYPs) 1A2, 2D6 and 3A4	Leaves; Stem	Biochanin 5,7,4′-Trimethoxykaempferol Protocatechuic acid Pectolinarigenin	3-Eicosyne Succinic acid, 2,4-dimethylpent-3-yl ethyl ester (2,4,6-Trimethylcyclohexyl) methanol 4-Penten-1-ol, propanoate 3-Methyl-2-butenoic acid, 2-ethylcyclohexyl ester	[67–69,73]
	In vivo	Serum	Decreases AST, ALT, ALP, and TB levels; Improves hepatic architecture in carbon tetrachloride-induced toxicity	Leaves	Biochanin 5,7,4′-Trimethoxykaempferol Protocatechuic acid		[67,69,72]

reported. Erukainure et al. [15] first reported the DPPH (1,1-diphenyl-2 picrylhydrazyl) radical scavenging activity of *C. volubile*, which he attributed to its total phenolic and flavonoid contents. This was also confirmed by Adefegha and Oboh [36,70], Ogunwa et al. [16], and Molehin et al. [38]. DPPH is a readily available commercial transient free radical used in the study of scavenging potentials of natural products [77]. Ogunwa et al. [16] and Molehin et al. [38] also reported the ability of the leaf extract to scavenge another commercial free radical, ABTS (2, 2-azinobis (3-ethylbenzo-thiazoline-6-sulfonate)). The leaves have also been reported to scavenge $O_2^{\bullet -}$, $OH^{\bullet}$, and H_2O_2 [16,36,38,70]. Molehin et al. [38], and Adefegha and Oboh [36] attributed these activities to the identified phenolics (Table 6.2). Phenolics have been recognized as strong antioxidants [7]. This has been attributed to their hydroxyl groups, which donate an electron to the free radical. This leads to the formation of a more chemically stable product, as well as interaction of the hydroxyl groups with π-electrons of the benzene ring, leading to delocalization of the radical [7]. These reactions quench the effect of the free radical, thus attenuating the oxidative damage in the cells.

The antioxidant properties of phenolics have also been attributed to their ability to chelate metal ions involved in redox metabolism [78]. Metals such as iron (Fe), copper (Cu), chromium (Cr), and cobalt (Co) have been shown to exacerbate the formation of $O_2^{\bullet -}$ and $OH^{\bullet}$ via the Fenton reaction, leading to peroxidation of the membrane lipids and fragmentation of DNA [79]. Adefegha and Oboh [36,70] reported the antiperoxidative effect of the phenolic rich extract of *C. volubile* leaves against Fe^{2+}-induced lipid peroxidation in rat liver and pancreas. Oboh et al. [37] further reported a similar effect in rat brain. The reducing and chelating properties of *C. volubile* leaves on iron have also been reported [15,36,38,70], thus demonstrating the protective potential of the leaves against iron toxicity. The prooxidant properties of iron can be attributed to its ability to exist in both ferrous (Fe^{2+}) and ferric (Fe^{3+}) ionic states [80].

The ability of *C. volubile* leaves to mitigate oxidative stress in disease states and toxicity has also been reported. Erukainure et al. [74] reported an increased GSH level, SOD and catalase activities, and decreased malondialdehyde (MDA) in breast cancer cells treated with the leaf extract. The antioxidative protective properties of the leaf extract were also reported in diabetic oxidative pancreas and serum [69], which was attributed to the isolated flavones, biochanin and 5,7,4′-trimethoxykaempferol. Molehin et al. [72] reported an aggravative GSH level, SOD, catalase, and GPx activities with a concomitant reduced MDA level in carbon tetrachloride-induced hepatotoxicity. These activities can be attributed to the ability of the hydrophobic benzenoid rings and hydroxyl groups of phenolics to interact with proteins (Fig. 6.3) [7].

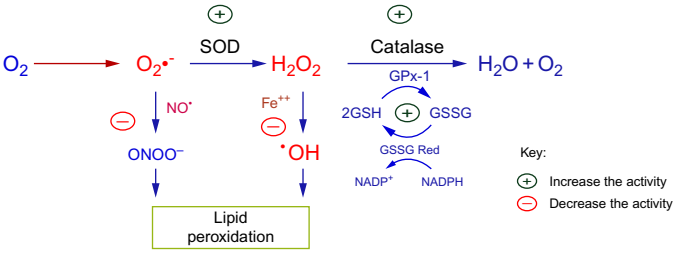


FIG. 6.3 Antioxidative mechanism of *C. volubile* leaves.

4.2 Antihypertensive Activity

Hypertension is the most common cardiovascular disease and has been recognized as a major contributor to global morbidity and motility, thus representing a global health epidemic [36,81,82]. It is characterized by high blood pressure, which could lead to other cardiovascular complications, kidney failure, neurodegeneration, and other complications [81,83].

Activation of angiotensin-I converting enzyme (ACE) has been implicated in the progression of hypertension owing to its regulation [84]. It is activated after stimulation of the angiotensin 1 receptor, leading to an increased level of angiotensin II, a potent vasoconstrictor [70,84]

Adefegha and Oboh [36,70] reported the ability of *C. volubile* leaves to inhibit ACE, which they attributed to the total phenol content and identified phenolics of the extracts (Table 6.2). Erukainure et al. [69] further confirmed this ability and attributed it to the isolated flavones, biochanin and 5,7,4′-trimethoxykaempferol. The molecular interactions of these compounds with ACE were further investigated in silico: 5,7,4′-trimethoxykaempferol interacted with ACE by forming a hydrogen bond with the residues HIS 387, TYR 523, LYS 511 [69], while biochanin interacted via HIS 383, GLU 411, HIS 387, TYR 523 [69]. These results demonstrate the antihypertensive properties of *C. volubile* leaves, which can be attributed to the phenolic contents (Fig. 6.4).

An inverse relationship has been correlated with increased phenolics intake and decreased hypertension [82,85]. Phenolics have been shown to inhibit ACE by chelating its hydroxyl groups with the Zn^{2+} moiety of ACE [81]. Structure-inhibition studies on their relationship revealed an increased inhibitory activity with increasing hydroxyl groups and less structural steric hindrance [81].

4.3 Antidiabetic Activity

Diabetes mellitus (DM) is a global epidemic with 425 million people reported with the disease in 2017, and sub-Saharan Africa (SSA) accounted for 3.76% [86]. It is a metabolic disease that affects carbohydrate, fatty acid, and lipids metabolism owing to inability of the pancreatic

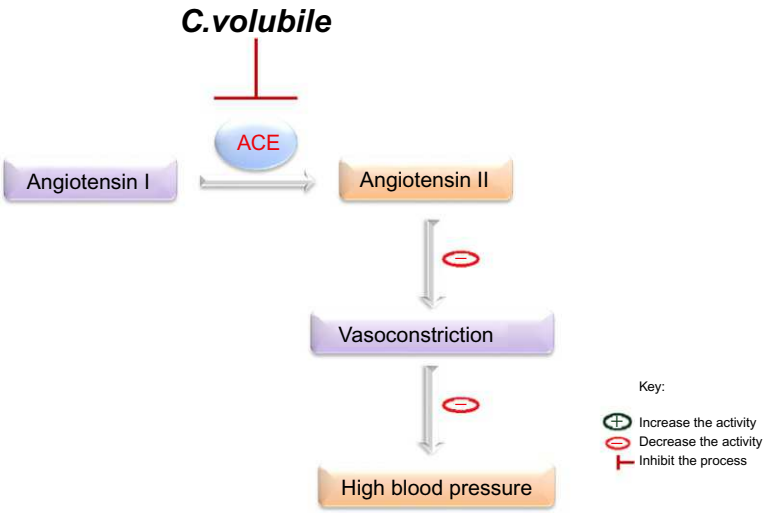


FIG. 6.4 Antihypertensive activity of *C. volubile* leaves.

β cells to secrete insulin (type 1 diabetes) or the body not being able to effectively utilize the insulin secreted (type 2 diabetes [T2D]) [87]. Both types of diabetes are characterized by increased blood glucose, with T2D being the most prevalent [86]. Insulin resistance and β-cell dysfunction have been recognized as the hallmarks of T2D, leading to chronic hyperglycemia [88].

Owing to cost of treatment, there has been increased interest in the use of medicinal plants for the treatment and management of T2D. The leaves of *C. volubile* have been investigated for their antidiabetic properties. Adefegha and Oboh [36] investigated the ability of its phenolic rich extract to inhibit α-glucosidase and amylase, key enzymes linked to T2D. They concluded the extract exhibited a dose-dependent inhibitory effect of the enzymes, depicting an anti-type 2 diabetic potential. The observed activity was attributed to the HPLC-identified phenolics (Table 6.2). Similarly, Erukainure et al. [69] reported the inhibitory activities of the methanol extract and its fractions on α-glucosidase, which they attributed to biochanin and 5,7,4′-trimethoxykaempferol. Erukainure et al. [67,69] also investigated the molecular interactions of protocatechuic acid, biochanin and 5,7,4′-trimethoxykaempferol with α-glucosidase in silico. Protocatechuic acid interacted with the active site residues ASP 146 (4.3 Å), GLY 149 (3.6 Å), and LYS 164 (5.3 Å) via hydrogen bonding with the two meta and para oxygen atoms of its two free hydroxyl groups and oxygen of —OH of the —COOH [67]; 5,7,4′-trimethoxykaempferol interacted with residues ASP 203, ARG 526, and PHE 450, with the —OH and —COOH groups donating hydrogen bonds to ASP 203 and ARG 526, respectively [69]; while biochanin interacted with residues ARG 526, TRP 406, ASP 443, and HIS 600, with the —OH and —COOH groups donating hydrogen bonds to ASP 443 and ARG 526, respectively [69]. This reflects a structure-activity relationship. Unsaturation at the 2,3-position and the presence of the 4-carbonyl group have been reported to increase the inhibitory property of phenolics on α-amylase [89]. Similarly, hydroxylation and galloylation have been shown to improve their inhibitory effect [8]. Inhibition of α-glucosidase and α-amylase has been employed by most antidiabetic drugs, such as acarbose, in the reduction of blood glucose level [90]. These enzymes break down dietary carbohydrate to glucose, thereby increasing the postprandial blood glucose level. Their inhibition would therefore reduce the breakdown of dietary carbohydrate, thus reducing blood glucose level.

Erukainure et al. [67,69] reported the ability of the ethyl acetate and dichloromethane (DCM) fractions to reduce blood glucose level, and they attributed it to the isolated phenolics, protocatechuic acid, biochanin and 5,7,4′-trimethoxykaempferol. The fractions improved β-cell function and increased serum insulin level. Further investigation with the DCM fraction revealed an improved distribution of pancreatic β-cells as well as glucose tolerance [69]. The Ca^{2+} level of the pancreatic tissues was also increased (Fig. 6.5).

4.4 Immunomodulatory and Antiinflammatory Activity

Polymorphonuclear neutrophils (PMNs) are among the known phagocytes and the body's first line of defense against pathogen invasions [91]. Their ability to modulate both pro- and antiinflammatory activities indicates a major influence on the immune system [92]. They (PMNs) release large volumes of ROS via NADPH oxidase activity upon stimulation, a process known as respiratory oxidative burst [67,68,71]. This process has been recognized as an efficient microbicidal mechanism.

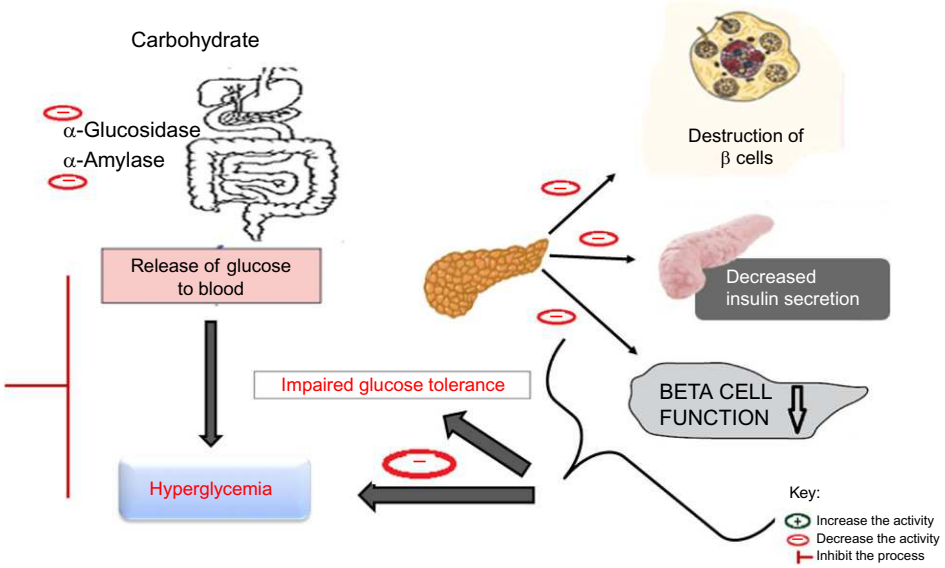


FIG. 6.5 Antidiabetic activity of *C. volubile* leaves.

Excessive oxidative burst has, however, been implicated in the recruitment of inflammatory cytokines, which leads to pathogenic inflammatory processes and related disorders with physiological consequences [92].

Over the years, medicinal plants have been employed as immunomodulators due to their antioxidant and anti-inflammatory properties [68]. Erukainure et al. [67,71,73] reported the ability of *C. volubile* leaves, stem, and flowers to quench PMN respiratory oxidative burst, thereby exhibiting an immunomodulatory activity. They attributed the activity to the isolated phenolics, protocatechuic acid and pectolinarigenin, which also displayed potent antioxidative burst activity. The authors demonstrated the ability of pectolinarigenin to suppress T-cell proliferation, portraying an antiinflammatory potential [68]. The leaves have been reported to reduce the linear circumference of rat paws, which also portrays an antiinflammatory activity [19].

Computational simulation of protocatechuic acid with T-cell showed that the para hydroxyl group hydrogen bonded to GLU 406, while the hydroxyl group (—OH) of the carboxylic acid (—COOH) bonded to PRO 437. Dual hydrogen bond interactions were observed on the carbonyl group with HIS 405 and HIS 415. There was a metal bond between the ligand and ZN 1. Electrostatic, covalent, and van der Waals interactions were also observed between the ligand and with residues GLY 349, LEU 350, HIS 409 and TYR 436 [67]. This corresponds with previous reports that the scavenging activities of phenolics are greatly influenced by the number of hydroxyl substituents [93]. Ciz et al. [93] further proposed that their C-2,3 double bond might play an influential role in the inhibition of phagocytic ROS production (Fig. 6.6).

4.5 Hypolipidemic Activity

Hyperlipidemia is a medical condition characterized by elevated levels of serum cholesterol and/or low-density lipoprotein (LDL) cholesterol as well as alteration of other lipid parameters such as triglycerides, very low-density lipoprotein (vLDL) cholesterol and high-density lipoprotein (HDL) cholesterol [94,95]. It has been recognized as a major cardiovascular risk factor, which could give rise to cardiovascular disease (CVD) and other atherosclerosis-related conditions [96].

It is divided into two subclasses, primary and secondary hyperlipidemia. The former is genetically related, while the latter arises from complications from other disease conditions [96]. Medicinal plants and diets have been used in the treatment and control of secondary hyperlipidemia; this has been attributed to their ability to modulate blood lipid profile, particularly reducing blood cholesterol and increasing HDL levels [94].

The hypolipidemic properties of the leaves of *C. volubile* have been reported. Akinpelu [21] reported the ability of the leaf ethanol extract to lower plasma cholesterol, LDL, vLDL, and triglyceride levels, as well as increase HDL level in Poloxamer-407-induced hyperlipidemic rats. Molehin et al. [72] reported similar effects in carbon tetrachloride-induced hepatotoxicity. Erukainure et al. [67,69] reported the ability of the leaf fractions to mitigate increased serum cholesterol, LDL, vLDL, and triglyceride levels, while increasing HDL level concomitantly in type 2 diabetic rats. This was attributed to the isolated phenolics, protocatechuic acid, biochanin, and 5,7,4′-trimethoxykaempferol [67,69].

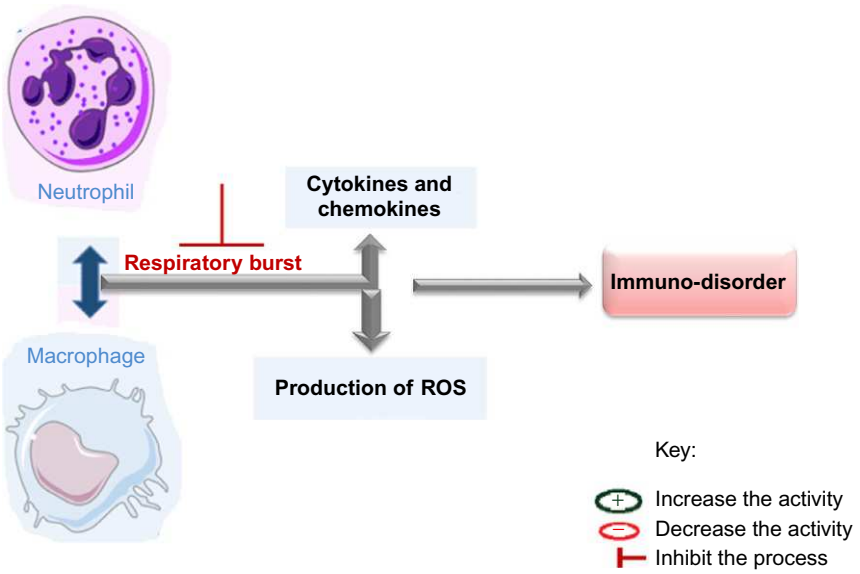


FIG. 6.6 Immunomodulatory activity of *C. volubile* leaves.

4.6 Toxicity

The safety of medicinal plants has been a major concern, owing to issues of standardization, characterization, and preparation [97,98]. Plants consist of a cocktail of phytochemicals that are extractable based on the type of extracting solvent. Different plant extracts exert different biological functions, which can be toxic [97].

The toxicity of different extracts of *C. volubile* leaves has been reported. Erukainure et al. [67,68,71] reported the cytotoxic effect of the fractions and isolated compounds of the leaves and flowers. They showed that DCM fractions were cytotoxic against CC-1 cells but the isolated compounds, biochanin, 5,7,4′-trimethoxykaempferol, protocatechuic acid, and pectolinarigenin, had no cytotoxic effect.

The toxicity of these isolated compounds was also predicted in silico. The toxicity classes of biochanin, 5,7,4′-trimethoxykaempferol, and pectolinarigenin were predicted to be V, while protocatechuic acid was IV. These classes further showed the relative safety of the compounds. Biochanin and 5,7,4′-trimethoxykaempferol were predicted as potent inhibitors of CYPs 1A2, 2C9, 2D6, and 3A4, thus suggesting caution in cousage with other prescribed drugs metabolized by the enzymes [69].

In vivo studies showed the ability of the leaf extract to lower serum hepatic (AST and ALT) and renal (urea) biomarkers level in type 2 diabetic rats [69]. Molehin et al. [72] reported similar effects in carbon tetrachloride-induced hepatotoxic rats. They further reported the ability of the methanol extract to improve hepatic architecture in carbon tetrachloride-induced hepatoxicity [72].

5 PROPOSED MECHANISM OF ACTION OF *C. VOLUBILE*

Based on the biological activities studied so far, *C. volubile* may bring about its health beneficiary effect by [1] modulation of the redox homeostasis, leading to decreased oxidative stress, which in turn decreases the pathogenesis and complications of diabetes, hepatotoxicity, cancer and neurodegeneration; [2] down-regulation of MMP-9 expression and concomitant arrest of the cell cycle, leading to decreased proliferation of cancer cells; [3] inhibition of ACE activity leading to decreased production of angiotensin II, thereby inhibiting vasoconstriction of the blood vessels; [4] inhibition of oxidative burst and inflammatory cytokines, thereby inhibiting immune disorders and inflammations implicated in the pathogenesis of pancreatic β-cell dysfunction and insulin resistance, which in turn leads to increased insulin production and glucose tolerance; [5] inhibition of key carbohydrate catabolic enzymes leading to decreased postprandial blood glucose level; and [6] inhibition of cholinergic and monoaminergic enzymes implicated in neurodegeneration.

This is summarized in Fig. 6.7.

6 CONCLUSION

Studies so far on *C. volubile* authenticate its folkloric use in the management and treatment of various ailments. These health benefits can be attributed to the phenolics identified so far in the plant. However, cautions should be considered when using the plant with other

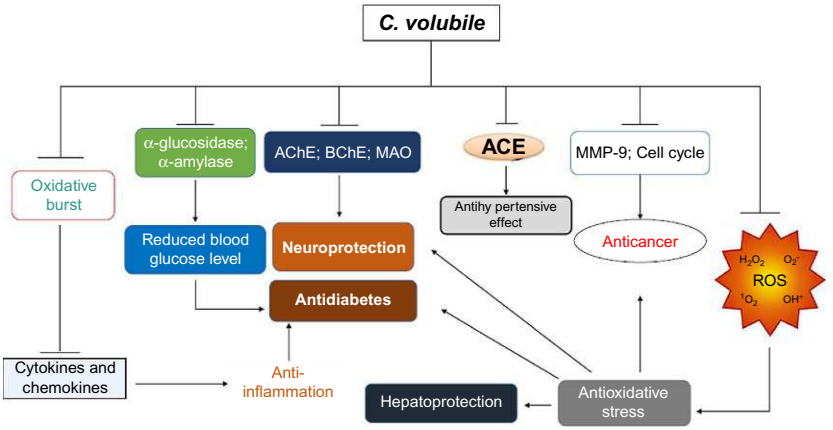


FIG. 6.7 Proposed mechanism of action of *C. volubile* and its medicinal activities.

drugs metabolized by CYPs 1A2, 2C9, 2D6, and 3A4. More studies should be conducted on the molecular mechanisms behind the pharmacological properties of the plant.

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Conflict of Interest

The authors report no conflict of interest.

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CHAPTER

7

Eryngium campestre L.: Polyphenolic and Flavonoid Compounds; Applications to Health and Disease

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Abbreviations

A 40	beta-Amyloid Peptide A 40
A 42	beta-Amyloid Peptide A 42
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ATCC	American Type Culture Collection
Cd	cadmium
Co	cobalt
Cr	chromium
Cu	copper
DPPH	1,1-diphenyl-2-picryl-hydrazyl
Fe	iron
FRAP	ferric reducing antioxidant potential
GAE	garlic acid
IC_{50}	inhibitory concentration 50%
K	potassium
LDH	cytosolic enzyme lactate dehydrogenase
Mn	manganese
MRSA	methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
Na	sodium
NO	nitric oxide
Pb	lead
QE	quercetin
TNF-α	tumor necrosis factor-alpha
UPLC	ultra performance liquid chromatography
UV	ultraviolet
VCAM1	vascular cell adhesion molecule 1
Zn	Zinc

1 INTRODUCTION

Before the availability of synthetic drugs, humans were completely dependent on medicinal herbs for prevention and treatment of diseases. The use of medicinal herbs for curing diseases has been documented in the history of all civilizations. Plants were used in crude forms, such as expressed juice, powder, decoction, or infusion [1].

Commonly known as "Tabelwadha" or "Chouk el Abiod," *Eryngium campestre* L. (Field Eryngo), belonging to the family Apiaceae, is an important plant with several ethnomedicinal and nutritional values. Therefore it is used extensively worldwide as food as well as folklore remedies to treat various diseases. The plant is a perennial pale green herbaceous flowering plant growing to 60 cm. It grows in dry, rough grassland near the coast, roadsides, and waste places in North Africa and the southwestern areas of Asia and North America [2]. It is widely distributed in North Africa and Asia, where roots and aerial parts of this plant have long been used in traditional medicine for several ailments, especially to treat inflammation diseases. In Algerian folk medicine, it is used against urinary infections, cough, tonsillitis, and whooping cough. In addition to the widespread use of this herb for food and as an ethnomedicinal agent, many studies and reports have ascribed various biological and/or pharmacological activities to this plant and previous phytochemical investigations on *E. campestre* (roots and aerial parts) have revealed the presence of bioactive secondary metabolites (SMs), including saponins, phenolic acids, flavonoids, coumarins, and essential oil.

Because of the interesting biological effects of *Eryngium campestre* L. extracts and isolated compounds, the present study is carried out to summarize and to highlight the present state of our knowledge of its phytochemical, pharmacological, and biological properties and possible relevance for human health.

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2 ABOUT THE PLANT

Eryngium Campestre L. has other names around the word such as Chardon roulant, common eryngo, cardo panical, and Bo⊠adikeni. It is one of the ruderal, prickly plants that grow in lean, stony, and sunny pastures, wood edges and mantles, and dry meadows, on calcareous substrates, from 0 to 1600 m above sea level, often found in abandoned fields, roads, and margins of cultivated fields [2,3]. The plant is a glaucous perennial pale green herbaceous flowering plant growing to 60 cm [2]. *Eryngium campestre* L. is a hemicryptophyte perennial herb, with a taproot and erect hairless glaucescent stem, which is woody at the base. The leaves are spiny, thorny, more complex at the base and simple to the apex [2,3]. It flowers from June to September [3].

3 DISTRIBUTION

Eryngium campestre: this herbaceous, xeromorphic plant species shows a very peculiar distribution pattern. Overall, it is distributed in the Mediterranean (from Morocco to Portugal and eastwards up to Iran and Afghanistan), and in Central Europe, with South England as northwestern border. The northern German lowlands represent the northern distribution border, and the northeastern distribution border lies in Central Russia [4]. It grows in the southwestern areas of Asia, North Africa, Holland, Britain, and North America [2], in Spain, France, Balkan Peninsula, and in Africa and Asia as well [5].

4 TRADITIONAL USES

It is clear that traditional medicines have played an important role in the provision of health care for thousands of years and they continue to provide new remedies to humankind in many developing countries. Their use is also significant in developed countries. Worldwide, there is a history of using plants to supply food, fuel, cosmetics, and medicine. It is estimated that 90% of the African population are still dependent on traditional medicine [6]. However, in the middle of the 20th century, the use of medicinal plants was reduced because researchers favored the use of synthetic chemicals for curing diseases. Nevertheless, now the trend is changing and people favor medicinal plants, as they contain natural products, which are effective, chemically balanced, and have fewer side effects as compared to synthetic chemicals [7].

The genus *Eryngium* (Apiaceae) is used in traditional medicine for different therapeutic purposes [8]. Some species, such as *Eryngium foetidum* L., *Eryngium maritimum* L., *Eryngium campestre* L. and *Eryngium creticum* L., have been used in traditional medicine worldwide as a folk remedy for the treatment of various inflammatory disorders [9,10]. *Eryngium campestre* and *E. maritimum* were in the past popular medicinal herbs used in folk medicine for their antiscorbutic, diaphoretic, diuretic, expectorant, antiinflammatory, and aphrodisiac properties. Nowadays, European herbal medicine recommends them as diuretics for treating renal disorders (cystitis, urethritis, chronic prostatitis, painful urination) and for preventing kidney stone formation [9,11].

In Turkish folk medicine, various species of the plant are used for a wide range of ailments; particularly, roots are used against various inflammatory disorders, edema, sinusitis, urinary infections or inflammations, etc., and snake or scorpion bites or goiter; roots and leaves for infertility and herbs for wound healing, as well as food while fresh [8]. Infusions of aerial and root parts of this species are used in folk remedies as antitussive, diuretic, appetizer, stimulant and aphrodisiac [12], and against scorpion venom [13]. In North America, the genus has an ethnomedicinal use as an antitoxin and antiinflammatory agent; also *Eryngium* has been reported as a topical antidote to scorpion envenomation [14].

The traditional uses recorded for this herb are numerous and mainly medicinal. Table 7.1 recaps the different uses of *E. campestre* around the world; according to Kikowska et al. [28], *E. campestre* is used in the treatment of many diseases instead of *E. maritimum*. The medicinal parts are dried roots, leaves, and flowers. Eryngo root is administered in tea mixtures, extracts, decoctions, liquids, and tinctures for the treatment of bladder and kidney stones, renal colic, kidney and urinary tract inflammation, and edema. It is also used for coughs, bronchitis, skin and respiratory disorders. The herb of *E. campestre* is administered as an extract and in homeopathic dilutions in the treatment of urinary tract infections and as an adjuvant to treat inflammation [28]. The dried leaves, flowers, and roots are used to make an herbal tincture that assuages dysuria [29].

Beside its medicinal use, field eryngo is consumed as food; *Eryngium campestre* L. new leaves are stored in brine and eaten as a vegetable. It is sweet smelling and aromatic. It is able to warm, and expels urine and the menstrual flow. Taken as a drink, it dissolves griping and gaseousness. It is good with wine for liver complaints, those bitten by venomous creatures, and as an antidote for those who have taken a deadly drink. It is taken in a drink for the most part with one teaspoonful of pastinaca seed. It is said that, used as a personal ornament or rubbed on someone, it dissolves tubercles [growths]. The root (taken as a drink with honey water) is said to cure tetanus and epilepsy [30].

TABLE 7.1 Ethnomedicinal Uses of *Eryngium campestre* L. Around the World

Country	Parts used	Ethnobotanical uses
European folk medicine	Aerial and root parts	*Eryngium campestre* L. is one of the plants found in four renaissance herbals (books were among the most important European herbals of the 16th and 17th centuries: Matthioli, 1590; Tabernaemontanus, 1591; Tabernaemontanus, 1687; and Zwinger, 1696) to treat malaria (internal application) (a).Infusions of aerial and root parts of *Eryngium* species have been used as antitussive, diuretic, appetizer, stimulant, and aphrodisiac [15]. *E. campestre* has been used in European herbal medicine as an infusion to treat whooping cough, kidney and urinary tract inflammation [16]
Turkey	Aerial and root parts	*Eryngium campestre* is known in Turkish folk medicine as *Bogadikeni*. It has been used in folk medicine as an infusion to treat cough, whooping cough, urinary infections, disturbed functions of kidney, increased urine secretion, eliminating out stones and sand from kidney and bladder, against water-retaining and other conditions in urinary tract, for regulation of the function of the prostate. The root is known as antispasmodic, aromatic, diaphoretic, diuretic, expectorant, galactogogue, and stimulant, as antitussive, diuretic, and appetizer [17–19]. It promotes free expectoration and is very useful in the treatment of debility attendant on coughs of chronic standing in the advanced stages of pulmonary consumption. There are no known hazards of *Eryngium campestre* [4]; young shoots and roots are edible parts of the plant which can be cooked and used as an asparagus substitute and as an easily digested vegetable [4]; fresh root and stem has been used as a food (b, c). Aqueous extract of the roots of *Eryngium campestre* is used orally and applied as a poultice for a remedy of scorpion stings in the Balıkesir-Turkey. Similar utilizations were found in the old documents from the medieval ages, such as against wounds and burns, pains, hemorrhoids, and sexual diseases [20–22]
Romania	–	*Eryngium campestre* was in the past a popular medicinal herb used in folk medicine for its antiscorbutic, diaphoretic, diuretic, expectorant, antiinflammatory and aphrodisiac properties. Nowadays, European herbal medicine recommends it as diuretic for treating renal disorders (cystitis, urethritis, chronic prostatitis, painful urination) and for preventing kidney stone formation [11]. *E. campestre* is also known for antiepileptic and expectorant characteristics [23]
Bulgaria	Roots	Infusion of roots is used as prostatitis, diuretic and spasmolytic (d)
Italy	Leaves, roots, young buds	Leaves, young buds can stimulate appetite, used as an aperitif, laxative and as food in salads [24]. Tinctures and decoctions of roots are used as an antiedema, diuretic, cholagogue, choleretic, and to promote perspiration (d), milk production, emmenagogue, urinary infection [25]. Decoction of leaves for treating eye inflammations and nowadays leaves are used as food [26]
Iran	Aerial and root parts	*E. campestre* a notable therapeutic plant, implantations of the airborne and root parts are utilized as a diuretic, antitussive, sexual enhancer, stimulant, and hors d'oeuvre (e). Fresh stem are used against kidney stones, as carminative and appetizer (f)
Palestine	Roots	Roots are used in folk medicine for treatment of bladder stones, dropsy, skin disorders, diuretic, urinary tract infections, jaundice, abdominal colic, delirium, whooping cough; also used for promoting menstrual discharge as decoction, as well as its infusion used as appetizer, stimulant, antitussive, and aphrodisiac [2]
Medieval Ottoman al-Sham	–	The plant is used to treat hemorrhoids and sexual diseases, pains, wounds and burns (g)
Algeria	Roots	Fresh roots of *E. campestre* was one of plant species reported as medicinally used by Hilton-Simpson, used against toothache (h). Roots of this plant (fresh, decoction) are used against urinary infections, cough, tonsillitis, and whooping cough [27]
Spain	Aerial and root parts Branches, inflorescences	*Eryngium campestre* is a species with greater therapeutic uses (external and oral use of branches and roots), as most of the remedies are related to the treatment of unimportant ailments, referring to disorders of the skin, and the respiratory and digestive systems, rheumatism and inflammations, injury and ulcer, toothache, pancreatic problems, hemorrhoids, skin and subcutaneous tissue problems (i). Branches have an external use as they are applied as a poultice on stings, very effective to alleviate (j); aerial part decoctions are used in the treatment of edema; inflorescence to treat sores and roots decoctions are used in the treatment of circulation problems, digestive disorders, hypertension (freshly ingested), hemorrhoids, and eczema (k)

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5 PHYTOCHEMISTRY

Plants synthesize a vast range of organic compounds that are traditionally classified as primary and secondary metabolites, although the precise boundaries between the two groups can in some instances be blurred. Primary metabolites are compounds that have essential roles associated with photosynthesis, respiration, and growth and development. Other phytochemicals, many of which accumulate in surprisingly high concentrations in some species, are referred to as secondary metabolites [31].

Although ignored for a long time, their function in plants is now attracting attention, as some appear to have a key role in protecting plants from herbivores and microbial infection, as attractants for pollinators and seed-dispersing animals, as allelopathic agents, UV protectants, and signal molecules in the formation of nitrogen-fixing root nodules in legumes. Secondary metabolites are also of interest because of their use as dyes, fibers, glues, oils, waxes, flavoring agents, drugs, and perfumes, and they are viewed as potential sources of new natural drugs, antibiotics, insecticides, and herbicides [31,32]. In most plants, synthesis and accumulation of SMs is regulated in space and time. As a rule, vulnerable tissues are defended more than old, senescing tissues. Organs that are important for survival and multiplication, such as flowers, fruits, and seeds, are nearly always a rich source of defense chemicals. The specific localizations of SMs make sense if their role as defense and/or signal compounds is accepted [33].

At the end of the 20th century, from the new 3500 novel chemical structures identified, 2619 were isolated from superior plants. In the United States, 25% of the market is based on plant-based compounds. Most of the prescribed drugs of plant origin were initially used in popular medicine [34]. So far, over 4000 such phytochemicals have been recognized and about 150 phytochemicals have been studied in detail [32].

Based on the review of phytochemical studies performed on *Eryngium* species up to now, this genus contains various bioactive compounds. The reported phytochemicals in genus *Eryngium* are triterpenoid saponin, flavonoids (namely kaempferol and quercetin glycosides), phenolic acids (rosmarinic acid, chlorogenic acid, and caffeic acid) [10,28,35], coumarin, acetylenes, steroids [13,36], as well as saponins [37], terpenoids, monoterpenes and sesquiterpenes [17,38], oligosaccharides [13], and essential oil [39,40].

Phytochemical studies show that *Eryngium campestre* L. contains a wide variety of active compounds that impart medicinal properties of this medicinal plant. The constituents of *E. campestre* vary depending on the place of origin and whether the studied parts are roots or aerial parts. The chemistry of field eryngo has been the subject of various studies since the 20th century [41,41a] up to the present day [23a,28,40]. Several studies concerning the chemical compositions of solvent extracts and essential oil from *E. campestre* reported the presence of phenolic acids, flavonoids, essential oils, acetylenes, saponins, steroids, terpenoids, and coumarins as the major phytochemical groups.

In addition to the presence of secondary metabolites, a study conducted by Nebija [4] reported that aerial parts and roots of *Eryngium campestre* L. from Kosovo contained mineral salts, namely eight microelements (Cd, Co, Cr, Cu, Fe, Mn, Pb and Zn) and two macroelements (Na and K). It is remarkable that different parts of *E. campestre* (aerial parts and roots) accumulate different amounts of investigated minerals [4].

5.1 Polyphenol Compounds

Polyphenols, one of the most widely occurring groups of biologically active substances, are distributed in plants as secondary metabolites. These substances are of considerable physiological and morphological importance in plants, providing color and flavor to different plant parts and also playing an important role in growth and reproduction, resistance against various microbial pathogens, and protection against radiations and toxins. Many of the recent studies have mainly focused on polyphenols and their diverse biological effects [42–44]. Polyphenols are natural compounds occurring in plants, including foods such as fruits, vegetables, cereals, tea, coffee, and wine [45]. Polyphenols have attracted increasing interest in recent years because of evidence of their beneficial health effects on humans, as a high level of such compounds may reduce the risk of development of several diseases due to their antioxidant power [43], their abundance in the diet, and their credible effects in the prevention of various oxidative stress-associated diseases [46]. Their regular consumption has been associated with a reduced risk of a number of chronic diseases, including cancer, cardiovascular disease, and neurodegenerative disorders [47,48].

In the human body, polyphenols are antioxidants and they exhibit a wide range of physiological activities, such as antidiabetic, anticancer, antiinflammatory, cardioprotective and vasodilatory, osteoprotective, neuroprotective, antiasthmatic, antihypertensive, antiaging, antiseptic, cerebrovascular protection, antiallergenic, antiartherogenic, cholesterol lowering, hepatoprotective, antithrombotic, antifungal, antibacterial, and antiviral properties [42,49,50].

The total phenolic content of the methanol extracts of the leaves and the flowers of *E. campestre* (growing in Turkey) was determined by Güneş and his collaborators using the Folin-Ciocalteu reagent method. The amount of total phenolic compounds found in the methanol

extract from flowers of *E. campestre* was 116.69 mg GAE/L of plant extract and 109.62 mg GAE/L of plant extract in the leaves extract [22]. In another study realized by Bouzidi et al. [27], the total phenolic content has been established using the same method as the butanol, ethyl acetate and water extracts of both roots and aerial parts of Algerian *E. campestre*; the results showed that the polyphenol content depends on the polarity of the extract and the nature of the studied part. However, the highest concentration was recorded in the ethyl acetate extract of aerial parts: 27.77 μg GAE/mg extract [27].

5.1.1 Flavonoids

Flavonoids are a broad class of low molecular weight, secondary plant metabolites characterized by the benzo-*γ*-pyrone structure. They are widely distributed in fruits, vegetables, nuts, leaves, seeds, bark, and flowers of plants and are an integral part of the human diet [51–54]. In plants, these compounds afford protection against ultraviolet radiation, pathogens, and herbivores [54]; flavonoids comprise the most abundant class of plant polyphenols with more than 6000 structures identified [55].

Besides their relevance in plants, they exhibit interesting and beneficial medicinal effects on human health. They have been reported to exhibit a wide range of biological effects, including antioxidant [54,56,57], antibacterial, antiviral [51,57], antiinflammatory [56,57], hepatoprotective, antithrombotic [57], antiallergic [51,57], vasodilatory, immune-stimulating, anticancer [56,57], antidiabetic, and antihyperlipidemic activities [58]. In addition, flavonoids inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and the activity of enzyme systems, including cyclooxygenase and lipoxygenase [47,51,54].

These compounds have been reported to possess a wide range of activities in the prevention of common diseases, including coronary heart disease [59], cancer, neurodegenerative diseases, Alzheimer's disease, and Huntington's disease; they are reported to decrease the risk of stroke, epilepsy [60], and gastrointestinal disorders and prevent angiogenesis and tumor cell invasion [47]. Flavonoids also improve endothelial function and reduce blood pressure [60,61], among other benefits.

In a comparative study, the total concentrations of flavonoids of extracts from both roots and aerial parts of *E. campestre* (Kosovo) were determined by employing the aluminum chloride colorimetric method. The results of determination of total flavonoid content in aerial parts presented percentages of total flavonoids (0.12%–0.14%) expressed as total quercetin [4]; for the same purpose another study was carried out on the total amount of flavonoids in three extracts of roots and aerial parts (butanol, water and ethyl acetate). However, maximum flavonoid content was obtained from aqueous extract of *E. campestre* roots (7.543 μg QE/mg extract) [27]. In the study accomplished by Güneş et al. [22], *E. campestre* flower extract had higher phenolic contents, 110.58 mg QE/L of plant extract, when 108.37 mg QE/L of plant extract was the total amount of the flavonoid contents found in the methanol extracts from leaves [22].

In a phytochemical investigation on *Eryngium campestre* L. that grows in Egypt by Hawas et al., eleven flavonol compounds were isolated and identified as three quercetin glycosides: quercetin 3-*O*-β-glucoside, quercetin 3-*O*-β-glucuronide-4′-methylether, and rutin; five isorhamnetin glycosides: isorhamnetin 3-*O*-β-glucoside, isorhamnetin 3-*O*-β-galactoside, isorhamnetin 3-*O*-α-rhamnoside, isorhamnetin 3-*O*-β-rutinoside, isorhamnetin 3-*O*-β-glucoside-7-*O*-α-rhamnoside; and three myricetin glycosides: myricetin 3-*O*-β-glucoside-3′-methylether, myricetin 3-*O*-β-glucoside-4′-methylether, and myricetin 3-*O*-β-galactoside-4′-methylether [13,16].

Hohmann et al. [62] reported the isolation and characterization of a new acylated flavonol defined as kaempferol 3-*O*-β-D-(2′-*p*-*Z*-hydroxycinnamoyl)-glucopyranoside and its trans-isomer Kaempferol 3-*O*-β-D-(2′-*p*-*E*-hydroxycinnamoyl)-glucopyranoside, together with five known flavonoids: kaempferol 3-*O*-β-D-glucoside-7-*O*-α-L-rhamnoside, rutin, kaempferol, and quercetin and isorhamnetin from the dried above-ground parts of the plant (Hungary) [16,17,28,62]. Quercetin-3-*O*-rutinoside (rutin), kaempferol 3,7-di-*O*-α-L-rhamnopyranoside, kaempferol 7-*O*-α-L-rhamnopyranoside, isoquercitrin, luteolin 7-*O*-β-D-glucopyranoside, and quercitrin have been identified in the aerial parts of *Eryngium campestre* L. from Germany [16,17,63].

Astragalin, naringenine 7-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-*β*-D-glucopyranoside [17] and tiliroside [16] were also identified in the aerial parts of the plant. Two flavonoids were isolated for the first time from the aerial parts of *E. campestre* growing in Algeria: 3′-*O*-methyl quercetin 3-rutinoside or isorhamnetin 3-*O*-rutinoside or narcissin and 4′-methoxy-3-(β-D-glucopyranosyl) kaempferol [64].

5.1.2 Phenolic Acids

Like flavonoids, phenolic acids are secondary metabolites extensively spread throughout the plant kingdom. Phenolic compounds confer unique taste, flavor, and health-promoting properties found in vegetables and fruits [65].

Phenolic acid compounds and their functions have been the subject of a great number of agricultural, biological, chemical, and medical studies [65]. Phenolic acids may be about one-third of the phenolic compounds in the human diet. It is known that these substances and their esters have a high antioxidant activity, especially hydroxybenzoic acid, hydroxycinnamic acid, caffeic acid, and chlorogenic acid, although other characteristics contribute to the antioxidant activity of phenolic acids and their esters [43]. In addition, phenolic acids have been reported in the literature to exert an antibacterial effect;

bacterial protein/enzyme inhibition and disinfectant activity of phenolic acids are all well documented. Phenolic acids are directly involved in the response to microorganisms. Indeed, their concentration in plants rises after infection [50].

The dominant phenolic acids present in *Eryngium* are caffeic acid derivatives, mainly rosmarinic and chlorogenic acids. Rosmarinic acid (a caffeic acid ester of 3,4-dihydroxyphenyl-lactic acid) has many biological and pharmacological activities, e.g., antiviral, antibacterial, antiseptic, antiphlogistic, and antiinflammatory. Chlorogenic acid is an ester of caffeic acid and quinic acid. The research reported various biological and pharmacological activities such as antiviral, antibacterial, antiinflammatory, antiallergy, antioxidant, and lowering blood glucose level [14].

In a recent study, phenolic acids from the aerial parts of *E. campestre* collected from Romania were identified and quantified by reversed phase high-performance liquid chromatography. The results revealed the following phenolic acids: chlorogenic acid, caffeic acid, rutoside, p-coumaric acid, and ferulic acid were detected in *Eryngium campestre* tincture [23]. Caffeic acid, chlorogenic acid [13,62], R-(+)-3′-*O*-β-D-glucopyranosyl rosmarinic acid [17], and rosmarinic acid [64] were isolated and identified from aerial parts of *E. campestre* around the world. Root parts of *E. campestre* have been the subject of a chemotaxonomic investigation carried out using two phenolic acids: R-(+)-rosmarinic acid and R-(+)-3′-*O*-β-D-glucopyranosyl rosmarinic acid. Roots of *E. campestre* showed a modest content of both phenolic acids when compared to other *Eryngium* species with 6 mg/g of R-(+)-rosmarinic acid and 4 mg/g of R-(+)-3′-*O*-β-D-glucopyranosyl rosmarinic acid [35].

Eryngium campestre L. leaves were characterized in comparison to roots by the presence of different level of phenolic acids. A previous UPLC analysis indicated that phenolic acid content (rosmarinic and chlorogenic acids) varies between organs of the same species: 5.32 mg/g for leaves, 3.69 mg/g for roots [15,28].

5.1.3 Other Phenolic Compounds

The first coumarin derivatives were isolated from *E. campestre* (root parts) by Sticher & Erdelmeier [41]. These simple coumarins are aegelinol benzoate, agasyllin, aegelinol, and grandivittin [13,17,28].

6 BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES

The results of a number of published studies show a broad spectrum of biological and pharmacological activities of *E. campestre*, which are attributed to different parts, extracts, and several volatile and nonvolatile constituents of this plant, through selected in vitro and in vivo models that are discussed in the following sections, including antibacterial and antifungal activities; antileishmanial and antimalarial effects; antiinflammatory, antitumor, and antioxidant activities; cytotoxicity; anti-Alzheimer activity; antinociceptive and antipyretic properties. One major limitation of these tests is that they were done in vitro or on animal models and therefore lack the clinical data to determine their appropriateness and effectiveness for human use.

Those pharmacological and biological properties are probably explained by the presence of different classes of bioactive compounds in all parts of *E. campestre*, such as triterpenoid saponins, sterols, coumarins, and especially by the presence of polyphenols (flavonoids and phenolic acids) in root and aerial parts of this medicinal plant.

6.1 Antioxidant Activity

Antioxidant activity of the methanol extract of *E. campestre* aerial parts and the isolated flavonols were evaluated using free radical DPPH scavenging assay and reducing power assay. *E. campestre* methanol extract exhibited relatively high DPPH scavenging activity (66.3%). Among the isolated compounds, rutin showed the highest DPPH free radical scavenging activity (56.2%). It was found that *E. campestre* extract revealed significant reducing power (1.8). Quercetin 3-*O*-β-glucuronide-4′-methylether, isorhamnetin 3-*O*-β-glucoside-7-*O*-α-rhamnoside, and rutin showed relatively similar reducing power activity (1.47, 1.43, and 1.34 absorbance at 700 nm, respectively) [16].

In recent research, the antioxidant activity of essential oil distilled from the aerial parts was assessed using three different assays (DPPH, FRAP, ABTS); essential oil seemed to act as a moderate reducing agent (FRAP assay), but on the other hand, no activity was observed for *E. campestre* essential oil in both DPPH and ABTS assays [40].

In a study by Nebija et al. [4], ethanol extracts from the aerial parts and roots were tested for in vitro antioxidant activity by four various testing systems: DPPH assay, inhibition of production of hydroxyl radical, β-carotene bleaching assay, and inhibition of lipid peroxidation. The results suggest that, in the DPPH system, ethanol extract of root of *E. campestre* exhibited higher radical-scavenging activity ($IC_{50}=0.72$ mg/mL) compared to the extract of the aerial part ($IC_{50}=1.14$ mg/mL). On the other hand, aerial part ethanol extract has exhibited stronger inhibition capacity on the production of hydroxyl radicals in the deoxyribose system than the root extract (50% and 45%, respectively). However, both ethanol extracts of *E. campestre* exhibited low antioxidant activity in β-carotene bleaching assay, as well as low capacity for inhibition of spontaneous lipid peroxidation in rat liver homogenate.

In our previous study [27], the antioxidant properties of various extracts (butanol, water and ethyl acetate) from both roots and aerial parts of Algerian *Eryngium campestre* L. were investigated using DPPH radical scavenging and β-carotene linoleic acid assays. According to the results of our investigation, butanol extract of the aerial parts showed the lowest IC_{50} value (16.140 μg/mL). However, roots aqueous extract showed the highest inhibition of oxidation (89.78%).

6.2 Antiinflammatory Activity

The antiinflammatory activity of ethanolic extract of *E. campestre* was performed in the biological assay using murine brain endothelial cells (inhibition of nitric oxide synthesis in cytokine-activated murine endothelial cells); the ethanolic extract of *E. campestre* (100 mg/mL) showed the ability to almost completely inhibit nitrite accumulation in the medium. Furthermore, the plant antiinflammatory effect was examined on murine monocyte/macrophage-like cell line P388D1; *E. campestre* extract significantly inhibited nitrite accumulation in LPS-stimulated P388D1 cells. Additionally, plant ethanol extract showed very strong inhibition of the synthesis of TNFα. *Eryngium campestre* (extract and fractions) inhibited TNFα-stimulated expression of VCAM1 on the surface of MBE-SV cells [66].

The antiinflammatory activity of the aerial parts of *Eryngium campestre* extracted in ethanol was reported in rats, the acute inflammation being induced by turpentine oil. The effects were evaluated by measuring: serum nitrites and nitrates, total oxidative status, total antioxidant activity, index of oxidative status, total leukocytes count, and differential leukocytes count. *E. campestre* extract showed a significant antiinflammatory action on bone marrow acute phase response by lowering the total leukocyte number by decreasing the neutrophils percentage, lymphocytes and monocytes. NO synthesis was decreased by a similar effect to diclofenac. In addition, the ethanol extract significantly decreased the oxidative stress by increasing total antioxidant status [11].

Küpeli et al. [20] studied antiinflammatory potentials of two different extracts obtained from either aerial parts or roots of *Eryngium campestre* L. against the carrageenan-induced hind paw edema and the TPA-induced ear edema in mice. In both tests used, *E. campestre* extracts were observed to possess a modest antiinflammatory activity; roots ethanolic extract showed a better antiinflammatory effect [20].

An ethanolic tincture of the aerial parts was the subject of a study assessed by Conea and his collaborators; the in vivo antiinflammatory effects were evaluated on a rat ligature-induced periodontitis in male Wistar rats by the histopathological examination of periodontal biopsy and serum total nitrites and nitrates, total oxidative status, total antioxidant response, and oxidative stress index. The results provide evidence that *Eryngium campestre* extract exerted antiinflammatory activity in experimental rat periodontitis by reducing the leucocyte infiltration and the nitro-oxidative stress. *E. campestre* significantly lowered total leukocyte count ($p<0.01$) due to the reduction of neutrophils and monocytes. NO synthesis was also decreased ($p<0.05$) by *E. campestre* tincture [23a].

Using another antiinflammatory test (fresh egg albumin-induced acute inflammation), we evaluated the antiinflammatory effects of butanol extracts from both roots and aerial parts of *E. campestre* at a dose level of (250 and 500 mg/kg of body weight); the study showed that both extracts had a significant effect on inflammation and clearly reduced the swelling [27].

6.3 Antitumor Activity

Antitumor activity of all different extracts prepared with three types of solvents (water, ethanol, and methanol) was assessed with the potato disc method; strong antitumor activity was observed with *E. campestre* extracts. Methanolic extract of *E. campestre* exhibited very strong tumor inhibition (80.6% tumor inhibition). Other tested extracts of *E. campestre* (aqueous and ethanol) showed a good level of antitumor activity: 61.1% and 52.8% tumor inhibition, respectively [38].

6.4 Anti-Alzheimer Activity

Hawas et al. examined the methanol extract of the aerial parts of *Eryngium campestre* L. anti-Alzheimer activity. H4 human neuroglioma cells expressing the double Swedish mutation (K595N/M596L) of human APP (APPsw) were used and treated with increasing concentrations of the *E. campestre* extract, and were analyzed for A 40 and A 42 levels in culture medium by sensitive sandwich enzyme link immunosorbent assay (ELISA). *Eryngium campestre* extract showed significant inhibition of the β-amyloid A 42 ($IC_{50}=155.75\pm7.43$ ng/mL) without significant reduction in total A (A 40 + A 42) levels in human H4 cell line. In H4 cells, a 73% reduction in the A 42 supernatant concentration was achieved at concentration of 300 ng/mL [16].

Authors suggested that those results indicate that *E. campestre* extract may be used as a potent candidate drug for the treatment of Alzheimer's disease or may at least ameliorate or delay the bad effects of this disease [16].

6.5 Cytotoxicity

The essential oils distilled from the aerial parts of *Eryngium campestre* L. were tested against a panel of human cancer cells, namely A375 (human malignant melanoma), MDA-MB 231 cells (human breast adenocarcinoma), and

HCT116 cells (human colon carcinoma), by the MTT assay. The oils resulted highly cytotoxic on the tumor cells, with IC_{50} values 1.57–2.99 μg/mL for *E. campestre*, comparable or close to those of the anticancer drug cisplatin [40]. Unlike the results of this research, none of the extracts (dichloromethane and methanol) from the aerial parts of *E. campestre* was cytotoxic when tested on a mammalian kidney fibroblast (Vero) cells (monkey kidney fibroblast) up to a highest concentration of 47.6 μg/mL [67]. The same results were obtained by [16]: no toxicity was detected by both standard MTT assay and LDH assay in H4 cells treated with methanol extract concentration up to 500 ng/mL.

6.6 Antibacterial and Antifungal Activities

The antibacterial and antifungal activities of extract fractions of different parts of *Eryngium campestre* L. were evaluated using various in vitro methods against a large spectrum of bacterial strains (Gram-positive, Gram-negative) and fungal species (yeasts, dermatophytes, and molds). Table 7.2 recaps the results of these studies.

TABLE 7.2 Anti-Bacterial and Anti-Fungal Activities of *Eryngium campestre* L

Part	Type of extract	Excremental model	Results	Reference
Aerial parts	Aqueous Ethanol Methanol	Gram-positive bacteria: *Streptococcus pyogenes* (ATCC 19615) *Staphylococcus aureus* (ATCC 25923) *Staphylococcus epidermidis* (ATCC 12228) Gram-negative bacteria: *Escherichia coli* (ATCC 25922) *Pseudomonas aeruginosa* (ATCC 27853) *Klebsiella pneumoniae* (ATCC 13883)	Ethanolic or methanolic extracts of *E. campestre* displayed strong antibacterial activity against at least one of the tested Gram-negative bacteria; *P. aeruginosa* was most susceptible to ethanolic extract of *E. campestre*. *E. coli* showed best sensitivity to ethanolic extract of *E. campestre*. Tested extracts of *E. campestre* exhibited antibacterial activity against *S. epidermidis*, *S. pyogenes*, *P. aeruginosa* and *E. coli* in this study	[38]
Leaves roots	Ethanol	Tested by the method of series dilutions against: Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 *Bacillus subtilis* ATCC 6633 Yeast; *Candida albicans* ATCC 10231 *Candida glabrata* clinical strain *Cryptococcus neoformans* clinical strain Molds; *Aspergillus niger* ATCC 16404 Dermatophyte; *Trichophyton mentagrophytes* clinical strain	The crude ethanolic extracts of both leaves and roots of investigated *Eryngium* species showed a significant antifungal activity and moderate antibacterial activity only against *Staphylococcus aureus*	[15]
Aerial parts	Ethanol	Gram-positive and Gram-negative bacterial: *Escherichia coli* ATCC 25922, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Salmonella enterititidis*, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 *Staphylococcus epidermidis*	The screening of antimicrobial activity confirmed a moderate effect on *Staphylococcus aureus* and *S. epidermidis*, as well as a high bacteriostatic effect on *Pseudomonas aeruginosa*. The effect of the *E. campestre* tincture was the most pronounced (inhibition zone; 25 mm)	[23]
Leaves and roots	Methanol extracts and fractions	*Staphylococcus aureus* ATCC 4163, *Pseudomonas aeruginosa* ATCC 6749, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal/ Fungicidal Concentration were determined of selected extracts	Both methanol extract from *E. campestre* roots and leaves showed a good antimicrobial activity. The strongest antibacterial activity was exhibited by the 80% fraction of roots	[28]
The entire plant	Aqueous and organic extracts	Gram-positive: *Staphylococcus aureus* ATCC6538P, *Staphylococcus epidermidis* ATCC12228, *Bacillus subtilis* ATCC6633. Gram-negative: *Escherichia coli* ATCC8739 *Pseudomonas aeruginosa* ATCC 9027 Yeast: *Candida albicans*	*E. campestre* showed a broad spectrum of activity against both Gram-positive and Gram-negative microorganisms, but more activity against Gram-positive bacteria, mainly *Bacillus subtilis*, which had the greatest diameter inhibition zone in the first and the second extracts of (18 mm) and (14 mm)	[2]
Aerial parts	Essential oil	Gram-positive clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) strains, MRSA 1, MRSA 2, MRSA 3, MRSA 4, MRSA 5, MRSA 6, MRSA 7, MRSA 8 and MRSA 9	*Eryngium campestre* showed a week antibacterial activity towards Methicillin-Resistant *Staphylococcus aureus* strains	[19]

TABLE 7.3 Some Biological and Pharmacological Activities of *Eryngium campestre* L

Activities	Part of plant	Type of extract	Excremental model	Results	Reference
Antipyretic activity	Root and aerial parts	Butanol	yeast-induced pyrexia in Wistar albino rats	Butanol extracts of *Eryngium campestre* L. possessed a significant antipyretic effect in the maintaining of normal body temperature; the activity was time and concentration dependent	[27]
Antinociceptive activity	Root and aerial parts	Aqueous Ethanol	benzoquinone-induced writhing test in Swiss albino mice	The results showed that the ethanol extract had a modest activity while the aqueous extract had no effect	[20]
Antimalarial activity	Aerial parts	Dichloromethane Methanol	This activity of the extracts was determined in vitro on chloroquine-sensitive and resistant strains of *Plasmodium falciparum*	Extracts had no significant antimalarial activity	[67]
Antileishmanial activity	Aerial parts	Dichloromethane Methanol	Antileishmanial activity was tested in vitro on a culture of *Leishmania donovani* promastigotes	Both methanol and dichloromethane extracts showed interesting activity against *L. donovani*; IC_{50} values were 15 and 36 μg/mL respectively	[67]
Acetylcholinest-erase inhibitory activity	Aerial parts	Essential oil	Reading was recorded at 450 nm. The inhibitory activity was calculated in comparison to the negative control	*E. campestre* showed a weak inhibition activity, the enzyme inhibition activity was about 10.5%	[40]
Inhibitory effect on the glutathione-*S*-transferase, glutathione peroxidase and catalase enzymes	Flowers leaves	Methanol		Results obtained show that the maximum glutathione-*S*-transferase enzymes inhibition activities were observed with methanol extract from flowers of *E. campestre* with IC_{50} value of 363 ng/mL. In this study, none of the extracts evaluated showed any reasonable glutathione peroxidase and catalase inhibition	[22]

6.7 Other Activities

Other evaluated activities of *E. campestre* extracts such as antipyretic activity and antinociceptive activity are presented in Table 7.3.

7 CONCLUSION

The present review sought to document and summarize the research publications that have appeared in recent years on *Eryngium campestre* L. and its constituents, which have been rarely reviewed. We have tried to focus on ethnomedicinal, modern phytochemical studies and pharmacological screenings on this valuable medicinal plant with several medicinal properties and health benefits. The in vitro and in vivo pharmacological studies have provided numerous biological activities, but further trials in humans are required to determine the safety and efficacy of *E. campestre* extracts and/or its bioactive compounds.

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CHAPTER

8

Antioxidant Activity of Anthocyanins in Common Legume Grains

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Acronym and Abbreviation

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
FAO	Food and Agriculture Organization
GAE	gallic acid equivalent
GPx	glutathione peroxidase
MDA	malondialdehyde
NADPH	nicotinamide adenine dinucleotide phosphate
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
USDA	The United States Department of Agriculture

1 INTRODUCTION

Legumes have long been consumed as rich sources of protein in human diets all over the world. Usually, people consume legumes in various cooked forms (boiled, fermented, fried) and they are served as either main or side dishes. In addition, legumes are also popular to be eaten as a snack (e.g., peanuts, soybeans) in many Asian and African countries. Legumes are plants belonging to the family Leguminoceae. Among many, soybean, peanut, pea, common bean, lentil, lupin, mesquite, carob, alfalfa, and clover are several examples of the best-known and most-consumed legumes in the world.

Recently, recommendations to increase the consumption of legumes have been widely promoted based on many chemical, biochemical, clinical, and epidemiological studies which show that there are positive correlations between consuming legumes and decreasing the incidence of various degenerative diseases, such as cancer, diabetes, coronary heart disease, and obesity. The ability of legumes to reduce the incidence of such diseases is believed to be attributed to biologically active compounds in legume grains. Among others, phenolic constituents including anthocyanins have been deeply explored in relation to their health-promoting properties. Therefore, in addition to their high nutrient profile, legumes are also rich sources of bioactive compounds, especially phenolics; recent research studies are comprehensively focusing on the role of phenolic constituents such as anthocyanin as antioxidants in human diet and their role in promoting human health, as well as in preventing the incidence of various diseases.

The increased rate of degenerative diseases has been investigated by numerous researchers who have suggested that factors such as unhealthy lifestyle, unbalanced diet patterns, and pollution are responsible as the trigger for incidence of degenerative diseases. One of these diseases is coronary heart disease, which is seen as the number one killer in the world. Changes in diet patterns, with more vegetables and legumes and less meat, are believed to have a favorable impact on human health and could decrease the risk of coronary heart disease, as well as other diseases.

2 FREE RADICALS AND ANTIOXIDANTS

The ability of legume grains to decrease the rate of disease or to promote human health is believed to be a result of a synergical effect between the nutrient content, which is rich in proteins and low in fat, with the bioactive compounds found in legumes, such as phenolics and anthocyanin. Bioactive compounds in legume grains play an important role as antioxidants or free radical scavenging compounds, which could balance the number of free radicals in the human system.

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Free radicals are molecules with unpaired electrons and are very reactive in nature. Therefore, they can damage surrounding molecules. In the human system, lipid, protein, DNA, and carbohydrate are molecules susceptible to attack by free radicals, resulting in diseases such as atherosclerosis, coronary heart disease, and cancer. In the normal condition, a certain level of free radicals is found everywhere in the human body, produced as the result of metabolic processes. Free radicals are also useful because they play a role in several biological processes—for example, the phagocytic work by white blood cells—and also they are believed to act as a cellular messenger in a biological process called redox signaling. However, unhealthy lifestyle and diet are believed to create an excessive number of free radicals. The free radical will initiate a chain reaction of the oxidation process, which causes damage to macromolecules. Degenerative diseases are believed to be a result of such severe oxidative stress.

Antioxidants are therefore needed to balance the number of free radicals in the human system, thus preventing damage to macromolecules due to oxidative stress. An antioxidant is any substance that has the ability to inhibit the oxidation of other molecules when present in low concentrations. The human defense system actually produces its own antioxidant substances, which are called endogenous antioxidants, in the form of enzymes (superoxide dismutases, catalase, glutathione peroxidase) and nonenzyme antioxidants (alpha lipoic acid, coenzyme Q10, metal-binding proteins). However, under abnormal/diseased conditions, the number of free radicals exceeds the production of antioxidants in the human system. Thus the body needs the intake of exogenous antioxidants from the diet. Enhancing the antioxidant capacity of the human system by optimizing the dietary intake of natural antioxidants is one of the best strategies to balance between free radical and antioxidant activity in the human system.

3 ANTHOCYANINS

Among many antioxidant compounds found in legume grains, anthocyanins are a bioactive compound that has been widely investigated due to its ability to scavenge free radicals and also to chelate metal due to its chemical structure. Therefore, anthocyanin also possesses a strong inhibitory effect against lipid oxidation [1]. Anthocyanins are representative of a wide group of flavonoids and are derivatives of 2-phenylbenzopyrylium, which is responsible for the attractive colors (blue, violet, purple, and even black) of the plant parts, including flowers, fruits, vegetables, and seeds [2]. Various food products of fruit and vegetable origin are the major sources of anthocyanin compounds. Anthocyanins can also be found in legumes that have black, purple, blue, and red color, for example, red kidney bean, pea, and black soybean [3–5]. The basic chemical structure of anthocyanin is shown in Fig. 8.1.

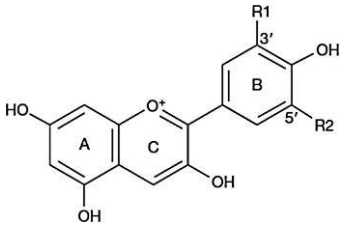

FIG. 8.1 Structure of anthocyanin.

4 LEGUME ANTHOCYANIN AS ANTIOXIDANT

Legume seeds are included as pulse crops, which belong to the *Leguminoceae* family. It has been cultivated in many areas and consumed as a staple or complementary food in traditional and modern diets. The nutritional value of legumes has been broadly investigated. Legumes are rich in proteins, fiber, vitamins, amino acids, polypeptides, flavonoids, and phenolic compounds, which are considered as a good ingredient of a functional food formulation. A number of researchers have reported the potentiality of legumes as a good source of antioxidants and also their ability to prevent diseases. The major compounds of legumes responsible for their high antioxidant activity are believed to be the phenolic and flavonoid compounds.

Anthocyanin, which belongs to the flavonoid group, are a natural pigment that is broadly dispensed in plants that are used in the human diet, including fruits, vegetables, and colored legumes [6]. The anthocyanin occurring in legumes appears as a darker color, ranging from red, purple, greenish, blue/gray, to black, in legume seed coats. Anthocyanins in legumes are correlated with a broad range of biological activities, such as antioxidant, anticancer, and antiinflammatory agent. Its mechanism in preventing some diseases through the role of antioxidant has been investigated. Likewise, the effect of food processing on the antioxidant activity of anthocyanin has been examined intensively. Regarding the development of legumes as functional food ingredients, legume anthocyanin's promising ability as an antioxidant is still an interesting topic in recent studies.

4.1 Black Soybean

Soybeans (*Glycine max* L Merril) have been widely used as a food source around the world due to their

TABLE 8.1 Black Soybean-Based Product

Products	Description	Countries of production	References
Natto	Fermented soybean paste, produced through the fermentation of soy with *Bacillus subtilis*	Japan	Park et al. [11]
Tofu	Soybean curd made by coagulation of heated soya milk with coagulant, followed by moulding and pressing the curd to draw the whey	Asian Countries	Gartaula et al. [12]
Chunjang	Black soybean paste, fermented by *Bacillus* species	Korea and China	Bai et al. [13]
Jajang	Black soybean sauce, made from fried chunjang, fermented by *Bacillus* species	Korea and China	Bai et al. [13]
Tempeh	Fermented soybean with fungus, such as *Rhizopus oligosporus* and *Rhizopus oryzae*	Indonesia	Chang et al. [14]
Kinema, peruyyan, and hawaijar	Fermented food with sticky texture, gray tan color, with slight ammoniacal flavor produced by natural fermentation	India	Tamang [15]

various nutrient and functional compounds, such as proteins, lipids, vitamins, fiber, and phytochemicals. Soybeans are popular in China, Japan, Korea, India, and Southeast Asia nations [7]. Particularly in Asia, soybeans are used in the making of soya paste, soya curd, tempeh, tofu, and oil [8]. Soybean consumption's beneficial health effects include prevention of cancer, early aging, diabetes, and cardiovascular disease [9].

One of the soybean varieties is black soybean. Black soybean is commonly consumed as a food ingredient, especially in fermented products. In Korea, black soybean is utilized to make *chujang* and *jajang*. Natto is also popular as a black soybean-based fermented food in Japan. In Indonesia, black soybeans are used for the production of tempeh [10]. The utilization of black soybean for food production is presented in Table 8.1.

Recent research shows that black soybean has been found to possess a high amount of anthocyanins along with strong biological activity [16]. Pigmentation of black soybean seed coats is contributed by anthocyanin. Anthocyanins in the black soybean seed coat are reported to have antioxidant activities and abilities for α-glucosidase inhibition, regulation of adhesion molecules, protection from ischemia, reperfusion of heart injury, stimulation of wound healing in fibroblasts, and prevention of inflammation in endothelial cells [17]. Anthocyanin may be as essential as isoflavone in determining black soybean characteristics and its functional source. Moreover, the radical scavenging effects of anthocyanin against DPPH and ABTS radicals were stronger than other isoflavones [9].

The anthocyanin compositions are diverse among different black soybean varieties. A study of the correlations between antioxidation and the contents of total phenolics and anthocyanin in 127 accessions of black soybean in China has been reported [18]. Major differences existed among 127 types of accessions, which came from difference geographical regions. The average values of total antioxidant capacity of black soybean were remarkably higher than for yellow soybean, indicating that active material contents of black soybean are apparently superior to yellow soybean, due to its anthocyanin content [19]. Related to anthocyanin content, the best type of black soybean was the autumn sowing type, followed by the summer sowing type and the spring summer type [18]. The anthocyanin content may either be indicated by genetic means or planting conditions and ecological environments.

A recent study showed that nine anthocyanins were found in soluble phenolics that are isolated and identified from the extract of black soybean [9]. Three major anthocyanins, cyanidin-3-glucoside, delphinidine-3-*O*-glucoside, and petunidin-3-*O*-glucoside, respectively constitute more than 90% of the total anthocyanin content, but the predominant anthocyanin in black soybean was cyanidin-3-glucoside [20]. Some animal model studies revealed that cyanidin-3-*O*-glucoside is the most bioactive anthocyanin in avoiding diabetes and obesity-related diseases [21]. Cyanidin-3-*O*-glucoside content in the black soybean seed coat ranged from 6.275 to 19.808 mg/g, approximately 75% to 96% of total anthocyanin content [9]. Anthocyanin content and composition may be highly influenced by the distinct cultivar, genetics, and environmental stresses, as shown in Table 8.2 [20].

Anthocyanin showed obvious antioxidant capacity in many in vivo and in vitro studies, as shown in Table 8.3. in vitro antioxidant capacity of anthocyanin of black soybean varieties in Indonesia by monitoring TBARS formation has been reported [10]. All of the anthocyanin extracted at various levels from black soybean was effective to inhibit low density lipoprotein (LDL) oxidation. A greater inhibition of LDL oxidation was found through black soybean consumption than for yellow soybean [26,27]. The seed coat of black soybean, which contains anthocyanins, was reported to have higher antioxidant activity than the yellow soybean coat. The lag time of LDL oxidation by the black soybean seed coat extract was almost four times longer than the lag time of LDL

TABLE 8.2 Total Anthocyanin Contents of Different Black Soybean Varieties

Black soybean varieties	Country of origin	Total anthocyanin content (mg/g)	References
Geomjeongkong 2	Korea		Man et al. [9]
Crop year: 2009		20.030	
Crop year: 2010		23.043	
Seonheukkong			
Crop year: 2009		8.885	
Crop year: 2010		12.697	
Cheongja 2			
Crop year: 2009		10.948	
Crop year: 2010		10.904	
Cheongja 3			
Crop year: 2009		10.894	
Crop year: 2010		14.072	
Dongbeichun	China	0.98	Xu et al. [18]
Beifangchun		0.1	
Nanfangchun		0.83	
Nanfangxia		0.88	
Malika	Indonesia	13.6	Astadi et al. [10]
Cikuray		14.5	

TABLE 8.3 Health Promoting Properties in Several Black Soybean Products

Soybean product	Health promoting properties	References
Black soybean natto	Hypocholesterolemic therapy for treating the male reproduction system	Gofur and Lestari [22]
Black soybean seed coat extract	Anti-inflamatory and antifibrotic effects on penile plaque formation in rat peyronie disease models	Kim et al. [23]
Fermented black soybean with *Bacillus* spp.	Inhibition of Angiotensin Converting Enzyme (ACE)	Juan et al. [24]
Roasted black soybean powder	Improve cholesterol metabolism, insulin resistance, and alleviate oxidative damage in Non-Alcoholic Fatty Liver Disease (NAFLD)	Jung and Kim [25]
Thai Fermented soy bean (*thua nao*)	Enhance free radical-scavenging activity and ferric reducing antioxidant power, inhibit LDL oxidation	Dajanta et al. [26]

oxidation by the yellow soybean seed coat [27]. Total polyphenol content of black soybeans was also higher than the yellow soybeans. The LDL protection of anthocyanin may be due to metal-chelating and radical scavenging capacity, but the mechanism by which the extract inhibits LDL oxidation in vitro remains unclear. Another research study on in vitro antioxidant capacity of anthocyanin has revealed that the multiple carbon ring structure that belongs to anthocyanin, which is similar to cholesterol, has a greater opportunity to protect cholesterol from oxidation. It may potentially contribute to preventing the inflammation related to cardiovascular and other chronic diseases [28].

Fermented black soybean has a higher antioxidant content compared to nonfermented soybeans [22]. Natto, as a fermented product of black soybeans, has fibrinolytic activity due to its nattokinase enzyme. Natto consumption decreased total cholesterol level in hypercholesterolemia rat model serum [11,22]. It was also reported to have antithrombotic effects and inhibit coagulation of platelets, which was generated by the increasing of prothrombin time and the euglobulin clot lysis time in rat models fed with 750 and 1500 mg/head/day natto dried powder. There were no statistical differences in antithrombotic effects between those rats fed with natto dried powder and the control group, which received 100 mg/head/day aspirin. The dried natto powder contains not only nattokinase but also other biologically active compounds. This may confirm that normal dietary intake of natto offers potential health benefits in preventing cardiovascular disease. Moreover, black soybean natto consumption could affect the reproduction system of hypercholesterolemic male mice by increasing the density and motility of sperm and the testosterone level, as compared to a high-fat fed mice group [22].

Another research study on the inhibitory effect of black soybean supplementation on nonalcoholic fatty liver disease (NAFLD) found that black soybean had inhibitory effects on the cholesterol metabolism and insulin resistance of mice receiving a high cholesterol diet (HCD) [25]. Mice receiving black soybean supplementation had lower blood fasting glucose and insulin levels compared to the group of mice that received HCD. Black soybean supplementation also decreased liver total cholesterol and triglyceride levels in mice compared to the HCD group. The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, which were analyzed to evaluate liver function, were significantly increased in the HCD group compared to the normal cholesterol diet (NCD) group, but there were no statistical differences among all the experimental groups receiving black soybean supplementation. The antioxidant activities of black soybean were analyzed using hepatic antioxidative enzyme activities, including SOD, GPx, and catalase. The results found that the SOD, GPx, and catalase activities were significantly decreased in the HCD group and were

significantly higher in all the experimental groups supplemented with black soybean than in the HCD group. The Malondialdehyde (MDA) and nitrate levels of groups receiving black soybean supplementation was lower than the HCD group, indicating that black soybean could regulate antioxidative enzyme activities in the liver of NAFLD mice. The serum adiponectin, which is involved in insulin resistance in NAFLD, was significantly lower in the HCD group than in the NCD group and largely increased with black soybean supplementation in a dose-dependent manner. Black soybean influences insulin sensitivity and regulation of blood glucose by improving fatty acid oxidation, glucose intake, and insulin resistance via adiponectin secretion. Therefore, black soybean supplementation may have effectively prevented NAFLD, due to its capacity to inhibit fat digestion, fat adsorption, and insulin resistance [25].

TABLE 8.4 Anthocyanin Content From Common Beans Based on Seed Coat Color

Seed coat color/varieties	Anthocyanin content	References
Cream	0.055%[a]	Díaz et al. [34]
Yellow	0.046%[a]	
Brown	0.048%[a]	
Pink	0.062%[a]	
Red	0.161%[a]	
Purple	0.049%[a]	
Black	0.25–0.47 mg/g	Akond et al. [37a]
Cream mottled	0.05–0.16 mg/g	
White	0.06–0.15 mg/g	
Pink with black spot	0.16 mg/g	
Light cream	0.14 mg/g	

[a] *Expressed as delphinidin-3-glucoside equivalents.*

4.2 Common Bean

Common beans (*Phaseolus vulgaris* L.), which are extensively consumed throughout the world, play a key role in the traditional human diet. They have been recently developed as a food ingredient in several food products, mainly because of the high content of protein, starch, dietary fiber, micronutrients, and bioactive compounds with low levels of fat [29–31]. The greatest varieties of leguminous plant can be found in tropical and subtropical areas [32]. There are more than 40,000 varieties of common beans in the world. It has one of the highest levels of variation in growth habit, seed characteristics, maturity, and adaptation [33].

Dry beans, the primary product of common beans, are considered to be exceptional commodities related to their long storage life, good nutritional properties, and simple storage for eating. The health benefit of consuming beans has been established in the later epidemiological studies, especially related to the antioxidant capacity, which could be observed by analyzing the amount of anthocyanins, isoflavones, and other polyphenol compounds [34]. Those bioactive components are responsible for the antioxidant activity of common beans. Similar to the black soybean, the seed coat is also responsible for the antioxidant capacity of common beans, as shown in Table 8.4. Determination of color could be a good indicator to predict the antioxidant capacity of beans. The color features of dry kidney bean are related to the anthocyanin content and antioxidant activities of 26 kidney beans cultivated in China [35]. Some individual anthocyanins observed were cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. The color feature of bean coat was analyzed using a colorimeter. The results showed that some of the kidney beans with the lighter seed coat color contain smaller anthocyanin content if compared to the darker seed coat. There was a strong correlation between the delphinidin content and the seed coat color of kidney bean. The 3-*O*-glucosides of delphinidin were found to be the major anthocyanin of black beans [34–36]. The total anthocyanin contents in the seed coat ranged from 0 to 5.84 mg/g and was reported to be higher than some fruits and vegetables with dark color, such as blueberries and red cabbage. The highest total anthocyanin content was found in cultivar Honghuayuanzhongheizibaijia (5.84 mg/g), followed by the Honghuaheizibaijia (4.76 mg/g) and Jiuliqing (4.55 mg/g). The anthocyanin composition reported in common beans varied compared to cowpea [37].

Another legume cultivated in Asia is red adzuki beans (*Vigna angularis*). This legume was traditionally used for making red bean paste [38]. The adzuki bean seed coat contains a high amount of polyphenols identified as catechin and epicatechin glycosides, quercetin glycosides, myricetin, anthocyanin, and procyanidin dimers [39]. The role of adzuki bean bioactive components in preventing certain diseases has been recently reported. Adzuki bean seed coats (ABSC) treatment on hypertensive rats has attenuated vascular oxidative stress and inflammation in spontaneously hypertensive rats. Polyphenol-containing adzuki bean seed coats restrained the elevation of rat's systolic blood pressure throughout the treatment period. The $NADPH^-$ stimulated O_2^- level and the NADPH oxidase subunits decreased significantly in the aorta of spontaneously hypertensive rats which were treated by ABSC, compared to the untreated ones, suggesting that ABSC suppressed excess O_2^- production in the aorta during progression of hypertension. Thus, ABSC treatment could be useful as a preventive strategy for hypertension and atherosclerosis [40].

Food processing has effects on the antioxidant capacity of common beans. The health benefit of common bean consumption depends mainly on their thermal processing [41]. Anthocyanin-containing beans such as black beans, kidney beans, and pinto beans are usually cooked or thermally processed before being consumed. Anthocyanins are sensitive to heat but there was no significant degradation of the total phenolic content of canned and open-pan-cooked black beans compared to crude black beans [41]. However, the total phenolic content was not always related to the antioxidant capacity. Total phenolics and flavonoid content of common beans increased after toasting, rather than boiling or cooking by autoclaving [42]. Thermal processing may release more bound phenolic acids from the breakdown of the cellular constituents. The stability of antioxidant products such as phenolics and flavonoids during heating may be due to the formation of Mailard products, such as hydroxymethylfurfuraldehyde, that also exhibit high antioxidant capacity [42,43]. Soaking dry beans before cooking could leads to the softening of wall tissues and increase the solubility of bound polyphenols that may later leach into the soaking water during the process. Nevertheless, the amount of polyphenol compound leach was not significant.

Another processing method that can be used to increase the utilization of common beans as a good source of antioxidants in food production is fermentation. *Bacillus subtilis* and *Lactobacillus plantarum* strains were used as microbial agents in solid state and liquid state fermentation using kidney beans var. Pinto [44]. The results indicate that the solid state extract from the fermentation using *Bacillus subtilis* contains high soluble phenolic compound content (31–36 mg/g) and antioxidant activity (508–541 μg trolox equivalent/g), while liquid state fermentation extract using the *Lactobacillus plantarum* strain showed potential antihypertensive activity related to high levels of γ-aminobutiric acid (GABA) and angiotensin converting enzyme inhibitory (ACEI) activity (90%). Thus, this revealed that fermented kidney bean var. Pinto can be a good source of bioactive compounds. The fermentation process yields water-soluble functional extract that can be used as functional ingredients in novel foods and neutraceuticals to prevent cardiovascular diseases [44,45].

Despite the antioxidant activity and other health benefits of common beans, there is still limited information on the transformation of those biological components associated with the food matrix during gastrointestinal digestion, especially in the form of a food product. Later studies have simulated the whole digestion process, from mouth to colon, to estimate bioaccessibility and small intestine permeability of free phenolic compounds in a corn cooked-common bean flours chips [46]. The DPPH and ABTS antioxidant capacity of the chips in in vitro gastrointestinal digestion was found higher at the mouth stage in comparison to methanolic extract. As the gastrointestinal digestion progresses, the antioxidant activity increases until reaching the large intestine, where the value decreases. The lowest antioxidant activity reported in the large intestine might be due to some phenolic compounds that cannot cross the epithelial intestine barrier and reach the colon, where colonic microbials ferment them and modify the antioxidant capacity [46,47]. Apparent permeability (Papp coefficient) was also investigated to define the rate of accumulation of a compound in a receptor chamber normalized by superficial tissue area and it has been used as an *ex vivo* measurement related to human absorption coefficient [46,48,49]. They found that the Papp coefficient for phenolics compounds were high, suggesting that the model applied is similar to an in vivo model [46].

Numerous studies on functional bioactive compounds in common beans have focused on the amount of the component, the health benefit in a crude form and in food mixture or food products, and the effects of processing on the antioxidant capacity. However, studies of the in vivo bioavailability and bioaccessibility through gastrointestinal models are still limited. With regard to the importance of such topics, more research related to the bioavailability and bioaccessibility of common bean antioxidant compounds needs to be conducted.

4.3 Cowpea

Cowpea (*Vigna unguiculata*), also known as black-eyed peas, southern peas, and crowder peas is a kind of pulse grain which is the most widely produced pulse grain behind common dry beans (*Phaseolus vulgaris*) and chickpea (*Cicer arietinum*) [50,51]. It can grows well in an area which usually usuitable for most other legumes because of its heat and drought tolerance, making cowpea as an environment and climate-change friendly crop. West Africa is the world largest cowpea productions, accounts for more than 87% of the world production and use. Cowpea were more popular than common legume grains to be used in Africa [52].

Cowpea contains bioactive compounds including polyphenols which are condensed in the seed coat. As in found on soybean and common beans, the composition of phenolics in cowpea varieties are also diverse, mainly be affected by seed coat color. The phenolic compounds are responsible for most of the coloration observed in cowpea varieties, ranging from white, read, cream, bronze, purple, to black [50,53]. The seed color, polyphenol structure and composition are directly affects specific mechanism for disease prevention and also influence nutrient bioavailability of cowpea varieties.

The major phenolic compound found in cowpea is the phenolic acids [53]. Red cowpea phenotypes tend to have

the highest phenolic acids compared to other cowpea varieties. The phenolic content of cowpea was ranging from 19.1 to 48.3 mg/100 g for a set of 15 commonly consumed cowpea varieties, considerably higher than the phenolic content of common dry beans (*Phaseolus vulgaris*). The phenolic acids are possibly an important contributors to health benefits related to phenols in cowpea and other pulses [50].

Anthocyanin is a bioactive compound that also found in cowpea. It found only in a specific phenotypes, usually in the black pigmented seed (1.7–3.9 mg/g) and concentrated mostly in the seed coat, showed as more than fivefold relative to the entire seed [50,54,55]. Seed coat also contained approximately 10 times more flavonoid compared to whole seeds [56]. Anthocyanin found in black, grey, navy blue, green, and black/grey varieties of cowpea, dominated by delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside which account for 68%–74% of all the pigments [37]. Besides delphinidin and cyanidin, other anthocyanins presented in cowpea are petunidin, peonidin, and malvidin, as shown in Table 8.5. Other pigmented red, maroon, and brown seed coat varieties contain no measureable anthocyanins [6,37]. Regarding the fact that anthocyanin were concentrated in the seed coat, processing technology that remove the seed coat will unwittingly eliminate the benefit of anthocyanin, as well as removing valuable other phenolic compound and fiber. Similar to common beans, thermal process have limited effect on the profile of phenolic compound. Additional work is needed to uncover the metabolism of bioactive compound in cowpea and their specific properties in order to be able to utilize cowpea in food formulation and products such as snack, cereals, and baked goods with high bioactive compound content and antioxidant activities.

TABLE 8.5 Anthocyanin Composition of Cowpea Seed (Awika and Duodu [50]; Chang and Wong [55]; Ojwang et al. [54])

Anthocyanin compound	Proportion (%)
Delphinidin-3-*O*-glucoside	26–33
Delphinidin-3-*O*-galactoside	8–11
Cyanidin-3-*O*-glucoside	24–27
Cyanidin-3-*O*-galactoside	1–8
Petunidin-3-*O*-glucoside	11–14
Petunidin-3-*O*-galactoside	<2
Peonidin-3-*O*-glucoside	2–3.5
Peonidin-3-*O*-malonylglucoside	<2
Malvidin-3-*O*-glucoside	10–13
Malvidin-3-*O*-acetylglucoside	3

4.4 Peanut

Peanuts (*Arachis hypogaea*) is one of the Leguminoceae family which are rich in oils, protein, vitamins, and other bioactive component such as phenolic compounds [57]. Peanuts was widely consumed across the world due to its unique flavor and flexibility in processing. Peanut can be eaten raw, boiled, roasted, and used in recipes not only in traditional food but also made into modern food ingredient [58]. Recent research shows that peanut was rich in antioxidant, phenolics, and other phytochemicals such as flavonoids, proanthocyanidins, and anthocyanins [58–60]. Those phytochemicals were found having role in preventing cancer, coronary heart diseases, degenerative nerve disease, Alzheimer, and viral/fungal infections [58]. The polyphenols and flavonoid in peanuts were responsible on its antioxidant capacity, antimutagenic capacity, and anti-proliferative effects [60].

Similar to other legumes, seed coat color in peanuts varieties were also rich in antioxidant. Peanut seed coat was varied from colorless/white, red, to deep purple/black. Seed coats were very light, contributed only 2%–7% of the total kernel weight. However, the total phenolic content (TPC) of seed coats (97.3–133.5 mg GAE/g) was significantly higher compared to raw kernel (3.28–9.20 mg GAE/g) and cotyledon (0.88–1.85 mg GAE/g) for 6 varieties of peanuts found in China [58]. Moreover, seed coats also contributed to the majority of TPC (67%–87%) presented in raw kernel, while only 12%–18% of TPC is allocated to the cotyledon. The same trend was also found in the total flavonoids content (TFC), condensed tannin content (CTC), monomeric anthocyanin content (MAC), and antioxidant capacity analyzed with DPPH and FRAP, which indicated that seed coats were the major source of antioxidant found in peanuts. Those trends represented in both for the red and the black seed coat peanuts, but the MAC for black seed coat peanuts appeared higher than that observed in red seed coats. The study also revealed that boiling may leads to significant losses in phytochemical contents and the antioxidant capacities of colored-seed coat peanuts.

Regarding the function of peanuts varieties as the source of antioxidant, the seed coat were not the only important one which define the total antioxidant capacity. The colorless peanuts varieties also have potential of being a good source of antioxidant. The antioxidant capacity of peanuts is not always directly related to the intensity of their seed color [60]. The results indicated that the Israeli peanut cultivars that have pale pink seed coat were discovered to have very high TPC, TFC, and FRAP antioxidant capacity, although their Total Anthocyanin Content (TAC) value was not very high. It may cause by the colorless flavonoids which could be the major source of antioxidant activity in colorless-seed coat peanuts and that colored anthocyanins plays only a secondary role.

Peanut skins have been used as fortificants in developing new food products to improve the content of bioactive compound. The aim of food fortification is to improve the health benefit of food. Peanut skins contains high proanthocyanidin content which have beneficial effect, however it may cause bitterness and astringent mouthfeel. Therefore, the formulation must be taken into account in order to produce food product with acceptable sensory characteristic. Peanut skins have been used as an ingredient for chocolate cookies fortification in order to increase their polyphenol content [61]. Peanut skins was added at 1.3%, 1.8%, and 2.5% to cookies in order to increase their polyphenol content. The result showed that the addition of peanut skins in those three different levels increased the total phenolic content and DPPH scavenging activity of the product by 12%–50% and 67%–250%, respectively. The antioxidant evaluation carried out using ABTS radical cation scavenging activity, H_2O_2 scavenging activity, and hydroxyl radical scavenging activity indicated similar trend as the DPPH assay. Moreover, the data showed that heating process did not have any destructive effect on either TPC or scavenging activity. However, the heating process did affect the product sensory characteristic due to an unexpected food color that has been developed after heating the polyphenol-rich materials in the cookies. Nevertheless, the color change did not affect the acceptability of the cookies in sensory evaluation since the color underlined the chocolate flavor which added to the cookies in order to mask the bitterness and astringent mouthfeel of the peanut skin.

Another food that could be fortified by peanut skins was peanut butter. The addition of ground peanut skins into peanut butter in certain levels resulted in a concentration-dependent increase in the total phenolic content and antioxidant activity. Adding peanut skins was effectively increased the total phenolic content, antioxidant capacity, and fiber content of the fabricated peanut butters in a concentration-dependent manner, at the same time maintaining the USDA's standard of identity for peanut butter. The sensory evaluation of the peanut skin-fortified peanut butters was also greatly acceptable [62].

The positive effects of consuming peanuts and its product, such as peanut oils, peanut flour, and peanut seed coats, are well established. Peanuts have healthy fatty acid profile and being good sources of components that are capable of scavenging free radicals [63]. The finding of recent studies has already shown that peanuts and its by-product can be potentially used as an ingredient in functional food formulations. Improvement of existing product may lead into product diversification of established brand available in the market.

4.5 Lentils

Lentils (*Lens culinaris* L.) is one of the oldest crops cultivated by humans and the most consumed leguminous seeds in the world together with peas (*Pisum sativum* L.), chickpeas (*Cicer arietinum* L.) and common beans (*Phaseolus vulgaris* L.). Lentils are not only contains high amount of macronutrients, such as protein, fatty acids, and carbohydrates, but also contain phytochemicals, such as phenolic acids, flavonols, saponin, phytic acids, and condensed tannin, which can be used as a source of antioxidant [64]. Some studies about lentils have shown that the phenolic contents and antioxidant capacity of lentils was one of the highest among the other leguminoceae family [32,65–67].

Lentils are generally canned or dry-packaged for retail sale or processed into flour [67,68]. It used in soups, stews, salads, snacks, and vegetarian dishes. It may also be used as a meat substitute or extender due to their high protein content [68]. Lentils flour can be used as gluten-free ingredient to be added in bread, cake, and baby food formulation. The variety of lentils can be represented by their seed color which ranged from yellow to red-orange, green, tan, brown, and black, as a solid or speckled in color, while the cotyledon is red, yellow, or green. The diversity of lentils phenotype indicated the intricate phytochemical characteristic and bioactivities among lentil varieties [64,68]. The color differences between lentils indicated that several pigments such as anthocyanins may be existed in lentils. A research that investigated the anthocyanin content of 11 lentils grown in northern part of USA, showed that the French Green lentil which have greenish brown/yellow seed coat exhibit much higher anthocyanin content (665.6 μg/g) than the Pardina (157.3 μg/g) that have greyish brown/yellow seed coat [30]. The anthocyanin content of the other lentil varieties were undetectable by HPLC, but still had the relatively high content of total flavonoid which reflected in their antioxidant assay [30].

The antioxidant activity were significantly differed amongst the various legume extract [32]. The result proved that lentils had the highest total phenolic content, total antioxidant capacity, DPPH scavenging activity, and total reducing power compared to other legumes (Table 8.6). The total antioxidant capacity, DPPH scavenging activity and total reducing power were positively correlated with total phenolic content. The scavenging effects of the legume extract on the 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) were shown in Fig. 8.2. Bioactive compounds in lentils that responsible for the high ability to act as antioxidant are the phenolic compounds, including procyanidins (69%), flavonols (17%), flavanones (5%), hydroxybenzoic (5%) and hydroxycinnamics acids (4%) [67].

Three different parts were recognized in the legume seeds: seed coats or testa, cotyledon, and embryonic axe which represent 10%, 89%, and 1%, respectively of the seed content [71]. The cotyledon consists the major supply substances and non-flavonoid compounds, which is primarily proteins and carbohydrates, while in the seed coat or testa are located the majority of flavonoid

TABLE 8.6 Total Antioxidant Activity of Different Legume Grain

Legume grain	Total phenolic content (mg/g)	Total antioxidant capacity	References
Pinto bean	33.4±3.0	567±93 U/g	Zhao et al. [32]
Cowpea	15.2±0.7	222±26 U/g	
Baby lima beans	9.5±1.0	116±3 U/g	
Lentils	47.6±5.3	721±51 U/g	
Chickpea	21.9±2.8	648±18 U/g	
Small red beans	45.7±1.8	622±32 U/g	
Red kidney beans	27.1±3.0	516±61 U/g	
Black kidney beans	32.9±0.1	601±12 U/g	
Navy beans	11.6±0.01	215±28 U/g	
Mung beans	26.7±1.4	304±23 U/g	
Pigeon Pea	0.73	52.1%	Uchegbu and Ishiwu [69]
Yellow Pea	3.45	10.36 μmol TE/g	Oomah et al. [70]
Green Pea	0.65–0.99	1.73–9.95 μmol TE/g	Xu et al. [18]
Adzuki beans	90	1.76 μmol Trolox/mg	Amarowicz et al. [39]
Faba beans (14 varieties)	16.98–67.47[a]	0.88 μmol Trolox/mg	Amarowicz et al. [39]; Chaieb et al. [70a]

[a] *Expressed as mg GAE (Galic Acid Equivalent)/100 g dried beans.*

compounds in lentils. Although the seed coat exhibit only small portion of the total weight of lentils, the seed coat flavonoid, such as cathechins, procyanidin, flavonols, and flavones, are the main source of total phenolic content in the lentil seeds. The cotyledon, which represent a big portion of the total weight of lentils, provide low concentration of phenolic compound, that mainly detected as cinnamic and benzoic compounds.

Antioxidant activity of lentils was positively correlated with their phenolic content [64]. That might be developed as an ingredient of functional foods. Lentils proceeded to form micronized flakes can be a promising ingredient to replace oats in snack bar formulations due to its nutritional benefit [72]. Recent research showed that extrusion processing on lentil flour fiber-enriched formulation could increase polyphenol fraction [73]. Extrusion process may hydrolyze polyphenol bounds to fiber and protein which may lead to the increase of antioxidant capacity and bioaccessibility. The antioxidant activities of functional components in food product were depended not only on the level of the bioactive compound, but also the composition of the bioactive compound. The antioxidant activity of extruded flour samples which determined using DPPH assay exhibited higher values than the non-extruded flour samples. Extrusion promoted an increase in soluble fiber, total phenolic, hydroxycinnamic, and hydroxybenzoic acids in the lentil-based, fiber-enriched analyzed flours. The flour can be a good alternative to create a functional food with a balance nutritional and phytochemical composition. Those results confirmed the potential of lentils to be used as functional food ingredients. However, research on the effect of lentils consumption in both raw and processed form in preventing degenerative diseases should be intensified in order to evaluate the beneficial effects of lentil's consumption.

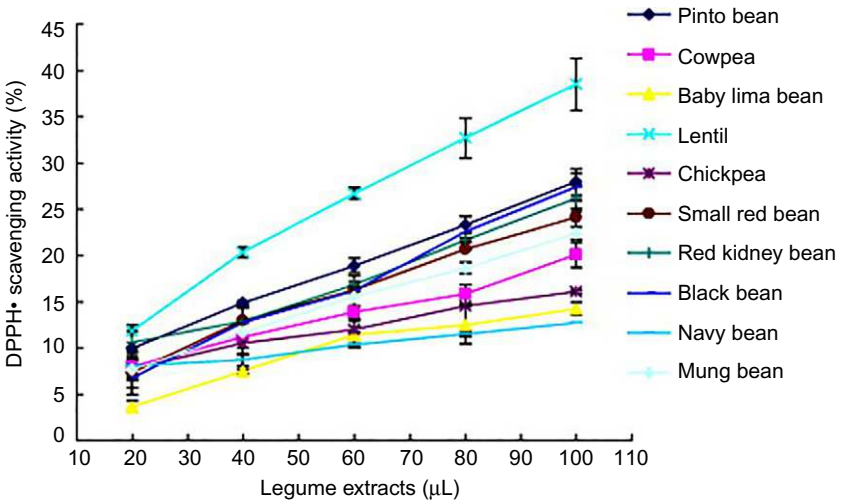

FIG. 8.2 Scavenging activity of the legume extract on the 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) as measured by Zhao et al. [32].

5 CONCLUSION

Anthocyanin are predominantly found in the seed coat of dark colored legumes such as black soybean, common bean, cowpea, peanut, and lentils. Among other anthocyanin, cyanidin 3 glucoside is the major anthocyanin found in legumes. Beside the high antioxidant activity, anthocyanin from legumes have been reported exhibit anti-hypertension, anti-inflammatory, anticarcinogenic, antitumor, and antimutagenic activities. It also enhances spatial memory, enhances cognition, and inhibits LDL oxidation. Increase the consumption of legumes for human daily diet should be recommended due to their beneficial properties for health. Moreover, further research on the application or incorporation of legumes in snacks or other food products in industrial scale should be intensified in order to optimized the intake of anthocyanin and other bioactive compound in legumes.

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CHAPTER

9

Tomato Polyphenolics: Putative Applications to Health and Disease

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Abbreviations

ACE	angiotensin converting enzyme
ADP	adenosine diphosphate
AIN	American Institute of Nutrition
AP-1	activator protein 1
APX	ascorbate peroxidase
cAMP	cyclic adenosine monophosphate
CAT	catalase
COX-2	cyclooxygenase 2
CRP	C-reactive protein
DHAR	dehydroascorbate reductase
EFSA	European Food Safety Authority
EGF	epidermal growth factor receptor
GR	glutathione reductase
HER-2	human epidermal growth factor receptor-2
HUVECs	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
IGF-1	insulin like growth factor-1
IKK	IkB kinase
IL	interleukin
ILSI	International Life Science Institute
INF-γ	interferon-γ
LDL	low density lipoprotein
LOX	lipoxygenase
MCP-1	monocyte chemotactic protein 1
MDHAR	monodehydroascorbate reductase
MED	minimal erythema dose
MMP	matrix metalloproteinase
NF-κB	nuclear factor kappa-beta
NO	nitric oxide
PF4	platelet factor 4
PPARα	peroxisome proliferator activated receptor alpha
ROS	reactive oxygen species
SOD	superoxide dismutase
TNF-α	tumor necrosis factor alpha
TRAP-6	thrombin receptor activator for peptide 6
UVR	ultraviolet radiation
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WHO	World Health Organization

1 INTRODUCTION

Epidemiological studies have shown that diets rich in fruits and vegetables provide unmatched health benefits. Tomato in particular is one of the most important vegetables worldwide, and its products, including sauce, juice, puree, paste, and diced, canned or dried tomatoes, have been shown to reduce the risk of various chronic diseases, including cardiovascular diseases, several types of cancer, and inflammation. These health-promoting effects of tomato and its processed products are associated with the presence of phytochemicals [1]. Tomato fruits are valuable sources of polyphenols, including naringenin chalcone, rutin, quercetin, chlorogenic acid, caffeic acid, naringenin, kaempferol-3-rutinoside, *p*-coumaric acid, ferulic acid, and kaempferol [2]. The structures of these phenolic compounds are shown in Fig. 9.1. An exhaustive study of the literature demonstrated that these compounds can provide effective protection by neutralizing free radicals, which are unstable molecules linked to the development of a number of degenerative diseases and conditions [4]. Although health benefits of tomatoes are usually linked to lycopene, significant polyphenolic content is also naturally present in this fruit. In parallel, some studies have indicated that consuming whole tomato was more effective than consuming lycopene alone, suggesting that

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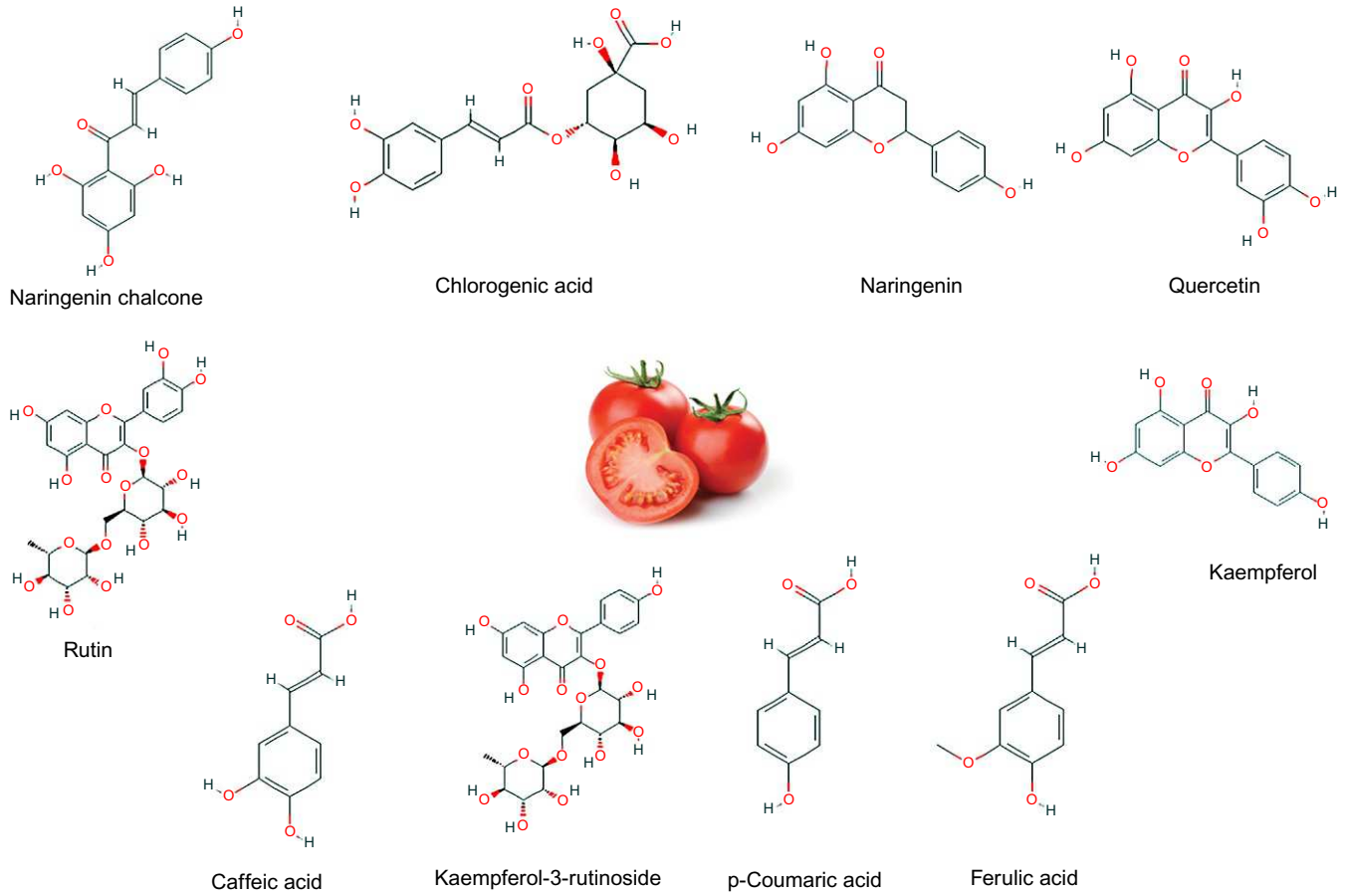

FIG. 9.1 Tomato polyphenols [3].

tomatoes contain important polyphenols [5,6]. Moreover, the antioxidant effect of tomato is most probably due to the synergism between several compounds, which are naturally present in whole fruits and vegetables, and it is not related to the lycopene content alone [7].

In this chapter, we have summarized and discussed the potential role of tomato polyphenolics on the prevention of chronic diseases including cardiovascular diseases, several types of cancer, and diabetes. In addition, health-promoting features of tomato polyphenolics, i.e., antioxidant, antiangiogenic, antiinflammatory, antibacterial, antifungal, and antiviral effects, are highlighted.

2 HEALTH EFFECTS OF TOMATO PHENOLICS

2.1 Cardiovascular Diseases

Currently, cardiovascular diseases are the leading cause of morbidity and mortality in the world regardless of race, ethnicity, or gender [8]. According to the PASSCLAIM consensus on criteria published by the International Life Science Institute (ILSI) in 2005, well-established biomarkers for changes in the risk of cardiovascular diseases are low density lipoprotein (LDL) cholesterol—the hallmark for cardiovascular diseases—and blood pressure. In fact, cholesterol is not considered to be a biomarker as such, in that its presence or absence reflects injury or damage, but it is an abundant and natural metabolite in blood whose quantitative variation reflects various metabolic states that are in turn reflective of cardiovascular diseases [9]. The endothelium plays a critical role in cardiovascular health, as endothelial dysfunction is associated with development of atherosclerosis and hypertension. Endothelial cells that line the internal lumen of blood vessels express molecules that are capable of regulating inflammation and blood flow [10,11].

A number of epidemiological studies, clinical trials, and animal experiments have demonstrated that polyphenols contribute to the prevention of various degenerative diseases, including cardiovascular diseases. The tomato polyphenols could also contribute to cardiovascular health as their beneficial effects have been reported in several in vivo and in vitro studies. Regarding in vivo studies, it has been shown that consumption of 200 g of tomatoes (equivalent to two tomatoes) per day for 8 weeks significantly lowered both diastolic and systolic

blood pressure and platelet aggregation response [12,13]. The role of tomatoes in prevention of cardiovascular disease is widely discussed in in vitro studies. Following, some recent reports on cardioprotective effects of tomato phenolics are highlighted.

Industrial processing of tomato generates a considerable amount of waste, known as tomato pomace. In a recent study [14], the antiplatelet and antithrombotic activities of aqueous extracts from red tomatoes, tomato-based products (sauce, ketchup and juice) and pomace were investigated. The major phenolics identified in these samples were chlorogenic, ferulic, *p*-coumaric, and caffeic acids. Extracts of red tomatoes, tomato products and by-products were able to inhibit the platelet aggregation induced by adenosine diphosphate (ADP), collagen, thrombin receptor activator for peptide 6 (TRAP-6), and arachidonic acid, but to a different extent. The highest activity was observed for ketchup in the case of ADP and collagen stimulation, whereas pomace showed the highest activity when TRAP-6 and arachidonic acid were induced. Overall, pomace extract presented the best antiplatelet activity because it inhibited platelet aggregation by different activation pathways. Considering these promising results, the authors further evaluated the effect of pomace extract on laser-injured thrombus formation in mice mesenteric artery in vivo. Pomace extract also presented antithrombotic activity. Therefore, in terms of primary prevention of cardiovascular disease, these results suggested that, given the ability of pomace extract to inhibit platelet function, it could be considered as a lead source candidate as an antiplatelet and antithrombotic agent [14].

Inhibition of antiangiotensin converting enzyme (ACE) has been considered to be one of the effective therapeutic approaches for the treatment of cardiovascular diseases. In the study of Biswas et al. [15], sugar-free tomato extract was tested for its anti-ACE activity using human plasma and rabbit lung pure ACE. In addition, its effect on human platelet aggregation induced by ADP, collagen, or arachidonic acid was also determined. The extract, which was rich in phenolics, inhibited ACE activity and platelet aggregation induced by different aggregating agents in a dose-dependent manner. The platelet inhibitory action of tomato extract was mediated in part by reduction of platelet factor 4 (PF4) release and by stimulation of cyclic adenosine monophosphate (cAMP) synthesis. Overall, the results indicated that aqueous extract of tomatoes contains anti-ACE factors in addition to antiplatelet factors [15]. In another recent study [16], the effect of tomato aqueous extract on endothelial dysfunction in human umbilical vein endothelial cells (HUVECs) was analyzed. HUVECs were activated with tumor necrosis factor alpha (TNF-α) in order to induce endothelial dysfunction. The results showed that aqueous extract of tomatoes was able to reduce endothelial dysfunction-associated expression of adhesion molecules (intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) in endothelial cells.

As indicated previously, in a variety of studies it was demonstrated that water-soluble components of tomatoes are capable of inhibiting platelet aggregation [14,15] and ACE [15] and relaxing the vascular endothelium [16]. Considering these beneficial effects, a water-soluble tomato extract containing the bioactive compounds was developed and given the trade name Fruitflow® [17]. Fruitflow is standardized using three compounds, adenosine, chlorogenic acid, and rutin, which are representative of active constituents and have been shown to inhibit both platelet aggregation and inflammatory pathways. Currently, Fruitflow is available in two ingredient formats: Fruitflow 1, a syrup of which more than 50% (w/w) comprises tomato-derived carbohydrates and ~3% (w/w) comprises known bioactive compounds, and Fruitflow 2, a low-carbohydrate powder, of which more than 55% (w/w) is composed of bioactive compounds. After conducting eight human studies, and seven nonhuman studies with Fruitflow, the European Union Commission authorized the health claim "water-soluble tomato concentrate I and II helps maintain normal platelet aggregation, which contributes to healthy blood flow." Thus, Fruitflow is now authorized by the European Food Safety Authority (EFSA) for daily consumption, with the conditions that 3 g Fruitflow 1 or 150 mg Fruitflow 2 must be included in either foods (e.g., fruit juices, flavored drinks, or yogurt drinks with a total volume up to 250 mL), or in powdered, tableted, or encapsulated food supplements (to be taken with up to 250 mL water) [17].

2.2 Cancer

Cancer, no doubt, is one of the major public health problems worldwide. The most common cancers to be diagnosed in men are lung, prostate, and colorectum, whereas lung, breast, and colorectum in women are most common. Moreover, in 2018, 1,735,350 new cancer cases and 609,640 cancer deaths are estimated to occur in the United States [18]. In general, smoking, alcohol use, overweight, obesity, and low fruit and vegetable intake raises the risk of these cancers [19]. Based on these findings, it has been an important goal to inhibit cancer and find novel and effective cancer treatments. However, conventional therapies such as surgery, chemotherapy, and radiotherapy are associated with adverse side effects. Therefore, cancer research is focused on developing new anticancer treatments using polyphenols [20]. Studies on humans and animals as well as in vitro studies conducted in the lab have clearly demonstrated that polyphenols have been active in cancer chemoprevention

[21]. A good example of a commonly used food crop with a high content of phenolic compounds is tomato, which contains kaempferol, rutin, caffeic acid, naringenin, and quercetin, etc. It has been shown that these compounds may inhibit carcinogenesis in three stages: initiation, promotion, and progression [22]. The effect of tomato polyphenolics on cancer is investigated both in in vivo and in vitro studies.

Cell growth effects of different tomato waste extracts were evaluated in HeLa (cervix epitheloid carcinoma), MCF7 (breast adenocarcinoma), and MRC-5 (fetal lung) cell lines. In all cell lines, antiproliferative effects were observed at higher concentrations ($\geq$6.3 mg/mL) [23]. A recent study tested the anticancer activity of the total metabolite extract containing flavonoids and other phenolic compounds from InsP 5-ptase expressing tomato fruits on a breast cancer cell line (MCF-7). They observed that the extract of tomato fruits expressing the InsP 5-ptase gene showed stronger anticancer activity compared to the wild-type tomato extract. The higher anticancer activity of InsP 5-ptase expressing tomato plants was explained by an increase in the accumulation of phenolic compounds in the fruits of the transgenic plants [22]. Another study showed the antiproliferative effects of quercetin, kaempferol, and naringenin present in tomatoes on Hepa-1c1c7 (a mouse liver cancer cell line), and LNCaP (a human prostate cancer cell line) cells. Moreover, they also investigated the inhibition of cancer cell growth with combination of the flavonoids to test for synergistic interactions. The study concluded that all single flavonoids decrease cancer cell growth in a dose-dependent way in all cancer cell lines. In addition, a combination of two flavonoids demonstrated significant synergistic antiproliferative effects on the inhibition of cancer cell growth [24]. Furthermore, each polyphenol showed different properties on cancer cells. For example, Wang et al. [25] observed that the glycoside rutin did not modulate AT6.3 cell growth, apoptosis, or insulin-like growth factor-1 (IGF-1)-regulated intracellular signaling events. These authors emphasized that the glycoside polyphenols must be hydrolyzed in vivo to demonstrate antiprostate cancer activity. Saunders [26] showed that all three varieties, with different levels of flavonoids and total phenolics, were able to inhibit the growth of colon cancer cells significantly. Choi et al. [27] found that the cervical HeLa and the liver HepG2 carcinoma cells were inhibited by the tomato extracts. In a recent study, whole tomato extracts impaired gastric cancer cells [28].

Although tomatoes and tomato products contain many phytochemicals, the research has mainly focused on lycopene. However, some studies have indicated an increased protective effect when consuming whole tomatoes compared to lycopene alone. Guil-Guerrero et al. [29] evaluated cytotoxicity effects of different tomato extracts on the HT-29 cell line. The study concluded that ethanol-water, petroleum ether, and in vitro-digested tomato extracts exhibited in vitro cytotoxicity against the proliferation of the cultured cancer cell line (HT-29). The authors emphasized that the degree of growth inhibition on cancer cells appeared to be dependent on the total phenolic content of the tomatoes rather than the levels of rutin and naringenin. Another in vivo study investigated the inhibition of colon carcinogenesis by lycopene and tomato juice on F344/NSlc rats. The colon cancer was significantly lower in the tomato juice group (21%–40%), but not in the lycopene group (33%–72%), than in the control group (54%–84%). These studies suggest that polyphenols, antioxidants, can quench or prevent the formation of reactive oxygen and nitrogen species, which play important roles in carcinogenesis [30]. Another possible explanation of cancer protection by flavonoids may include alterations in detoxification enzymes [24]. Similarly, phenolic acids and their analogs can inhibit tumor cells and induce apoptosis by inducing cell cycle arrest, regulating signal transduction pathways, inducing or inhibiting some enzymes, and enhancing detoxification [31].

Human and animal studies have highlighted the positive effect of consuming tomato and its products on cancer. In the in vivo study of Shirzad et al. [32], the effect of tomato extract on the growth of fibrosarcoma in mice was tested. They proposed that antioxidants of tomato extract have a role in the prevention and control of fibrosarcoma growth. Tomato juice led to significant inhibition on adenosine deaminase activity in the prostate tissues from cancer patients [33]. Edinger et al. [34] showed a decrease of 10.77% in prostate-specific antigen levels in patients who were subjected to daily ingestion of tomato paste. Another in vivo study on Dunning R3327-H prostate cancer indicated that intake of lycopene showed no changes in apoptotic rates in tumors, yet tomato consumption resulted in increased apoptosis and the combination of whole foods may be superior in slowing cancer growth [6]. Similarly, 194 male rats with prostate cancer fed with tomato powder had a significantly lower death rate compared to lycopene-fed animals and controls [5]. These studies indicated that tomato phytochemicals may act in synergy with lycopene.

Studies related to processing of tomato showed positive effects on the content of phenolic compounds. The increase in the phenolic compounds of the heat-processed tomatoes was explained by the increased amount of bound phytochemicals released from the matrix with thermal processing [35,36]. Similarly, Raiola et al. [37] reported that a cytotoxic effect of tomato extracts was observed on liver (HepG2), kidney (Hek293), and cervix (HeLa) cells only after processing. It has been reported that processing causes a significant increase in the flavonoid content. Another study conducted by Shen et al. [38] investigated the suppression effect of phenolics in fresh and heated tomatoes on the expression of cyclooxygenase

2 (COX-2), which is induced by growth factors, tumor promoters, oncogenes, and carcinogens. Cell studies showed that phenolic extracts of heated tomatoes resulted in increased suppression of COX-2 expression compared with that of fresh tomato. As the amount of clinical research on tomato polyphenols is low, further studies are needed to evaluate their anticancer activity.

Overall, both in vivo and in vitro studies highlighted that whole foods and combinations of phytochemicals are more effective in cancer treatments compared to individual polyphenols.

2.3 Diabetes

Diabetes mellitus is a major endocrine disorder, affecting approximately 5% of the world's population. The World Health Organization (WHO) estimates that almost 3 million deaths occur annually as a result of diabetes and that there will be 366 million cases of diabetes by the year 2030. Diabetes is characterized by abnormalities in carbohydrate, lipid, and lipoprotein metabolisms, which not only lead to hyperglycemia but also cause many complications such as hyperlipidemia, hyperinsulinemia, hypertension, and atherosclerosis [39].

Tomatoes are rich sources of polyphenols, which are reported to exert several biological activities, one of which is antidiabetic activity. The role of tomato polyphenols in prevention of diabetes is mainly studied on a compound basis. For instance, it has been shown that rutin (50 mg/kg), one of the most abundant flavonoids in tomatoes, decreased glucose levels by increasing insulin secretion in hyperglycemic rats [40]. Furthermore, kaempferol was suggested to prevent the onset of diabetes by preventing oxidative damage in pancreatic β-cells [41]. Similarly, it has been shown that in diabetic rats, treatment of kaempferol for 10 weeks ameliorated insulin via down-regulation of IkB kinase (IKK) and subsequent inhibition of nuclear factor kappa-beta (NF-κB) pathway activation [42]. Oral administration of naringenin (50 mg/kg a day) to diabetic rats for 21 days gave similar results to those produced by glyclazide, a standard drug for therapy of diabetes mellitus [43]. In another study, the protective effect of naringin, the glycosylated form of naringenin, on high glucose-induced injury of HUVECs was examined. Naringin was able to reverse the increases in reactive oxygen species (ROS) production, CX3CL1 expression, and AKT phosphorylation. In addition, low nitric oxide production and oxygen consumption rates were restored with naringin treatment [44].

Few studies have investigated the antidiabetic actions of tomato phenolics in processed products. In a recent study [45], the antiinsulin resistance effects of processed tomato vinegar beverage, containing 1.37 mg phenolics per 100 mL, was investigated. Oral administration of tomato vinegar beverage (14 mL per kg body weight) to high-fat diet-induced obese mice for 6 weeks reduced insulin resistance via AMPK/peroxisome proliferator activated receptor alpha (PPARα)-mediated glucose oxidation. In an earlier study, it was suggested that 13-oxo-9,11-octadecadienoic acid derived from tomato juice acts as a potent PPARα agonist in obese diabetic mice [46]. On the other hand, treatment of obese rats with polyphenol rich Mediterranean tomato-based *sofrito* did not cause significant alterations in glucose profile. The authors reported that obese Zucker rat may not be an appropriate model of diabetes or glucose metabolism alterations and probably a longer period of administration of the dietary approach would be needed to appreciate significant modifications of the analyzed parameters [47].

2.4 Antioxidant Activity

Tomatoes contain abundant health-promoting components such as lycopene, provitamin A, ascorbic acid, vitamin E, folate, and flavonoids and health effects are mainly associated with the presence of antioxidants, especially carotenoids, flavonoids, lycopene, and β-carotene, which makes tomato an effective antioxidative food [4].

The antioxidants of tomato fruits are commonly classified as hydrophilic and lipophilic antioxidants. Hydrophilic antioxidants contain soluble phenolic compounds and vitamin C, which is the most effective antioxidant in plants [48], and also an important phytochemical of tomato fruit [49]. Vitamin C showed a significant impact on total antioxidant activity (83%), while the lipophilic activity is conferred by carotenoids, vitamin E, and lipophilic phenols (17%) [50].

Phenolic compounds are secondary metabolites known for their antioxidant activity [51], particularly their ability to scavenge free radicals, to donate hydrogen atoms or electrons, or to chelate metal cations [52]. At the cellular level, phenolic compounds participate in protecting the cell against the harmful action of ROS, mainly free oxygen radicals, produced in response to environmental stresses, such as drought, high light intensity, or mineral nutrient deficiency [53].

Lodovici et al. [54] investigated the effect of phenolic acids on oxidative DNA damage in vitro and they discovered that these compounds provided protection against DNA oxidation induced by iron at low concentration. An important role of tomatoes has been also attributed to the conservation of DNA stability. Daily intake of a tomato drink significantly reduced (by about 42%) DNA damage in lymphocytes subjected to oxidative stress [55].

Plants can rearrange their antioxidant defense system in order to rapidly neutralize and scavenge ROS, with

enzymatic as well as nonenzymatic antioxidants [56,57]. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) are considered important antioxidant enzymes, and these enzymes extenuate cadmium stress in plants due to their ascorbate-glutathione cycle [58]. The application of cadmium to tomato seedlings resulted in the increment of total flavonoid and phenolic contents by 63.38% and 70.57%, respectively [56]. Similarly, Kaya and Ashraf [59] worked with tomato and have reported the protective role of nitric oxide (NO) against boron-induced oxidative damage to membranes via the reduction of free radical production, which was possibly related with the upregulation of the antioxidant system that rapidly eliminates ROS, including H_2O_2.

2.5 Antiangiogenic Activity

Angiogenesis is the process in which new capillary blood vessels are formed, also called neovascularization; it rarely occurs under normal physiological conditions, except for embryogenesis, corpus luteum formation, and wound healing. Angiogenesis is important for the progressive growth of solid tumors and also permits the shedding of metastatic tumors from the primary site [60]. The phenolic and polyphenolic compounds have been investigated for their antiangiogenic activity over the years [61].

Phenolic acids have a protection against DNA oxidation and antitumor activity against colon carcinogenesis [54,62]. Caffeic acid can block the biosynthesis of leukotrienes that are involved in immunoregulation [63].

Quercetin's ability to inhibit tumor growth was also studied in vivo [64,65]. The underlying mechanism for quercetin is suppressing angiogenesis by inhibition of COX-2 and lipoxygenase (LOX)-5, interference with the epidermal growth factor receptor (EGF), the human epidermal growth factor receptor-2 (HER-2) intracellular signaling pathway, and the NF-κB nuclear transcription protein [65,66]. In addition, it was shown that caffeic acid possesses antitumor activity in humans [62].

2.6 Antiinflammatory Activity

Chronic low-grade systematic inflammation seemed to be a central mechanism underlying pathophysiology of metabolic syndrome [67], which is a constellation of metabolic abnormalities (glucose intolerance, insulin resistance, central obesity, dyslipidemia, and hypertension) [68]. Cytokine production is a feature of immune stimulation, and alterations in the cytokine profile can influence both the iron status and red blood cell profile. The effects of cytokines on iron bioavailability are largely mediated through a reduction in duodenal absorption of iron and a shift in the handling of iron by macrophages in favor of iron storage [69–71]. The elevated C-reactive protein (CRP) group had significant increases in proinflammatory cytokines, including interferon-γ (INF-γ), TNF-α, interleukin (IL)-1β, IL-6, and IL-8.

Phenolic compounds are considered to possess antiinflammatory properties and therefore have been proposed as an alternative natural approach to prevent or treat chronic inflammatory diseases [72]. It has been reported that consumption of tomatoes could possibly reduce disease development by reducing inflammation, inhibiting cholesterol synthesis, or improving immune function [73].

Burton-Freeman et al. [74] reported that tomato consumption during a meal attenuated postprandial lipemia-induced oxidative stress and associated inflammatory response. Gerardi et al. [75] found that tomato purees enriched with black carrot exhibited an increased antiinflammatory capacity, as shown by the reduced expression of vascular cell adhesion molecule VCAM-1 in human cultured endothelial cells, under inflammatory conditions.

Some authors have reported the implication of post-transcriptional regulation of IL-8 and monocyte chemotactic protein 1 (MCP-1) expression by quercetin in airway epithelial cells [76] and in IL-8 production by caffeic acid [77] in Caco-2 cells stimulated, respectively, by TNF-α or IL-1. There are also some studies on the correlation between a series of phenolic acids with the inhibition of activator protein 1 (AP-1) transcriptional activity, implicated in the processes that control inflammation, cell differentiation, and proliferation [78].

Tomato juice intake partially returned rat liver to the metabolic pattern of a normal diet from a high-fat diet [79]. On the other hand, serum concentrations of the cytokines IL-6 and TNF-α decreased significantly in overweight female students ($n = 56$) but not in control females consuming 330 mL/day tomato juice, suggesting that increasing tomato intake may help reduce inflammatory diseases associated with the two cytokines [80]. It has also been reported that quercetin exerts significant antiinflammatory effects on IL-1β activated human astrocytes, reducing cytokines and chemokines and oxidative stress [81].

2.7 Antibacterial, Antifungal, and Antiviral Activity

In nature there are several types of antimicrobial compounds that play an important role in the natural defense of all kinds of living organisms. The antiviral [82] and antimicrobial activity [83] of phenolic compounds has already been documented in the literature. There is also

some information on the interrelationship between phenolic compounds with antibacterial and antifungal effects [84].

Rauha et al. [85] performed the antimicrobial screening of 13 phenolic substances by using diffusion methods with four to nine microbial species (*Aspergillus niger, Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Staphylococcus aureus* and *Staphylococcus epidermidis*). Flavone, quercetin and naringenin were effective in inhibiting the growth of the organisms. Puupponen-Pimiä et al. [83] investigated the antimicrobial activity of pure phenolic compounds and according to their results the phenolic acids (cinnamic acid, 3-coumaric acid, caffeic acid, ferulic acid, and chlorogenic acid) showed activity only against Gram-negative bacteria at high concentrations (500 lg well^{-1}). Chlorogenic acid was the weakest inhibitor, exhibiting activity only against *Escherichia coli* CM 871. Luteolin was slightly inhibitory to Gram-positive lactic acid bacteria but not to Gram-negative bacteria. No clear-cut inhibition zones were detected, but the density of bacteria (cfu) in agar plates containing luteolin was significantly less than in control cultures, indicating weak bacteriostatic effects.

Jogaiah et al. [86] investigated the role of *Trichoderma virens* spores or cell-free culture filtrate in the regulation of growth and activation of the defense responses of tomato (*Solanum lycopersicum*) plants against *Fusarium oxysporum* f. sp. lycopersici by the development of a biocontrol-plant pathogen interaction system. Two-week-old tomato seedlings primed with *Trichoderma virens* spores cultured on barley grains or with cell-free culture filtrate were inoculated with *Fusarium* pathogen under glasshouse conditions; this resulted in significantly lower disease incidence in tomato Oogata-Fukuju plants treated with barley grains than in those treated with cell-free culture filtrate, and they concluded that *Trichoderma virens* barley grains and cell-free culture filtrate differentially induced jasmonic acid and salicylic acid signaling cascades for the elicitation of *Fusarium oxysporum* resistance in tomato.

Juárez et al. [87] presented a model human antibody for passive protection against the enteric pathogen rotavirus in transgenically labeled tomato fruits. They concluded that purple-colored extracts from transgenically labeled purple tomatoes contain elevated levels of recombinant antirotavirus neutralizing human immunoglobulin A in combination with the enhanced amounts of health-promoting anthocyanins.

2.8 Effects on the Intestinal System

Digestion is the key step for delivering nutrients and bioactive substances to the body. The way different food components interact with each other and with digestive enzymes can modify the digestion process and affect human health.

Figueiredo-Gonzales et al. [88] focused on the toxicity against human gastric adenocarcinoma cells and the antioxidant activity of hydromethanol, acetone, and alkaloid extracts prepared from the leaves of tomato plant cultivars. Alkaloid, hydromethanol, and acetone extracts of all cultivars reduced cell viability, IC_{50} values ranging from 9 ± 2 to 55 ± 11, from 103 ± 25 to 171 ± 29, and from 291 ± 26 to 459 ± 14 mg mL^{-1}, respectively. Moreover, both hydromethanol and acetone extracts scavenged NO (IC_{50} values ranged from 0.87 ± 0.12 to 1.54 ± 0.23 and from 0.90 ± 0.01 to 2.23 ± 0.14 mg mL^{-1}, respectively), but only hydromethanol was able to scavenge $O_2^{\cdot-}$ showing IC_{50} values from 0.12 ± 0.01 to 0.43 ± 0.08 mg mL^{-1}. These results demonstrated that tomato leaves, a by-product of the tomato processing industry, are a valuable source of bioactive compounds, providing beneficial properties for human health. In another study, Jannat et al. [89] aimed to evaluate the ability of raw mini tomato (*Solanum lycopersicum* L.) juice powder to attenuate cytotoxicity in combination with juice powders from two orange species, *Citrus sinensis* (L.) Osbeck and *Citrus unshiu* Marcow and they concluded that the results of combinations of juice powders, particularly at a ratio of 2:1:3, are a potentially useful therapeutic source of phenolic compounds for the treatment of oxidative stress-related hepatotoxicity.

Rutin, quercetin, glycosides of quercetin, and resveratrol have been shown to exert intestinal antiinflammatory activity [90,91]. In particular, rutin and quercetin have been proposed to act as quercetin prodrugs, preventing premature absorption of aglycone in the small intestine and releasing it in the colon [92,93]. In addition to the strong beneficial effects of phenolic compounds on human health, they also have been associated with a negative impact on the digestion process. In general, most of the polyphenols remain in the gastrointestinal tract due to their low absorption and they can possess inhibitory effects on enzymes involved in the degradation of saccharides, lipids, and proteins. Especially, inhibiting digestion of proteins is not desirable due to reduced utilization of amino acids [94].

3 CONCLUDING REMARKS

Tomatoes are rich sources of diverse bioactive compounds including lycopene, β-carotene, lutein, vitamin E, vitamin C, rutin, quercetin, glycosides of quercetin, and phenolic acids, which have been reported to have a positive role in the prevention of several diseases such as cardiovascular diseases, cancer, diabetes, etc., as described in detail within this chapter. However, in the

case of tomatoes, most of the research related to the health effects of tomatoes has been mainly focused on lycopene, β-carotene, and other carotenoids, and the effects of polyphenolic compounds have been mostly underestimated. On the other hand, in some of the studies different forms of tomato samples or extracts have been used to investigate their health effects, but the mechanism of action or the compounds responsible for these effects have been ignored. In conclusion, more in vivo and in vitro studies need to be performed to fill this gap in the research.

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CHAPTER

10

Polyphenolic Compounds in Sweet Cherries: A Focus on Anthocyanins

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Acronyms and Abbreviations

CRP	C-reactive protein
CVD	cardiovascular diseases
EN-RAGE	extracellular newly identified ligand for the receptor for advanced glycation end products
ET-1	endothelin1
FDA	Food and Drug Administration
FW	fresh weight
GI	gastrointestinal tract
HPLC	high performance liquid chromatography
IL-1Ra	interleukin-1 receptor agonist
IL18	interleukin-18
IQF	individual quick freezing
LDL	low-density lipoprotein
ORAC	oxygen radical absorbance capacity
PAI-1	plasminogen activator inhibitor-1
PAL	phenylalanine ammonia lyase
QoL	quality of life
SD	standard deviation
TNFα	tumor necrosis factor alpha

1 AN OVERVIEW OF THE NUTRITIVE AND NONNUTRITIVE PROPERTIES OF SWEET CHERRIES

Cherries are classified as a fleshy drupe of the stone fruit family. Common varieties (cultivars) include but are not limited to sweet cherries (*Prunus avium L.*), sour or tart cherries (*Prunus cerasus L.*), and black cherries (*Prunus serotina Ehrh.*). Cherries are thought to be native to Europe and western Asia and are now commercially cultivated in more than 40 countries worldwide, mainly in countries with temperate climates. All cherries have a central stony-hard seed surrounded with fleshy fruit and skin, which together contain most of the nutritive and nonnutritive components of the fruit. Sweet cherry ranges in color depending on the variety and can either be described as dark red, red, blush, or white. The main characteristics of sweet cherry quality include color, sweetness, sourness, and firmness. Sweet cherries are nutritionally dense in terms of both macro- and micronutrients and contain a larger proportion of simple sugars (13 g/100 g) in comparison to tart cherries (8 g/100 g) [1]. The simple sugars provide sweetness and mainly comprise glucose and fructose [2]. The sourness of sweet cherries is primarily due to the presence of malic acid, with the total organic acid content ranging from 3.67 to 8.66 g/kg fresh weight (FW) [3]. Sweet cherries also contain a range of vitamins including vitamin C, B, A, E, and K, potassium and melatonin.

Cherries are a phytochemical-rich fruit. The total polyphenol content of raw sweet cherries is on average 174.90 mg total polyphenols per 100 g of fresh weight (range 75–339 mg, standard deviation (SD) ±77.04 mg) [4]. Polyphenol is a classification term referring to a vast range of compounds present in varying quantities in virtually all plants. Polyphenols can be divided into the subclasses flavonoids, lignans, stilbenes, and phenolic acids (Fig. 10.1). Flavonoids are a major class of polyphenols, and in plants are nonnutritive secondary metabolites [5], which are further divided into six major classes: anthocyanins, flavanols, flavanones, flavones, flavonols, and isoflavones (Fig. 10.1).

Sweet cherries contain several classes of polyphenols, which are highlighted in Fig. 10.1, with one example of a predominant compound for each subclass. Sweet cherries contain the flavonoid groups anthocyanins, flavanols, and flavonols (Fig. 10.1). The predominant phenolic acids are hydroxycinnamates including neochlorogenic acid

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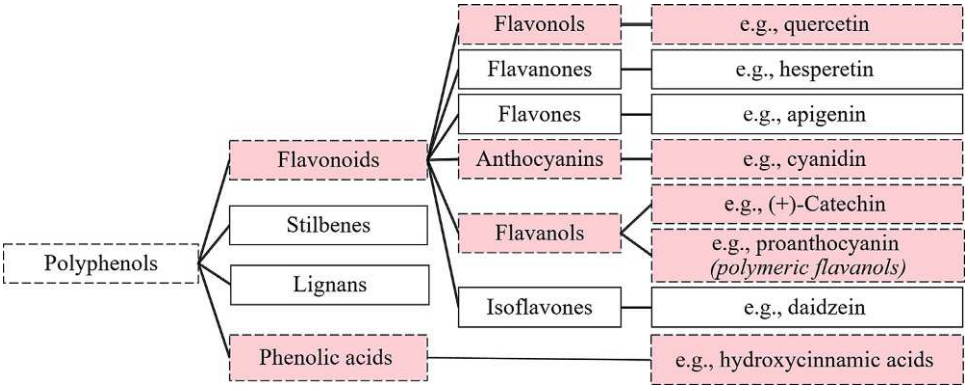

FIG. 10.1 Classification of polyphenolic compounds and examples of the major polyphenols in sweet cherries in the shaded boxes.

and *p*-coumarylquinic acid. Together in a synergistic manner, the polyphenols in sweet cherries act as secondary metabolites, in which they contribute to processes that allow the plant to adapt to its environment. These mechanisms include providing pigmentation and protecting the plant and fruit against pathogens and environmental stressors [6]. As a component of the human diet, secondary metabolites have been shown to provide potential health benefits over basic nutritional value [7].

2 A FOCUS ON ANTHOCYANINS IN SWEET CHERRIES

Anthocyanins (from the Greek terms *anthos* = flower and *kianos* = blue), are a subclass of dietary flavonoids (different from proanthocyanidins, which are polymers of flavanols, Fig. 10.1) responsible for the purple, red, and blue pigmentation in plant-based foods. Anthocyanins possess a positive charge, enabling the molecule to absorb light and therefore have color. It is inaccurate to use the terms anthocyanins and anthocyanidins interchangeably. Anthocyanins are glycosylated forms of anthocyanidins (aglycones) [8]. Anthocyanidins are initial degradation products in the gut during the microbial metabolism of anthocyanins, and they are highly unstable and consequently seldom found in nature [9]. Due to the instability of anthocyanidins, they are usually bound to a carbohydrate (sugar, usually glucose), which is esterified at the 3 position (Fig. 10.2). Approximately 95% of all anthocyanins are derived from six anthocyanidins: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, which exist in various glycosylated and acylated forms [9]. Anthocyanins share a common structure, comprising 15 carbons, with aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle (Fig. 10.2). In total, approximately 640 individual anthocyanins have been identified [10].

Anthocyanins constitute a significant proportion of the total polyphenol content of sweet cherries. The

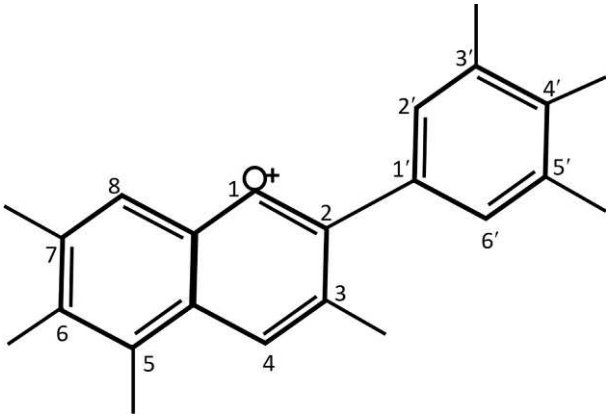

FIG. 10.2 Generic anthocyanin structure.

anthocyanin content of sweet cherries is outlined in Table 10.1, with data adapted from the Phenol Explorer Database [4]. These measures pool analytical data from several varieties of fresh sweet cherry, including 'Bing', 'Rainier', 'Royal Ann', 'Lambert', 'Summit', 'Van', and 'Stella'. A comparison with the anthocyanins comprising sweet and tart cherries is provided in Table 10.1. While tart cherries possess a larger total polyphenol content of 352.16 mg total polyphenols per 100 g (SD ± 61.74 mg), this is attributed to higher concentration of cyanidin-3-*O*-glucosyl-rutinoside specifically [11]. This anthocyanin accounts for approximately 70% of the total anthocyanins in tart cherries.

Anthocyanins have been shown to be concentrated in the skin of cherries [12] and, depending on the variety, may be limited to the skin or additionally distributed throughout the flesh. The red colors visible in sweet cherry skins and flesh are primarily due to anthocyanins, with darker fruit containing higher levels of anthocyanins [13]. Across cherry varieties, the anthocyanin content and profile differ significantly [14]. Cherries of similar color appearance may have different anthocyanin contents dependent on their harvest date and storage conditions. Anthocyanins have been shown to differ across growing

TABLE 10.1 Mean and Standard Deviation of Individual and Total Anthocyanins in Sweet Cherries and Tart Cherries in mg/100 g Edible Weight

	Sweet cherries	Tart cherries
Individual anthocyanins[a]		
Cyanidin-3-*O*-rutinoside	143.3±97.6	6.9±6.5
Cyanidin-3-*O*-glucoside	18.7±16.3	1.1±1.1
Cyanidin-3-*O*-glucosyl-rutinoside	–	43.6±57.4
Peonidin-3-*O*-rutinoside	7.4±7.5	2.7±0
Pelargonidin-3-*O*-rutinoside	1.2±1.3	–
Peonidin-3-*O*-glucoside	0.8±0.8	–
Total anthocyanins[b]	32.7±20.1	48.88±24.21

–, content not available. All data is expressed as mean content ± standard deviation in mg/100 g edible weight for raw fruit.
[a] *As determined by chromatography methods.*
[b] *As determined by pH differential method.*
Data adapted from Phenol Explorer database: Neveu VPJJ, Vos F, Crespy V, du Chaffaut L, Mennen L, Knox C, Eisner R, Cruz J, Wishart D, Scalbert A. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods, 2010.

FIG. 10.3 Example of visual variation in *red* pigment (which is correlated with anthocyanin content [16]) across 'Lapins' sweet cherries. *Photo source: Di Murch.*

regions [15], and even sweet cherries of a single variety grown in the same orchard within one season can possess different pigmentation (see Fig. 10.3). The evolution of color in sweet cherries closely correlates with anthocyanin levels, specifically for cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside and therefore cherries with differing pigmentation likely possess a different anthocyanin content.

In maturing cherries, anthocyanins play an important role with both defensive and physiological functions, which operate simultaneously [17]. Anthocyanins have been shown to act as visual attraction for pollinators and seed dispersers [18]. The astringent quality of anthocyanins may also deter animals from feeding on the fruit as it develops [18]. The role of anthocyanins in plant defense has been well reviewed (see Ref. [17]). As the anthocyanins in sweet cherries are secondary metabolites, their concentrations are influenced by many factors including in response to mechanical damage, pathogen attacks, and environmental factors such as climate, UV light exposure, and soil type. In addition, cherry variety, ripeness stage, harvest date, and storage conditions all impact on their anthocyanin content and profile [16]. The full impact of pre- and postharvest factors that impact on sweet cherry anthocyanins are explained in Section 5 of this chapter. In relation to human consumption, anthocyanins in sweet cherries add to sensory characteristics such as flavor, astringency, and color [19], influence their aesthetic value [18], and may significantly contribute to the observed health benefits of sweet cherries (see Section 10).

3 ANTHOCYANIN BIOSYNTHESIS IN SWEET CHERRIES

Anthocyanin biosynthesis and accumulation in growing cherries share an inverse relationship with the phenolic acids present in the fruit [20]. During early stage development cherries are high in phenolic acids and anthocyanin synthesis increases with ripening. The ripening process in cherries accompanies a change in color from green to red, which reflects the accumulation of anthocyanins and degradation of chlorophyll [1]. The accumulation of anthocyanins is shown to exponentially increase during ripening, and then their biosynthesis and accumulation is shown to slow down during the last part of the season [21].

Anthocyanin biosynthesis in plants occurs via the flavonoid pathway (see review [22] and see [21] for an overview of sweet cherry flavonoid biosynthetic pathway). Briefly, flavonoids are synthesized from phenylalanine (a product of the shikimate pathway) via the general phenylpropanoid and flavonoid pathway [22]. The flavonoid pathway branches from the general phenylpropanoid pathway, where 4-coumaroyl-CoA and malonyl-CoA are converted to tetrahydroxychalcone by the enzyme chalcone synthase. Tetrahydroxychalcone is later converted into flavanones, dihydroxyflavonols, and finally anthocyanins are synthesized [23].

In cherries, colorless dihydoflavonols are converted to colorful anthocyanin pigments in two steps [21]. Firstly, dihydroflavonol is converted to leucoanthocyanidin by dihydroflavonol-4-reductase. Secondly, leucoanthocyanidin dioxygenase oxidates leucoanthocyanidin to form anthocyanidins, which are then glycosylated. Various

glycosides can be produced depending on the dihydroflavonol substrate, the aglycone, and its conjugates. In addition to this process, further modification of the anthocyanin glycosides can occur through glycosylation, methylation, and acylation to elevate the number of different anthocyanins in cherries [21].

4 MEASUREMENT OF ANTHOCYANIN CONTENT IN SWEET CHERRIES

The anthocyanin content of sweet cherries is typically measured in two ways: either a colorimetric assay or by high performance liquid chromatography (HPLC). A comparison of the results of the two approaches in sweet cherries has shown that the total count of anthocyanins as determined by colorimetric assays was closely aligned with the sum of individual anthocyanins analyzed with HPLC [11]. This finding is important, and supports the practical application of colorimetric methods, which are quicker and cheaper to run. Once the anthocyanin profile (ratio of individual anthocyanins in a variety) has been established via HPLC, pigment evolution throughout the season or postharvest can be assessed by the more simple colorimetric approach [16].

Nonspecific measures of antioxidant activity, such as the oxygen radical absorbance capacity (ORAC) test, are sometimes favored by agricultural industries as a simple assessment of bioactive compounds in developing or harvested fruit. Although the ORAC assay is a preferable method for the assessment of antioxidant capacity and may reflect their biological relevance to in vivo with respect to antioxidant efficacy, in cherries antioxidant response may derive mostly from phenolic acids [24]. It is important to note that ORAC as a nonspecific test may reflect the anthocyanin content in sweet cherries in isolation, but is likely confounded by the presence of other antioxidant compounds such as vitamin C. For example, in one study the cherry fruit from variety 'Zio Peppino' contained low levels of total anthocyanins, but had a high antioxidant activity [25]. This result indicates that even though anthocyanins may not account for the total antioxidant activity of cherries, they may provide other significant bioactivities in vivo (see Section 10).

5 PRE- AND POSTHARVEST FACTORS THAT IMPACT ON ANTHOCYANINS IN SWEET CHERRIES

In sweet cherries, anthocyanin biosynthesis and pigment accumulation are mainly determined by the variety, but a range of preharvest factors including region, climate, and seasonal variations as well as orchard management decisions (such as plant density and the use of growth regulators) define the expression and manifestation of this potential. After the cherries have been harvested, critical factors such as transport, packaging, and storage conditions as well as manufacturing processes influence the levels of anthocyanins in sweet cherry fruit.

Cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside represent the major anthocyanins in sweet cherries. However, particular levels of individual anthocyanins are variety-specific and can be influenced by a range of internal and external factors. These factors include light intensity and spectrum, temperature, and plant hormones, which can affect the biosynthesis of anthocyanins through changes in the expression of structural and regulatory genes [26]. The complex molecular mechanisms behind the regulation of anthocyanin biosynthesis in sweet cherry have only recently been investigated [27,28]. Specific transcription factors (i.e., PavMyB10.1) have been identified and linked to the accumulation of anthocyanins, which starts approximately at the end of the pit-hardening stage and reaches its maximum at maturation [27], making the stage of ripeness and date of harvest critical factors for total anthocyanin content in sweet cherry [2]. A recent study found that the inherent anthocyanin composition and genetic variability of each variety may be a more significant factor than other environmental influences [29].

Not only variety, but interactions between variety and rootstock influence total anthocyanins in cherries. Rootstocks are the underground parts of the plant with a root system, onto which the target variety has been grafted. The average content of total anthocyanins over 2 years in the varieties 'Kordia' and 'Regina' grown on four different dwarfing rootstocks was shown to vary, ranging between (mean ± SD) 33.18 ± 4.51 and 39.85 ± 13.03 mg/100 g FW for 'Kordia' and between 17.53 ± 8.53 and 26.19 ± 12.98 mg/100 g FW for 'Regina'. In addition to the rootstock effect, a strong seasonal influence on total anthocyanins of both varieties was recorded [30]. This seasonal effect was also demonstrated using four sweet cherry varieties ('Burlat', 'Saco', 'Summit', and 'Van'), which showed significantly higher total anthocyanins in 2002 compared to 2001 [31]. Variations in seasonal climatic conditions such as average temperature and solar radiation can explain these differences. Induced changes in microclimate (the climate in a small or restricted area) have been shown to lead to earlier ripening and higher anthocyanin content in fruit grown in poly-tunnel systems, compared to fruit cultivated under field conditions [32].

Environmental conditions at ripening stage and agronomic decisions such as the application of preharvest treatments may impact on the anthocyanin content of sweet cherries. In a controlled environment trial, greater ultraviolet light exposure was associated with increased anthocyanin concentrations in sweet cherries [33]. If extrapolated to "real world" conditions, stronger

environmental ultraviolet light exposure to cherries during ripening may result in a higher accumulation of anthocyanins [33]. In addition, higher temperature has been shown to increase anthocyanin contents of fruits [34]. Preharvest application of growth regulators may assist the grower in regulating the ripening process. It has been shown that the application of preharvest methyl jasmonate (MeJA) delayed the ripening process and, dependent on variety, reduced the anthocyanin content at harvest [35]. Preharvest treatments with oxalic acid resulted in significantly higher levels of anthocyanins in 'Sweetheart' and 'Sweet Late' cherries at the time of harvest without compromising other fruit quality parameters [36]. The same varieties also showed enhanced anthocyanin contents after the preharvest application of salicylic acid and acetylsalicylic acid [37]. In addition to higher initial levels of anthocyanins at harvest, salicylic acid and acetylsalicylic acid had positive effects in preserving cherry fruit quality and enhancing anthocyanin levels during postharvest storage [38]. Understanding the changes in endogenous levels of growth hormones during fruit ripening and their impact on fruit quality and anthocyanin content is a crucial step in assisting growers to adjust the external application of growth regulators [39].

Orchard management decisions, especially the date of harvest, can influence anthocyanin content. One study showed that delaying the harvest of sweet cherries by 4 days maximized the nutritional (highest sugar and organic acid contents), sensory (greatest firmness and color development), and functional (greatest phenolics content, anthocyanins and antioxidant capacity) quality of sweet cherries [40]. This is in agreement with a study in which the varieties '0900 Ziraat', 'Regina', and 'Sweetheart' showed significantly higher levels of anthocyanins one week after the optimum date of harvest [35]. However, the optimum date of harvest may be significantly different between cherry varieties and needs to be determined individually on a seasonal basis to guarantee the best possible outcomes for functional properties and other economically relevant fruit quality factors.

The aforementioned agronomic decisions and environmental factors will also impact on anthocyanin levels in sweet cherries during postharvest storage and processing, where levels of anthocyanins are enhanced and maintained throughout storage [38]. Storage temperature and duration influence total anthocyanin levels in cherries; however, the relative amounts of individual anthocyanins remain stable [41]. Enzymatic studies have shown that the process of anthocyanin biosynthesis is still active during storage. 'Hardy Giant' cherries stored for 8 days at 20°C had significantly higher total anthocyanin levels and PAL enzyme activity (key enzyme in anthocyanin metabolism) compared to cherries stored at 1°C [42]. A comparable pattern was obtained for four varieties ('Burlat', 'Saco', 'Summit', and 'Van') stored at 15±5°C and 1–2°C; however, the extent of color and anthocyanin enhancement was variety-dependent. With only a few exceptions, anthocyanin levels after storage were higher than initial values [16]. In contrast, cold storage at 1°C for 15 days resulted in a significant decline in the contents of cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside in 'Sciazza' and 'Ferrovia' cherries [43].

Postharvest storage is an important consideration as cherries are often stored at low temperatures for several weeks before reaching consumers. Improvements in postharvest packaging, such as modified atmosphere packaging, have shown to be promising postharvest storage options for the maintenance of anthocyanin concentrations in sweet cherries under cold storage conditions [44]. The benefits of higher storage temperatures for functional fruit quality may compromise other commercially relevant quality parameters, such as fruit weight and firmness, which are best maintained at a storage temperature of 0°C [41]. Adjustments in storage temperature and duration may be a useful tool to influence cherry quality parameters for fresh consumption or the production of anthocyanin-rich value-added products.

Irrespective of efforts in optimizing pre- and postharvest management, anthocyanin concentrations in sweet cherries remain vulnerable to uncontrollable natural product variability as a result of the complex interplay of internal and external preharvest factors, harvest timing, and postharvest handling and storage decisions.

6 PATTERNS OF GLOBAL GROWTH AND ECONOMIC IMPORTANCE OF SWEET CHERRY PRODUCTION

Sweet cherry is becoming an increasingly important horticultural crop with over 3,156,000 tons of cherries (including tart) produced globally in 2016/17 (Table 10.2) with an increase to 4,000,000 tons forecast for 2020. Sweet cherries are an economically important fruit, with the global cherry market revenue increasing at approximately 2.4% per year over the last decade, with revenue now approaching almost $10 billion USD. The vast majority of sweet cherry is produced in the northern hemisphere with Turkey and the United States together accounting for almost one-third of the total output (Table 10.2). The southern hemisphere is a relatively small producer in comparison with Chile producing 124,000 tons and Australia producing 16,000 tons. The United States is the biggest exporter of cherries by volume (Table 10.2), where over 25% of their annual production is exported overseas. By contrast, Chile exports up to 80% of its annual produce to other countries, filling an important gap in northern hemisphere production. China and Hong Kong are the major importers of cherries corresponding with the greater trends in consumption observed for these countries.

TABLE 10.2 Five-Year Trends in Global Cherry Production by Region

	Year[a]	2012/13	2013/14	2014/15	2015/16	2016/17	2017/18
PRODUCTION OF FRESH CHERRIES IN TOTAL							
Region	European Union	622	724	758	751	730	576
	Turkey	500	510	465	565	525	500
	United States	418	401	462	417	458	495
	China	170	180	220	250	330	360
	Ukraine	245	282	280	280	280	280
	Russia	255	278	278	278	278	278
	Uzbekistan	126	140	140	140	140	140
	Serbia	97	126	126	126	126	126
	Chile	65	86	127	103	116	124
	Syria	82	62	62	62	62	62
	Other	105	117	115	115	111	116
	Total	2686	2906	3033	3087	3156	3057
FRESH CHERRIES FOR DOMESTIC CONSUMPTION							
Region	China	209	214	280	336	431	455
	European Union	377	401	422	424	424	389
	Russia	332	363	347	352	346	345
	Turkey	289	301	260	341	290	275
	United States	214	142	170	167	171	194
	Ukraine	93	126	129	129	129	128
	Uzbekistan	121	123	123	135	111	111
	Other	371	360	393	391	428	409
	Total	2006	2030	2123	2275	2329	2307
CHERRIES USED FOR PROCESSING INTO PRODUCTS							
Region	European Union	262	328	339	315	338	207
	United States	116	202	218	189	224	201
	Turkey	155	155	155	155	155	165
	Ukraine	150	152	150	150	150	150
	China	2	4	5	6	8	10
	Chile	4	4	8	6	5	5
	Canada	6	6	5	5	5	5
	Other	2	2	2	2	2	2
	Total	697	853	881	828	887	745
IMPORTED CHERRIES							
Region	China	42	38	65	92	109	105
	Hong Kong	46	32	58	63	100	75
	Russia	76	85	69	74	68	67
	European Union	47	50	42	37	58	50

TABLE 10.2 Five-Year Trends in Global Cherry Production by Region—cont'd

	Year	2012/13	2013/14	2014/15	2015/16	2016/17	2017/18
	Canada	37	25	30	24	24	30
	South Korea	10	9	13	13	14	19
	Belarus	3	8	12	44	18	15
	Taiwan	15	9	12	9	11	12
	United States	13	10	13	14	13	10
	Japan	11	7	5	5	5	5
	Other	4	7	3	4	9	9
	Total	304	279	323	378	427	397
CHERRIES EXPORTED							
Region	United States	101	67	88	75	76	110
	Chile	51	67	103	84	96	105
	Turkey	57	54	50	69	80	60
	European Union	30	43	39	48	25	30
	Uzbekistan	5	17	17	5	29	29
	Serbia	11	19	12	8	19	23
	Azerbaijan	7	4	10	12	15	14
	Canada	8	6	9	14	10	12
	Belarus	1	3	7	38	6	8
	Australia	3	3	4	6	3	5
	Other	15	18	12	6	7	7
	Total	288	301	351	362	366	403

All data expressed as units in 1000 metric tons.

[a] *For the northern hemisphere, the marking year commences in January while in the southern hemisphere, the marketing year commences in November, reflecting seasonal changes. Data adapted from Foreign Agricultural Service/USDA Office of Global Analysis [https://www.fas.usda.gov/data/stone-fruit-world-markets-and-trade].*

There are now hundreds of commercial varieties available. For most countries there are a few predominant varieties, with consideration for varietal selection based on harvest timing and market access, fruit quality and eating attributes, and shelf-life with the suitability for long-distance shipping [45]. Varietal choice for growers is primarily aimed at producing cherries that are visually acceptable rather than for their nutritional properties, such as anthocyanin content, or their perceived health benefits.

7 IMPORTANT SENSORY ATTRIBUTES AND TRADITIONAL QUALITY INDICATORS OF SWEET CHERRIES

Traditional fruit quality indicators and sensory attributes for sweet cherry are usually associated with fruit maturity, size, skin color, flavor (sweetness, sourness), firmness, and stem color [46]. Fruit size is an important contributor to the perception of a high-quality cherry and is of critical consideration for export grading (Fig. 10.4 depicts large, top-grade sweet cherries of the 'Lapins' variety). However, larger fruit size decreases the skin to volume area of the fruit, which may impact on the relative anthocyanin content of the fruit. Consumer preferences for cherry fruit color is related to consumer perceptions of sweetness, where darker red cherries were considered sweeter than lighter red fruit [46]. As darker red cherries contain higher levels of anthocyanins [31], this may have indirect benefits for consumers inadvertently choosing cherries with potentially greater nutritional benefit.

For sweet cherry, attributes of taste (sweetness and flavor), freshness, color, and size have been strongly correlated with consumers being willing to pay premium prices for higher quality cherries [47]. The sweetness of cherries is measured instrumentally by total soluble

FIG. 10.4 Fruit size is an important contributor to the perception of a high-quality cherry. *Photo source: Di Murch.*

solids and the tartness/sourness is measured instrumentally by titratable acidity/pH. Both of these quality outcomes are critical for consumer perceptions [46] where there is a strong relationship between the sweet-sour balance and sensory rating given by consumers. Anthocyanins influence mouthfeel and astringency of fruit, but more research is required to demonstrate a relationship between the anthocyanin content of fruit and consumer perceptions. Interestingly, fruit nutritional attributes are rarely considered in studies investigating consumer willingness to pay or consumer sensory studies. This may be due to a lack of awareness of which sweet cherries will be nutritionally richer or the failure of researchers/marketers to consider nutrition as an important influence of consumer purchasing behavior.

8 CONSUMER PREFERENCE FOR SWEET CHERRIES MAY NOT BE DRIVEN BY HEALTH

Perceived fruit quality is a strong driver of consumer purchasing behavior. In one of the largest studies of its type, consumer preferences for various sweet cherry attributes were investigated using choice experiments. The findings challenged the commonly held notion that the color of sweet cherry fruit is the strongest influencer of purchaser decision making [47]. Instead the authors reported that when a consumer is a frequent purchaser of sweet cherry, they will place the highest value on fruit they know has the greatest sweetness and flavor. This research also highlights that red color (and therefore anthocyanin content) is not a major driver for purchasing behaviors.

Yet, with such a strong focus on the fruit quality aspects of cherries, it appears there is a missed opportunity for marketing cherries for their health benefits. If cherry varieties with the highest anthocyanin content could be clearly identified and labeled, this may provide a competitive edge for growers. For other summer fruits, such as plums, increasing interest in nutraceuticals and functional foods has led plant breeders to initiate selection of crops with higher than normal phenolic antioxidant contents. However, for sweet cherries specifically, levels of consumer awareness on the health benefits are still largely unknown and even less is understood on how increased knowledge may influence consumer purchasing behavior.

More specific marketing strategies that attract consumers to cherries are required to meet a growing demand by consumers for potentially nutritionally superior foods. This demand is actively shaping both the cherry industry through variety choice and orchard management, in addition to the types of value-added products made from second-grade or waste cherries. There is a growing interest in functional foods and health recipes containing bioactive compounds recovered from cherry seconds [48]. As described in Table 10.3, research is continually developing new methodologies for creating cherry extracts containing bioactive compounds that aim to also contribute to the health and well-being of the consumer. These methods are favored by research to overcome seasonal variations in anthocyanin content of fruit, and the availability of fruits. Additionally, the fact that consumers can be quickly and easily provided with a standard "dose" may be useful once a dose-response relationship with sweet cherry consumption and bioactivity is further investigated and confirmed. Also, products like juices and extracts ensure that groups of people who cannot consume cherries due to health conditions, such as dysphasia, are able to include cherry products in their diets.

Of the fruit produced, approximately 60% of cherries are consumed fresh, with the remaining 40% either discarded or processed (Table 10.3) [45]. Cherries can be processed in a variety of ways, and these products help overcome seasonality restrictions, and also minimize food waste from the harvest as products can be made from lower-grade fruit. However, food processing of cherries into value-added products tends to lower the contents of valuable nutrients. Additionally, anthocyanins are unstable during processing, so maintaining

TABLE 10.3 Methods of Processing for Sweet Cherry Products, and the Advantages (✓) and Disadvantages (×) of Each Method

Product	Processing method	Advantages and disadvantages
Frozen cherries	**1.** Conventional freezing at −18°C **2.** Individual quick freezing (IQF) in a high-velocity blast freezer	× Conventional freezing may lead to loss of turgidity and firmness ✓ IQF enables more uniform rate of freezing and may prevent browning and/or flavor loss
Cherry juice	**1.** Cherries are crushed and extracted (using cold or hot extraction) **2.** Pectinase enzyme (0.1%) is added to increase extraction and juice is clarified	✓ Retention of anthocyanin content ✓ Consistent composition minimizes the nutritional differences between varieties and geographic origin ✓ Juices can be used to produce concentrates and extracts
Wine and distilled cherry products	**1.** Fermentation and distillation of cherries **2.** Application of different fermentation parameters to influence characteristic taste and mouthfeel of wine Cherry spirits contain 37.5%–70% ethanol (v/v)	✓ Cherry pits can be retained, as they contain amygdalin which degrades during the fermentation process leaving an almond-like flavor characteristic in the wine
Canned, jarred or brined cherries	**1.** For canned and jarred cherries, fruits are soaked with syrup before processing in an exhausting tunnel at high temperature **2.** For brined cherries, cherries are packed in a 1%–1.5% sulfur dioxide solution	× Both methods reduce cherry firmness, therefore temperature and duration are important considerations × Both preservation techniques may leach anthocyanins and phenolic compounds
Dried cherries	**1.** Dehydration of fully ripe cherries with high sugar levels using freeze-drying or air-drying with blanching treatment	✓ Retention of high phenolic content
Extracts (Crude, bioactive; mixture; purified)	Various processing methodologies include: **1.** microwave or ultrasonic assisted extraction **2.** subcritical water extraction **3.** ultrahigh pressure extraction **4.** homogenization and sonication with methanol as solvent	✓ Retention of bioactive components for further utilization in food or as pharmaceutical × Variation in anthocyanin recovery for different processing methodologies

Information adapted from Chockchaisawasdee S, Golding JB, Vuong QV, Papoutsis K, Stathopoulos CE. Sweet cherry: composition, postharvest preservation, processing and trends for its future use. Trends Food Sci Technol 2016;55:72–83.

anthocyanins through industrial handling is a challenge [12]. The anthocyanin content of cherry products is likely to be compromised in comparison to the fresh-fruit equivalent, and will depend on the specific processes.

9 SWEET CHERRY CONSUMPTION AND CONTRIBUTION TO HABITUAL ANTHOCYANIN CONSUMPTION

Despite various estimates of anthocyanin intake, there are limited data available to estimate sweet cherry intake and the relative contributions of cherries to total anthocyanin intake in humans. Economic data show that sweet cherry sales have increased over the past decade. In line with this, recent statistics from the United States suggest that the last decade has seen a per capita increase of fresh sweet cherries in the United States by over 150%, with annual consumption estimated to be 1.2 kg per person [49]. However, this occurred with marked decline in processed (frozen and canned) sweet cherry over the same period. Per capita consumption of cherries in Australia is significantly less than in the United States, with an average annual per capita consumption of 579 g.

Anthocyanins [50] are wide spanning across the human diet, with rich dietary sources including berries, such as blueberries and blackberries; fruits, such as cherries and plums; vegetables, such as red cabbage and red onion; pulses, such as black beans; and beverages, such as red wine and cranberry juice. Daily intakes of anthocyanins have been estimated in several populations with extensive variations, which may reflect actual differences in dietary patterns, or could be influenced by differences in dietary assessment methods utilized and reference food composition databases applied to the dietary data. A US estimation using large-scale dietary data reported an average intake of 12.5 mg/day [50]. Anthocyanin intakes in Greek and Spanish adults were 20.9 and 18.9 mg/day, respectively [51,52]. A much higher estimation of anthocyanin intake in Finish adults (47 mg/day) may be a reflection of higher habitual berry consumption [53].

The contribution of cherries to anthocyanin intake may vary according to the population in terms of both age and

geographical location, among individuals with different education, financial status, and culture [50]. In a recent study on flavonoid intake in older adults, it was reported that cherries contributed 10% to total anthocyanin intake in that population [54], and cherries were ranked the third highest contributor for anthocyanin intake and eighth for flavonoid intake overall. Another population-based study has estimated that habitually 0.46 g of sweet cherries are consumed daily, providing 0.56 mg of anthocyanins per day to the human diet [50]. This contributed less than 1% of anthocyanins provided by all fruits (in the same analysis blueberries contributed 38.74% anthocyanins provided by fruit). As the majority of sweet cherry consumption is from fresh fruit, there is likely to be significant seasonal differences in intake based on the availability of fruit.

The seasonality of cherries in the northern hemisphere depends on where the cherries are produced, with latitude, altitude, and import regulations having important influences on cherry availability [55]. Generally speaking, sweet cherries are available late spring through to the end of summer, with seasonal gaps in the market filled by imports from a range of countries. For example, in the nonproductive season, China imports over 90,000 tons of sweet cherries from Chile with additional supply from Australia and New Zealand to meet demands around Chinese New Year. In the southern hemisphere, the Australian market is fairly unique in that peak consumption is aligned with supply from the domestic market. Early season cherries are available from October with domestic supply (approx. 16,000 tons) continuing through to late February.

10 SWEET CHERRIES AND HEALTH OUTCOMES: POTENTIAL MECHANISMS OF ACTION

The role of diet is a central factor in the prevention and the protection against chronic disease. To understand how complex dietary patterns may impact on human health, it is important to also identify the unique aspects of individual foods and isolated dietary components, and how they may individually affect disease risk. Plant-based foods form an integral component of the human diet and their consumption is consistently linked to a range of improved health outcomes, including the prevention of a broad array of diseases. Early research attributed the health benefits of a plant-rich diet to nutrients and antioxidant vitamins. However, more recently, there is a growing body of literature supporting the role of phytochemicals and linking their consumption to observed health benefits in addition to nutrients. In particular, a high consumption of both total flavonoids, flavonoid subclasses, and individual flavonoids has been linked with a protection against chronic diseases, including cardiovascular conditions, by influencing vascular changes [56] and neurodegenerative diseases [57] by guarding areas of the brain against age-related decline [58,59]. However, the extensive body of evidence for various cancers shows conflicting or neutral results.

Recent reviews have linked anthocyanin-rich food consumption with improvements in various health outcomes, including cardiovascular disease [60] and cognitive outcomes [61]. However, each review recommends that further population-based and long-term human intervention studies are necessary to evaluate the role of anthocyanins in specific-disease pathologies. At present, it is difficult for intervention studies linking anthocyanin-rich food intake with health outcomes to be compared in a meaningful synthesis, due to the differences in food-based interventions used and diverse health-outcomes of interest.

This section will evaluate the available literature surrounding the health benefits for sweet cherries, specifically. Individualized study of cherries is justified due to the potential impact of their unique food matrices on the bioavailability of anthocyanins [62], and the inherent nutritional and non-nutritional differences between cherry varieties. For a recent review of tart cherries, please see Abla et al. [63]. Major factors from within the food matrix that impact on anthocyanin bioavailability include other antioxidants and macronutrients present in the food consumed [62], which consequently affect the absorption and antioxidant capacity of anthocyanins. As cherries are rich in other phytonutrients, macro- and micronutrients, they could undergo a distinctive metabolism, in comparison to other anthocyanin-rich fruits. For sweet cherries, a major factor limiting health research is a lack of understanding about the bioavailability and metabolism of anthocyanins from this unique dietary source. In addition, sweet cherry research is presented for the following outcome measures: bioavailability, inflammation, cardiovascular diseases, neurodegenerative diseases, and gut health. Lastly, the subjective tolerance to sweet cherry interventions by research participants is discussed.

10.1 Anthocyanin Metabolism: A Lack of Data for Sweet Cherries

The bioavailability of polyphenols varies widely across different subclasses and individual compounds. Additionally, the same polyphenolic compounds may possess differing bioavailability across their various dietary sources. In sweet cherries, the maturity state of the fruit has been shown to impact on the polyphenol bioavailability [64]. For anthocyanins, major developments in our understanding of their metabolism and

bioavailability reflect the rapidly growing evidence from human studies linking anthocyanin intake to improved health outcomes [65]. However, at present in vitro experiments constitute a large fraction of our current knowledge, and while these studies are fundamental to our understanding, they may be only partially representative of actual human metabolism and physiology.

In general, anthocyanins metabolism has been well reviewed [66], documenting their extensive metabolism following ingestion. Briefly, metabolism may begin in the oral cavity, with saliva (which possesses strong hydrolic activity) hydrolyzing anthocyanin glycosides [67]. The small proportion of anthocyanins to reach the upper gastrointestinal (GI) tract is directly absorbed (1%–2%) due to their molecular size. Anthocyanins absorbed through the gut are transported to the liver where they are conjugated, commonly by methylation, sulfation and glucuronidation [68]. Conjugated compounds are then released to the gut flora via the biliary route, where they undergo reabsorption in the colon [68]. However, the largest proportion of anthocyanins is passed through the intestine to the colon, where an abundance of colonic microflora metabolize the anthocyanins into a variety of phenolic and aromatic acids and other metabolites [69]. These metabolites are the main transport forms of anthocyanins in vivo [70]. Without considering anthocyanin metabolites, the estimated bioavailability of anthocyanins is very low, with most studies reporting less than 2% recovery in urine. If metabolites are included, at least 15% of anthocyanins consumed are detectable in biological samples [71]. This highlights the significant role of the gut microflora in the metabolism, absorption, and subsequent bioavailability of anthocyanins and their metabolites. The major metabolites that are bioavailable in biological tissues following anthocyanin ingestion and metabolism have been identified, with metabolites measured in plasma, urine, and stool samples.

For sweet cherries specifically, only one study has been identified that documents changes in phenolic acid metabolites after sweet cherry consumption [72] in humans. An acute pilot, cross-over study documented changes in plasma concentrations over 6 h for eight anthocyanin-related metabolites: ferulic, isoferulic, caffeic, p-coumaric, syringic, vanillic, 4-OH benzoic, and t-cinnamic acids [73] after consumption of 300 mL sweet cherry juice. Following sweet cherry juice intake, there was a documented increase in each biomarker, confirming the uptake and metabolism of anthocyanins from the cherry juice. However, this was not a comprehensive metabolic analysis, and therefore a full metabolic profiling approach is needed. Although the metabolism of anthocyanins may be similar to other fruits that possess similar anthocyanins, and these studies could inform target metabolites, there may be a unique bioavailability profile for sweet cherries due to synergistic interactions with the food matrix, and therefore an isolated investigation is justified.

10.2 Antioxidant Activity and Inflammation

Inflammation is the body's response to "injury" and can either be acute or chronic, with chronic inflammation related to a host of poor disease outcomes. The mechanisms of action by which flavonoids exert in vivo antiinflammatory actions have been hypothesized [74]. Both isolated anthocyanins and anthocyanin-rich interventions have been shown to influence DNA cleavage, estrogenic activity, enzyme inhibition, cytokine production, antiinflammatory activity, and lipid peroxidation [74].

While anthocyanins possess powerful antioxidant activity, depending to a large extent upon their chemical structure, they are biologically unstable and are rapidly metabolized after they are consumed. Therefore, it has been hypothesized that not the parent anthocyanins directly but the smaller metabolites may be responsible for a large proportion of their bioactivity [75]. This is despite possessing significantly smaller antioxidant capacity in comparison to their parent compounds. However, the antioxidant capacity of sweet cherries has been shown to be high [76]. Therefore, for the 1%–2% of anthocyanins that are absorbed intact, their antioxidant activity may be biologically relevant. An in vitro study has reported that intact anthocyanins from sweet cherries demonstrated a 47% inhibition of cyclooxygenase-I and cyclooxygenase-II (enzymes related to pain and inflammation) [77].

In a small sample of healthy adults ($n=18$), consumption of 280 g/day of 'Bing' sweet cherries for 4 weeks, delivering 99.68 mg of anthocyanins, was shown to reduce various serum biomarkers of inflammation [78] and chronic inflammatory biomarkers [79]. The biomarkers include C-reactive protein (CRP), ferritin, interleukin-18 (IL18), tumor necrosis factor alpha (TNFα), interleukin-1 receptor agonist (IL-1Ra), endothelin1 (ET-1), extracellular newly identified ligand for the receptor for advanced glycation end products (EN-RAGE), and plasminogen activator inhibitor-1 (PAI-1).

Gout is a form of inflammatory arthritis characterized by excess uric acid in the bloodstream and deposited in joint tissues. Cherry (and cherry extract) consumption has been shown to be associated with a decreased risk of recurrent gout attacks in the 2 days following consumption in a case-crossover observational study [80] over 1 year. One human intervention study was identified, in which a single 280 g serving of pitted 'Bing' sweet cherries containing a dose of 106.4 mg anthocyanins was administered to healthy women ($n=10$). The results of the acute trial indicated that sweet cherry consumption was linked to a reduction in circulating levels of serum urate [81], which may protect against a gout attack. Of interest, claims (based on unsubstantiated data) have been made by several cherry producers, who have consequently been issued warnings by the US Food and Drug Administration (FDA) [63].

10.3 Cardiovascular Diseases

Cardiovascular diseases (CVD), including stroke and coronary heart disease, are a major cause of mortality in developed countries and are closely associated with modifiable lifestyle factors including diet [82]. Mechanistic studies indicate that flavonoids, including anthocyanins, exhibit beneficial effects on biomarkers of CVD risk such as nitric oxide, inflammation, and endothelial dysfunction [83]. Anthocyanin-rich foods have been hypothesized to alter CVD risk factors including reducing ischemia, blood pressure, and blood lipids [74], although the results are inconsistent. The mechanisms of action may be varied [84], with reviews indicating a multiplicity of effects [85]. The antioxidant capacity of flavonoids has been postulated to reduce arterial stiffness through preventing the oxidation of low-density lipoprotein (LDL) cholesterol [86]. Another mechanism relates to a reduction in circulating free radicals, reducing thrombogensis and platelet aggregation in the body. The ability of flavonoids to modulate endothelial function and thus influence blood pressure has also been investigated and relates to the modulation of nitric oxide bioavailability to the endothelium, resulting in endothelium-dependent vasodilation [87–89]. It is possible that a culmination of all these effects may provide a protection against CVD.

For sweet cherries specifically, only one pilot study was identified that documented changes in blood pressure in human subjects ($n=16$) after consumption of sweet cherry juice (300 mL) [72]. Blood pressure was significantly reduced 2 h after consumption and returned to baseline levels 6 h after consumption. The reduction of blood pressure and heart rate measurements at 2 h is supported by data from other acute anthocyanin feeding studies [90], whereby anthocyanins reportedly reach the circulatory system within 0.25–2 h and are excreted by 4 h postprandially. This data indicates that intact anthocyanins (not their metabolites) may be responsible for these vascular effects. However, an adequately powered trial would be warranted to confirm these findings.

10.4 Cognition and Neurodegeneration

Of all flavonoids, anthocyanins have been shown to be among the most beneficial subclasses in the area of neuroprotection [59]. Initially, the biological actions of flavonoids on the brain were attributed solely to their antioxidant ability, scavenging free radicals and reducing inflammation. However, their concentration in brain tissue is very low, and their ability to cross the blood-brain barrier was questioned [91]. Therefore, the actions of flavonoids in the brain have been investigated further and a variety of mechanisms has been postulated alongside their antioxidant activity. These include an ability to protect vulnerable neurons, to increase the number and the strength of the connections between neurons [59], and to enhance existing neuronal function (via the modulation of signaling processes) [92]. Additionally, flavonoids have been shown to stimulate blood flow to the brain and induce neurogenesis in the hippocampus, the area of the brain associated with memory [91]. The culmination of these mechanisms has been theorized as the potential pathways by which anthocyanins may influence memory, learning, and cognition and prevent neurodegeneration.

Only a few studies utilizing sweet cherries have investigated their neuronal or cognitive benefits. One in vitro study investigated the impact of total cherry polyphenols on neuronal cell survival [11]. The study reported that sweet cherries protected neuronal cells (PC 12) from cell-damaging oxidative stress in a dose-dependent manner, which was attributed to the presence of anthocyanins [11].

Two human trials have investigated the acute and chronic cognitive effects of supplementing older adults (with and without dementia) with anthocyanin-rich Bing cherry juice. The acute trial found no cognitive benefit associated with consuming anthocyanin-rich cherry juice (300 mL) in three groups ($n=16$ in total) of younger adults, older adults, and people with dementia [93]. However, this was a pilot trial and may have been underpowered to detect cognitive changes. The longer-term trial [94] supplemented the diets of older adults with dementia ($n=49$) with either anthocyanin-rich cherry juice (138 mg anthocyanins), or a control juice with minimal anthocyanins for 12 weeks. Cognitive improvements for verbal learning and short- and long-term memory were documented for the intervention (cherry juice) group after 12 weeks, with an accompanying reduction in blood pressure as secondary outcome. These preliminary findings are promising but several more well-controlled trials are required to confirm the cognitive benefits of sweet cherries.

10.5 Gut Health

The microbiota is now considered a metabolic organ, and the relationship between anthocyanins and microbiota are twofold. Firstly, anthocyanins are subjected to metabolism by microbiota, and, secondly, anthocyanins and/or their metabolites may modulate growth of specific bacteria produced from the microbiota [95]. Human genetic differences impact on intestinal microorganism populations, which could lead to differences in absorption and metabolism of anthocyanins between individuals [69]. Research in relation to anthocyanins and microbiota is limited at present, and of that data only one investigation into sweet cherries has been identified.

It was recently hypothesized that, based on cherries' polyphenolic profiles, *Lactobacillus*, *Bifidobacterium* and/

or *Bacteroides* ("good bacteria") may be stimulated, and that the bioavailability of specific polyphenols in various cherry products may influence cherry impact on gut microbiota. This hypothesis has been evaluated in one animal model [96], with dietary supplementation with sweet cherry powder for 12 weeks. The intervention was shown to affect the microbiota, with supplemented mice presenting an increased abundance of the Bacteroidaceae family, in comparison to the control group [96]. These changes occurred in absence of differences in most biomarkers of inflammation and other parameters of gut health. However, further studies are needed to confirm these effects and to understand the mechanisms underlying the interactive effects of anthocyanins and other nutritive components from cherries.

10.6 Tolerance to Sweet Cherry Interventions

While pharmacokinetics studies have made substantial progress regarding understanding of bioavailability and metabolic fate of anthocyanins, to do this, often large doses of anthocyanins are administered to achieve a physiological response. Subsequent human intervention studies that administer an anthocyanin-rich food to assess an acute and chronic physiological outcome sometimes administer very large doses of foods to achieve a bioactive effect. However, the feasibility, acceptability, and tolerability of the food-based interventions is often not considered.

One study of sweet cherries [97] has investigated the "tolerance" and "safety" of a 4-week intervention where participants (middle-aged men) were provided with three 142 g servings of sweet cherries daily. The intervention totaled consumption of 426 g/day raw sweet cherries, which provided between 7.18 and 161.56 mcg/g of total anthocyanins (reflecting the changes in anthocyanins across cherry varieties and harvest dates). The subjective tolerance to the intervention was determined three ways: firstly, the MOS 36-Item Short Form Survey Instrument (SF-36) determined quality of life (QoL); secondly through tracking changes in bowel movements; and lastly through compliance to the study protocol (return of pips, uneaten cherries, etc.). The authors report that "the study dose of three cups (426 g) of sweet cherries per day was well tolerated and achievable, with 75% of the men consuming >400 g on average for the duration of the 4-week intervention" (p. 504). The contribution of the cherries to total energy intake was >15% and the cherries provided a median 6.8 mg total anthocyanins/day. While there was no change in measures of QoL or bowel movements, the feasibility of consuming >400 g of cherries daily over the longer term is questionable. Another acute human trial reported adverse participant events after consumption of 90 cherries, but no adverse events after consumption of 45 cherries [98]. The large portion sizes provided in these trials do not align with general population levels of consumption, and this therefore inhibits our extrapolation of the study findings into potentially meaningful results or translation into practical dietary messages.

11 CONCLUSION: MAJOR GAPS IN LITERATURE AND FUTURE RESEARCH DIRECTION

This review provides current evidence in regards to the anthocyanins in sweet cherries, and as a result the need for future research in this area is clearly evident. Several major gaps in the literature span both nutrition and agricultural research fields. In light of this, a collaborative link between the two areas is likely to produce research outcomes that support the priority areas of both fields. One collaborative area may relate to linking population level consumption data used to estimate sweet cherry intake, to determine the relative contributions of cherries to total anthocyanin intake. Additionally, the development of "value-added" cherry products, produced from second-grade or waste cherries, may support human health and should be an active area of collaborative research. These products make nutrition research easier by eliminating the issue of seasonality and providing a standardized dose of anthocyanins per serving. At the same time, they may increase profits for growers by utilizing fruit that may be unsuitable for the fresh market.

In terms of agricultural research, while considerable progress has been made on pre- and postharvest management to produce high-quality cherries with long shelf-life, more research is required to produce cherries and secondary products that retain the anthocyanin contents of fresh fruit. Research in this area is limited by the multitude of factors that influence the anthocyanin contents of sweet cherries, and the nutritional content of the sweet cherries is only one outcome of consideration. The breeding of new cherry varieties with improved characteristics relating to both traditional quality indicators and anthocyanin biosynthesis and retention are needed in order to produce sweet cherries with high consumer appeal. Consumer demand to date highlights that red color (and therefore anthocyanin content) is not a major driver for purchasing behaviors. However, a lack of consumer knowledge about the health benefits of cherries may be masking a great marketing potential.

In terms of nutrition research, more research that specifically utilizes sweet cherries is needed across the spectrum of health outcomes. As a precedence, studies that determine the metabolism and bioavailability of anthocyanins using a comprehensive metabolic profiling approach, in addition to (and in light of) the other

polyphenols comprising sweet cherries are needed. This will allow researchers to attribute the bioactivities of anthocyanin-rich food consumption to either intact parent anthocyanin compounds, or potentially bioactive anthocyanin metabolites. Future well-designed human trials are needed to complement in vitro research, which outline potential mechanisms of action. Lastly, human trials need to be conscious of designing studies with feasible sweet cherry interventions, as the study outcomes need to be generalizable to the wider "free-living" population and tolerable over the longer term.

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Conflicts of Interest

None to declare

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CHAPTER

11

Citrus Fruit Polyphenols and Flavonoids: Applications to Psychiatric Disorders

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1 INTRODUCTION

In 2005, the World Health Organization (WHO) coined the iconic slogan "No health without mental health" to highlight that mental health is an essential component of well-being, and that mental disorders make a substantial independent contribution to the global burden of disease [1]. Mental health is more than just the absence of mental disorders or disabilities. Worldwide, poor mental health is associated with multifaceted risk factors, comprising not only genetics, but also environmental and social factors, such as unhealthy lifestyles, stressful work conditions, rapid social change, and social exclusion. Among these, lifestyle factors provide a suitable target for addressing both prevention and treatment actions. There is accumulating evidence for the importance of diet and nutrition in maintaining psychological well-being, mainly via pathways linked to neuronal function and synaptic plasticity. Mechanisms underlying such actions of dietary factors have mostly been explained through antioxidant and antiinflammatory activities and signaling regulation at the molecular level. The aim of this chapter is to highlight the potential role of citrus polyphenols as "brain foods" and neuroprotective agents by showing their biological activities and mechanisms of action that support this effect, and their promising role for intervention in neurodegeneration, whether illness- or aging-related.

2 THE GLOBAL BURDEN OF MENTAL ILLNESS

A considerable number of the world's health problems in both high-income countries (HICs) and low-to-middle-income countries (LMICs) arise from mental, neurological, and substance abuse (MNS) disorders, whose prevalence continuously increases. Since the publication of data on disease burden from the first Global Burden of Diseases, Injuries, and Risk Factors Study [2], a comprehensive assessment of human health, the category of mental and substance use disorders have accounted for a significant proportion of the world's disease burden, as assessed by disability-adjusted life years (DALYs), a health metric that measures the nonfatal component of the disease as years lived with disability (YLDs), and the fatal component as years lost to premature mortality (YLLs). Successive GBD reports [3] have confirmed that mental illnesses, including depression, bipolar disorders, anxiety disorders, psychoses, and substance use disorders, continue to hold the sad record of being one of the leading causes of disease burden in the world; between 1990 and 2010, absolute disability-adjusted life-years (DALYs) due to MNS disorders rose by 41%, from 182 million DALYs to 258 million DALYs (the proportion of global disease burden increased from 7.3% to 10.4%) [4]. DALYs from MNS disorders were highest during early-to-mid-adulthood (15–49 years), explaining 18.6% of total DALYs in this group age, whereas DALYs from neurological disorders were highest in elderly people, thus suggesting that this substantial increase in MNS-related DALYs is consistently due to population growth and aging [5]. The significant contribution of MSN disorders to global burden is also characterized by gender differences, with men accounting for more DALYs from neurodevelopmental disorders, schizophrenia and other psychoses, substance use disorders, and Parkinson's disease, and women for all other disorders in this category.

Further evidence shows that people with MNS disorders have a significant reduction in life expectancy, and the risk of mortality proportionally increases with

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disorder severity and chronicity [5]. Since the first observation in 1937 by Malzberg [6], who reported that depressed psychiatric inpatients had a mortality rate six times greater than the general population of New York, the link between mental disorders and premature mortality has been constantly suggested by many studies. A recent meta-analysis [5] aimed at examining mortality among people with mental disorders across a range of diagnoses, and which included 203 studies conducted in 29 countries, showed that mortality rate in psychiatric patients is 2.22 times higher than the general population, with the population attributable risk (PAR) rate of 14.3%, which indicates 8 million deaths annually. Examining specific diagnoses, although pooled relative risks (RRs) of mortality due to psychoses were significantly higher compared with depression and anxiety, the latter conditions contributed to more deaths overall compared with psychoses, because of their high base prevalence. Although excess and premature mortality from these disorders is not direct, since they are rarely coded as the direct cause of death, the presence of mental disorders is strongly associated with risk factors for comorbid chronic diseases, including cardiovascular disease, diabetes, and infections [7]. The high rates of comorbidity in patients with MNS disorders are mainly due to unhealthy lifestyles that include tobacco smoking, physical inactivity, and poor diet, all behaviors that contribute to the observed increase in chronic medical conditions among this population. Furthermore, according to the cost-of-illness-approach, the total economic output lost to MNS disorders in 2010 was estimated to be $8.5 trillion globally, and the sole economic burden of depression, including major depressive disorder (MDD), bipolar disorder, and dysthymia, was estimated at $210.5 billion in 2010 in the United States, with 45%–47% attributable to direct costs, 48%–50% to workplace costs, and 5% to suicide-related costs [8]. In Europe, a study of the economic cost revealed that the total cost of MNS disorders was €386 billion for the year 2004 [9] and it rose to €798 billion in 2010, with direct health care cost being €295 billion, indirect cost €315 billion, and the nonmedical cost (i.e., nursing homes) €186 billion [10].

3 MOVING TOWARD INTEGRATIVE PREVENTION AND CARE: THE LINK BETWEEN NUTRITION AND MENTAL HEALTH

Although a wide range of evidence-based and effective treatments, including pharmacological, psychological, and social interventions, are available for preventing and treating MNS disorders, the strong contribution of these disorders to the global burden of disease, also in terms of premature mortality and total costs, continues to increase, and this gives an account of the reason why they have been defined as the biggest health challenge of the century [10], since they represent a global threat to social and health care systems worldwide. Mental disorders constitute risk factors for the development of both noncommunicable (coronary heart disease, diabetes, and stroke), and communicable (infectious disorders) diseases, also contributing to accidental and nonaccidental injuries, and to worse reproductive, maternal, and child health [11]. On the other hand, many clinical conditions also facilitate the rise of mental disorders, still contributing to lengthen the duration of mental illness episodes. This bidirectional comorbidity further complicates health promotion, diagnostic processes, care programs, treatment and compliance, quality of life, and even affects the treatment and the outcomes of physical diseases, leading to chronicity and higher mortality. Reducing the burden of these disorders requires more research, focusing on a better understanding of the mechanisms and common pathways that underlie interactions between mental health and other health conditions, with the primary aim of developing additional preventive and effective treatment interventions. Within this context, the detection and the quantification of modifiable risk factors that can underlie both mental and physical diseases should involve multidisciplinary efforts in order to develop better health care.

In many medical illnesses, such as cardiovascular disorders, cancer, and infections, the main contribution to health has been provided by preventive rather than treatment actions. In the field of psychiatry, more focus has been given to treatments at the individual level than to prevention at a public health level; the development of effective preventive approaches to mental disorders at a population level still remains an unmet need.

The relative risk of developing mental disorders involves interactions between genetic and environmental factors, particularly in the developmental period; epidemiological evidence suggests the hypothesis that modifiable lifestyle-related factors are associated with psychopathology, and they could be among the candidate targets for prevention and treatment. The importance of lifestyle factors for mental health has been underestimated for a long time; yet, it has been highlighted that differences in just four lifestyle factors (physical activity, diet, alcohol intake, and smoking) exert a major impact on morbidity and mortality [12]. Evidence points at the relative contribution of unhealthy lifestyle factors to multiple psychopathologies, along with the importance of healthy lifestyles, such as the maintenance of adequate levels of physical activity, optimization of nutritional intake, engagement in cognitive stimulation, alcohol consumption reduction, and smoking cessation, for promoting psychological well-being, preserving and even enhancing cognitive and neural functions, and for treating complex and often comorbid mental disorders [13].

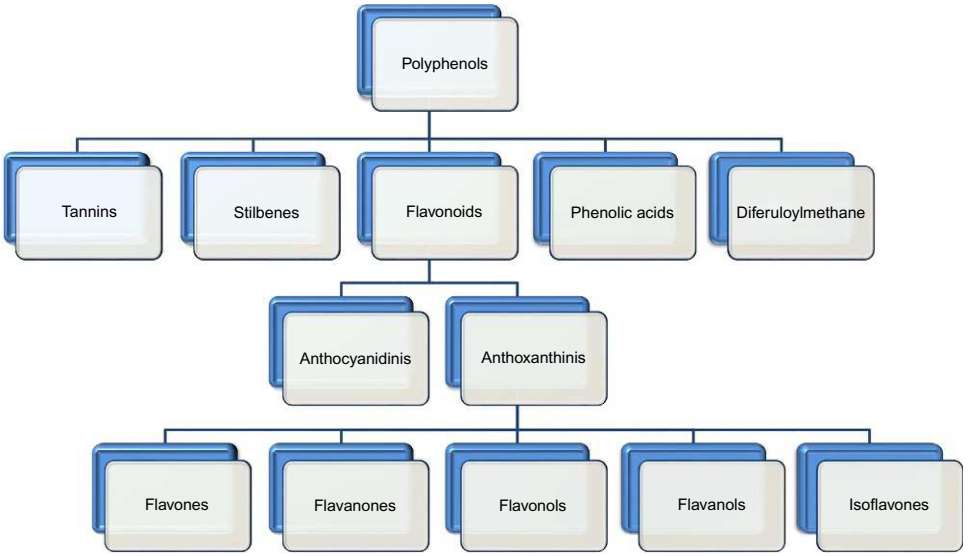

FIG. 11.1 Polyphenol classifications (orientation: horizontal).

According to the growing evidence from the literature, the nutraceutical approach might be a promising strategy for promoting mental health. It is now widely recognized that diet (food selection), nutrition, and nutrient supplementation are key targets in the field of psychiatric prevention and care [14,15]. It has been shown that healthy dietary patterns, characterized by high intake of fruits, vegetables, whole grains, nuts, seeds, and fish, and by low intake of processed foods, are inversely associated with the risk for depression, whereas high consumption of high-fat, high-sugar, and processed foods in adolescence and adulthood are positively associated with both anxiety and depression [16,17]. Further evidence suggests the role of diet quality in the antenatal/perinatal period and early childhood, demonstrating that poor maternal nutrition status and early-life diet are associated with antenatal and postnatal depression in women, and with emotional and behavioral dysregulation in children [18,19].

Diet quality and nutritional supplementation actually provide a suitable model for addressing an important modifiable risk factor for mental illnesses, and they have become the object of growing research, mainly in the areas of cognitive functions and neurodegenerative disorders [20]. For their wide range of activities, citrus polyphenols and flavonoids are promising agents for the development of general food-based neuroprotection and brain foods.

4 CITRUS POLYPHENOLS AND FLAVONOIDS

The genus *Citrus* belongs to the family Rutaceae, subfamily Aurantioideae; originated from southeastern Asia, *Citrus* species are actually cultivated and consumed almost worldwide.

Citrus fruits are an important source of high-quality bioactive compounds with health-promoting properties; besides the high rates of ascorbate (ASC) and carotenoids, whose content is highly variable from one species to another, they contain other nutrients and nonnutrients (minerals, dietary fibers, essential oils) that may offer a significant contribution to disease prevention for their wide range of activities. Among the naturally occurring components of citrus fruits, polyphenolic compounds are believed to be one of the most bioactive agents; however, the concentration of polyphenols is strongly dependent on citrus variety, growing conditions, and environmental stimuli. Polyphenols can be categorized into diferuloylmethanes, stilbenes, flavonoids, phenolic acids, and tannins [21]. The main class of polyphenols is flavonoids, which includes at least 6000 molecules whose chemical structure consists of a heterocyclic skeleton with two aromatic carbon rings benzopyran (A- and C-rings), and a benzene (B-ring); depending on the oxidation state of the heterocyclic (C3) ring, and on basic chemical metabolic substitutions (hydroxylation, glycosylation, methylation, sulfonation, acylation, and prenylation), flavonoids can be divided into subgroups (Fig. 11.1).

Citrus flavonoids, present in the glycoside or aglycone forms, consist of more than 60 individual flavonoids, which are included in four major subgroups: flavanones, flavones, flavonols, and polymethoxiflavones; anthocyanins and anthocyanidins, derived from flavones, are present only in blood oranges. Flavanones may be considered the signature compounds in citrus fruits, as they constitute approximately 95% of total flavonoid amount (up to 98% in grapefruits); however, flavanone content is higher in the whole fruit (pericarp,

flavedo, albedo, and seeds) than in its juice. Flavanones can exist as free aglycones but most of them commonly occur as C- or O-glycosides, sugars that affect the taste of citrus fruits; according to the glycoside forms, flavanones are classified as neohesperidosides and rutinosides. Neohesperidosides such as naringin, neohesperidin, neoeriocitrin, and neodiosmin are intensely bitter and are mainly found in grapefruits, bergamot, and bitter oranges. The rutinosides such as eriocitrin, hesperidin, narirutin, diosmin, and isorhoifolin are tasteless and mainly present in oranges (*Citrus sinensis* L.), lemons (*C. limon* L.), tangerines (*C. reticulate* L.), and bergamot. The flavones group includes apigenin, luteolin, and chrysin, which are mainly present in essential oils, flower extracts, and in juice (traces). The polymethoxyflavones such as nobiletin, sinesetin, and tangeretin are more present in oranges, lemons, and tangerines than in grapefruits [22,23].

Health effects of citrus polyphenols are to be found in the amount consumed and in their bioavailability. After ingestion, dietary polyphenols are available as esters, polymers, or glycosides that cannot be absorbed in those forms and need to be hydrolyzed by intestinal enzymes or by the colonic microbiota, and conjugated via methylation, sulfation, and glucuronidation for being absorbed. Possible interindividual differences in bioavailability of dietary polyphenols are probably determined by the variability in enzymatic patterns due to the peculiar composition of colonic microbiota. Moreover, for exerting potential beneficial effects within the brain, polyphenols need to cross the blood-brain barrier (BBB), thus their bioavailability is also a function of their stereochemistry, interactions with efflux transporters, and lipophilicity, with less polar polyphenols having greater brain uptake than the more polar ones (glucuronidated and sulfated derivatives) [24]. However, it is acknowledged that the majority of citrus polyphenols and flavonoids and their metabolites naringenine, hesperetine, and isorhamnetine, derived from naringin, hesperidin, and quercetine, respectively, are able to cross the BBB.

As a class, citrus flavonoids show a variety of biological activities that range from antioxidant and free radical scavenging activity, chelation of redox active metal ions, modulation of gene expression, to interaction with the cell signaling pathways [25]. These biological activities are responsible for the wide spectrum of beneficial effects on health that have been attributed to citrus flavonoids (Fig. 11.2).

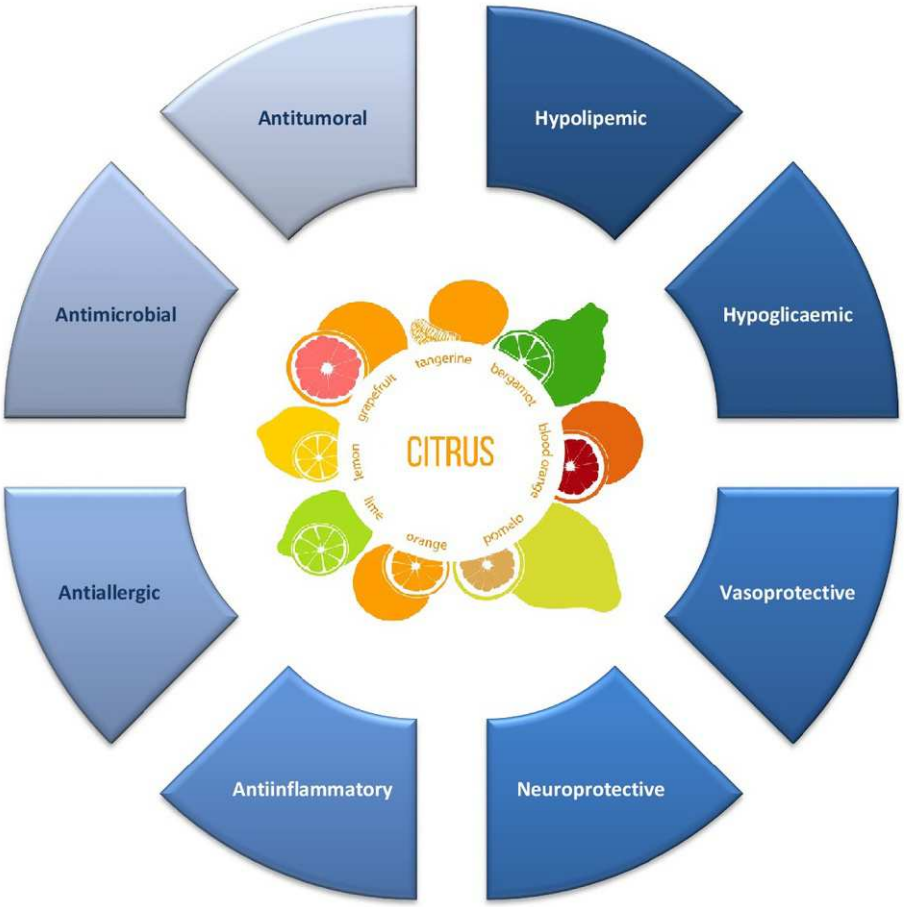

FIG. 11.2 Citrus fruit polyphenol biological activities (orientation: vertical).

5 CITRUS POLYPHENOLS IN MENTAL DISORDERS

A significant amount of evidence suggests that oxidative stress and activated immune and inflammatory pathways are involved not only in neurodegenerative disorders (Alzheimer's disease, Huntington's disease, and Parkinson's disease) and in normal aging, but also in mental disorders, including major depression, bipolar disorders, anxiety, stress-related disorders, and the spectrum of psychotic disorders [26]. In psychiatric disorders, the neurodegenerative component is mediated by a complex chain of events including the impairment of metabolic processes and of mitochondrial functions, excitotoxicity, and inflammation, and increased oxidative damage, which constitutes a main component of the process. Oxidative stress is a state in which there is a disbalance between production of free radicals, reactive oxygen, and/or nitrogen species (ROS/RNS) and the antioxidant defense system in favor of the former, leading to oxidative damage to proteins, DNA, and lipids (peroxidation), and, finally, to cell and organ damage [27]. With its high metabolic rate (20% of the oxygen consumption of the whole organism), the brain is particularly vulnerable to oxidative stress for a number of reasons. The high lipid (substrates for peroxidation) and redox-catalytic minerals (iron and copper) contents in the brain provide a potential for oxidative capacity that is not counterbalanced by the same amount of cellular defense systems (low activities of catalase, superoxide dismutase, and glutathione peroxidase); moreover, the potential for cellular regeneration in the brain is relatively limited when compared with that of other organs [28]. Additionally, microglial activation can amplify oxidative stress via proinflammatory cytokines (interleukin1 β-IL-1β and tumor necrosis factor-TNFα) and nitric oxide (NO) production which, in turn, enhances free radical formation and the subsequent lipid peroxidation, leading to cellular membrane damages, also including the membrane-bound structures, such as monoamine neurotransmitter receptors [29]. However, since microglial activation is not consistently observed in psychiatric patients and does not seem to be specifically associated with any single diagnostic category, it has been proposed as a marker of severity of psychiatric disorders [30].

Also mitochondrial dysfunction can contribute to oxidative stress and neurodegeneration by diminution of adenosine triphosphate (ATP) and free radical formation; the majority of ROS are produced as derivatives of ATP production. Mitochondria have a significant role in modulating inflammation either directly (inflammasome activation) or indirectly, by activating redox-sensitive transcription factors such as NFκB, and inhibiting regulator factors that limit the expression of proinflammatory cytokines [31]. Proinflammatory cytokines, in turn, can reduce mitochondrial density and damage mitochondrial oxidative metabolism with the consequence of increased ROS production [32].

High levels of oxidative stress can also trigger intracellular signaling pathways promoting proinflammatory gene expression, thus amplifying and maintaining the vicious cycle of the inflammatory response in the central nervous system (CNS). Inflammatory and immune cascades are also involved in the metabolic modulation of those neurotransmitters, which regulate cognition and mood, including serotonin, norepinephrine, and dopamine; dopaminergic, glutamatergic, and noradrenergic hyperactivity have been shown to further promote cytotoxicity also via oxidative stress [33].

These complexes and interdependent relationships between inflammatory, immune, oxidative, and neurotransmitter pathways further suggest the role of low-grade inflammation and oxidative stress in the pathogenesis and maintenance of mental disorders, providing a useful pathophysiological framework with strong biological underpinnings, and the possibility that this state of disequilibrium may be addressed by improving the total antioxidant capacity of CNS.

The neuroprotective effects of citrus polyphenols can be attributed to a range of bioactive mechanisms that include antioxidant, redox modulating, and antiinflammatory properties, along with the regulation of signaling pathways that influence neuronal development, survival, regeneration, or death (Fig. 11.3).

Antioxidant compounds can prevent, inhibit, or repair damage caused by oxidative stress. The antioxidant capacity of citrus polyphenols is attributed to the suppression of ROS formation by inhibition of enzymes and/or chelating metals involved in free radical production, to the scavenging of ROS, and to the enhancement of antioxidant defenses. The suppression of ROS formation is realized through the inhibition of the enzymes responsible for superoxide anion production (xanthine oxidase, protein kinase C) and of those involved in free radical generation, such as glutathione S-transferase, cyclooxygenase, lipoxygenase, and microsomal monooxygenase. Although essential for many physiological functions, from bioconstituents to enzymatic cofactors, trace metals, such as iron and copper, participate in the generation of ROS, enhancing the generation of highly aggressive hydroxyl radicals; flavonoids chelating properties allow to remove free metal ions and to render them inactive and unavailable for free radicals formation.

The direct scavenging of oxygen-derived free radicals exerted by citrus polyphenols implies the donation of a hydrogen atom from the hydroxyl groups, resulting in the creation of more stable oxygen products, R-H, and of phenoxyl radicals that can be stabilized by additional

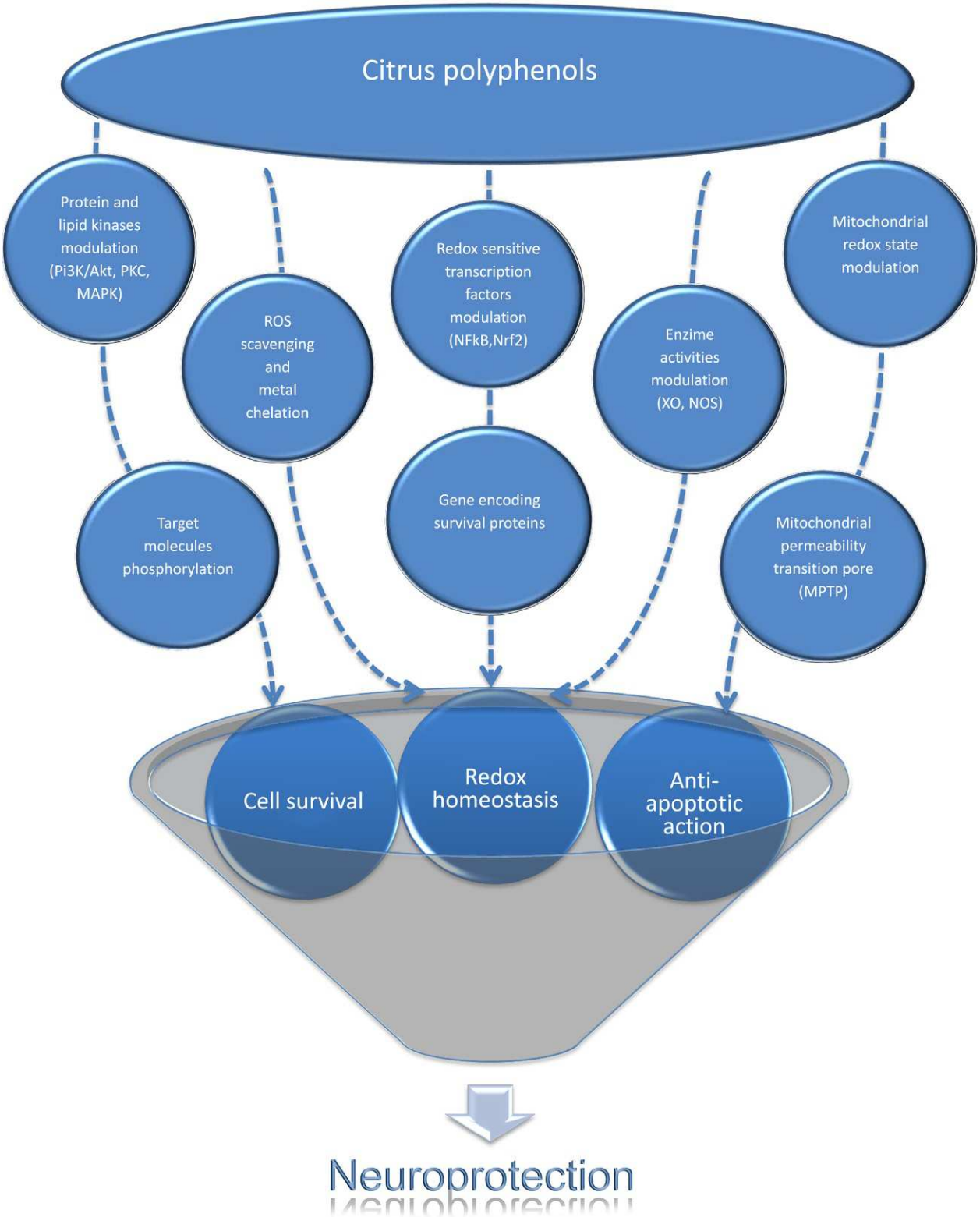

FIG. 11.3 Possible neuroprotective mechanisms of citrus polyphenols (orientation: vertical). *MAPK*, mitogen-activated protein kinase; *MPTP*, mitochondria permeability transition pore; *NFkB*, nuclear factor-kB; *Nrf2*, nuclear factor (erythroid-derived 2)-like 2; *Pi3K/Akt*, phosphoinositide 3-kinase/protein kinase B; *PKC*, protein kinase C; *ROS*, reactive oxygen species; *XO*, Xanthine oxidase.

reactions; moreover, delocalization of unpaired electrons from conjugated double bonds of phenol groups further stabilize free radicals. The radical scavenging activity of polyphenols depends on the structure and the substituents of the heterocyclic and B rings, and it has been shown that the scavenging activities of citrus glycosides are weaker than those of the aglycons; hesperidin, hesperetin, neohesperidin, naringin, and naringenin, have moderate antioxidant activities [34].

Regarding the enhancement of antioxidant defenses, flavonoids are able to influence antioxidant gene expression by modulating redox-sensitive transcription factors, and this can result in the induction of genes encoding for antioxidant and prosurvival enzymes, such as super oxide dismutase and glutathione peroxidases. Flavonoids have shown inhibitory action on those enzymes (tyrosine and serine-threonine protein kinases) that are involved in the generation of inflammatory responses and in immune cell activation processes, by the competitive binding to ATP at catalytic sites on the enzymes. The antiinflammatory activity of flavonoids is also exerted via the inhibition of the expression of inducible nitric oxide synthase, lipoxygenase, and cyclooxygenase, which are responsible for the production of a wide range of proinflammatory mediators, such as cytokines, adhesion molecules, NO, leukotrienes, prostaglandins, and thromboxane A2. Moreover, flavonoids also inhibit phosphodiesterases involved in cell activation. Among citrus flavonoids, both diosmin and hesperidin showed antiinflammatory activity by blocking the synthesis of arachidonic acid derivates, and diosmin and apigenin inhibited NO formation and TNF-α release in activated microglia. Recently, naringenin has been found effective in inhibiting the release of NO and proinflammatory cytokines, as well as the expression of cytokine signaling 3 in microglial cells [35].

Besides their antioxidant and antiinflammatory properties, there is evidence that citrus flavonoids exert their neuroprotective actions also as signal molecules in influencing the expression of genes that encode antioxidant enzymes, cytoprotective proteins, and neurotrophic factors that contribute to neuronal stress adaptation and survival. Flavonoids have a role in the modulation of several signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAP kinase), tyrosine kinase, and protein kinase C (PKC) signaling pathways. In a model of neuronal damage, the protection induced by citrus flavanones (hesperetin and 5-nitrohesperetin) was rather due to the activation of prosurvival Akt and extracellular signal-regulated kinase (ERK) signaling pathways than to their antioxidant potential [36]. In particular, the role of polyphenols as signaling molecules in the modulation of those intracellular signaling cascades involved in neurogenesis, synaptic plasticity, neuronal functions and survival, and, finally, in cognitive functioning and neurodegeneration, has expanded research to evaluate their neuroprotective potential as putative therapeutic agents against neurodegeneration [37].

Flavonoids also exhibit a protective activity on mitochondrial dysfunction associated with increased oxidative stress. Such dysfunction may contribute to neuronal cell degeneration and apoptosis by depletion of cellular ATP and changes of the mitochondrial permeability transition pore (MPTP), leading to the release of cytochrome C. The ability of several flavonoids, such as quercetin, apigenin, and kaempferol, to enter and accumulate into the mitochondria, seems to be related to their protective effects against oxidative damage and mitochondrial-linked pathologies, mainly via the modulation of mitochondrial redox state and of MPTP permeability [25].

5.1 Depression

Depression is the leading cause of disability worldwide, and is a major contributor to the overall global burden of disease. The etiopathology of depression is multifactorial and involves genetic, biological, psychological, psychosocial, and environmental factors. The leading monoamine theory of depression postulates that decreased levels of monoaminergic neurotransmitters, mainly serotonin, noradrenaline and dopamine, are predominately responsible for the syndrome; nevertheless, treatment of depression is less effective than expected, with about one-third of patients not responding to antidepressant therapy.

Knowledge of the neurobiology of depression is expanding, and an increasing body of evidence from several lines of research points to the fact that inflammatory, immune, and oxidative stress pathways play a major role in the pathogenesis of the disorder. Depression is characterized by significant relationships between the acute phase of inflammation (increased production of interleukin-1β - IL-1β, IL-6 and TNFα) and cell-mediated immune activation with monocytic activation, and with T and T helper (Th)-1-like cell activation [38]. The inflammatory response system in depression is related to the hyperactivity of the hypothalamic-pituitary-adrenal-axis (HPA), in which proinflammatory cytokines may have a role; then, serotonergic and monoamine disturbances should be the plausible consequence of cell-mediated immune activation which induces low plasma L-tryptophan levels through activation of indoleamine 2,3-dioxygenase (IDO) [38]. Depressive disorders are also characterized by increased activity of ROS-generating enzymes (catalase, SOD, xanthine oxidase (XO)), high production of ROS (peroxide plasma levels) and very low amounts of enzymatic (paraoxonase 1, PON1) and

nonenzymatic (Coenzyme Q10, vitamin C, vitamin E, and glutathione) antioxidants [39]. Furthermore, mitochondrial structure and function have been shown to be abnormal in depressed patients; mitochondrial dysfunction leads to apoptosis, and to metabolic injury to oligodendrocytes and myelin turnover [40].

With this background, for their roles in neurogenesis/neuroplasticity, neuroinflammation, and in the monoamine reuptake process, citrus fruit polyphenols have been evaluated in preclinical studies, using animal models of behavioral despair and chronic mild stress (CMS), such as the forced swim (FST), tail suspension (TST), and open-field tests, that should mimic the human depressive condition. Hesperidin is one of the most studied flavonoids; its effects are mediated via activation of the brain's monoaminergic system, modulation of kappa opioidergic receptors, interaction with the serotoninergic 5-HT1A receptors, increase of brain-derived neurotrophic factor (BDNF) and decrease in nitrate/nitrite (NOX) levels in the hippocampus [41–44]; neuroprotective effects with suppression of oxidative-nitrosative stress are displayed also by quercetin [45]. The antidepressant-like effect of apigenin was shown in several behavioral despair test models (forced swim test, chronic mild stress) in mice [46,47]; although the antidepressant mechanisms of apigenin are not yet fully understood, its modulatory actions on central dopaminergic, serotoninenergic, and noradrenergic activity, along with the inhibition of monoaminooxidase A (MAO A) seem to be involved. Naringenin has been shown to restore the stress-induced down-regulation of BDNF, to enhance the BDNF expression in the hippocampus, and to increase serotonine and noradrenaline levels [48,49]. Similarly to hesperidin and naringenin, also nobiletin induced antidepressant-like effects by interacting with the main monotransmitter systems [50], whereas luteolin acted via the potentiation of the gamma-aminobutyric acid (GABA) receptors [51]. Also, 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF) resulted effective in attenuating corticosterone-induced depressive-like behavior, and in enhancing neurogenesis and neuroplasticity in hippocampus via the induction of BDNF expression [52].

Finally, in a prospective, 10-year follow-up study [53] aimed at examining possible relationships between estimated habitual intakes of dietary flavonoids and depression in a cohort of 82,643 women aged 36–80 years, inverse associations between flavonol, flavone, and flavanone intakes and depression risk were observed. Participants who consumed $\geq$2 servings citrus (oranges and grapefruits) fruit or juices/day compared with those who consumed $<$1 serving/week had an 18% reduction in depression risk. Both citrus fruit and juices were individually associated with lower depression risk (P-trend$=$0.001 and 0.05, respectively); however, although moderate consumption of citrus fruits was related to lower depression risk, the significant association between citrus juice and lower depression risk was observed only at the highest consumption amount. Among late-life participants ($\geq$65 years at baseline or during follow-up), higher intakes of all flavonoid subclasses, except for flavan-3-ols, were associated with significantly lower depression risk, whereas flavones and proanthocyanidins showed the strongest associations only at the highest consumption amount.

In sum, citrus polyphenols may act as antidepressant compounds via multiple mechanisms of actions, increasing central neurotransmission and neurotrophic factors, limiting monoamines reuptake by synaptosomes, reducing oxidative and nitrosative stress, and modulating the neuroendocrine and GABA systems.

5.2 Schizophrenia and Psychotic Spectrum Disorders

Schizophrenia is a severe psychiatric disorder with an estimated point prevalence up to 1.0%; when considering the narrow psychotic spectrum disorders, which includes schizoaffective disorders, delusional disorder, brief psychotic disorder, schizotypal personality disorder, schizophreniform disorder, as well as psychosis associated with substance use or medical conditions, the prevalence inevitably rises. Beyond the alleged neurodevelopmental origin, genetic vulnerability, influence of socioeconomic factors (social isolation, urbanization), and neurodegenerative potential ("Dementia Praecox," as formerly named by Kraepelin), evidence documents a strong association between schizophrenia and neuroinflammation. Neuroinflammatory mechanisms implicated in schizophrenia include oxidative and nitrosative stress, increased levels of proinflammatory molecules, microglial activation, and astroglial loss, all contributing to neurotransmitter dysregulation (dopaminergic hyperactivity within the mesolimbic system and hypofunction in the mesocortical tract), probably mediated by different, although interacting, pathways, such as the increase of astroglial synthesis of kynurenic acid, and the up-regulation of excitatory amino acid transporter expression [29]. These cascades of events result in the typical neuropathologic changes observed in schizophrenia, such as reduced neuropil elements, synaptic number and dendritic arborization, progressive loss of cortical gray matter, as indicated by the frequent layer-specific reduction of neurons, even with the first psychotic episode.

Preclinical studies have examined the possible utility of polyphenols in schizophrenia and psychotic spectrum disorders; the results need to be translated for potential applications in the human biomedical field. Citrus flavonoid HMF was found effective in reducing locomotive hyperactivity on the Y-maze test induced by MK-801, an antagonist of *N*-methyl-D-aspartate (NMDA)

glutamatergic receptors used to provide behavioral and cognitive symptoms reflective of schizophrenia in animal models; it was suggested that this effect may be associated with the restoration of MK-801-induced alterations in ERK [54]. Based on the premise that hesperidin, tangeretin, nobiletin and naringenin are strong inhibitors of inflammation caused by activated microglia, that low content of these flavanones in natural products limits further research, and that several antipsychotic agents containing a piperidine group have antagonistic effects on dopamine receptors, a research group designed and synthesized new compounds with the piperidine group linked to flavanones, with the aim of investigating in vitro preliminary antipsychotic effects of the multitargeted compounds on neuroinflammation and on the dopamine D2 receptor, and in mouse models of schizophrenia (hyperactive behavior). Synthesized flavanone derivatives demonstrated a potential antipsychotic effect via the inhibition of dopamine activity, an antiinflammatory effect on activated microglia, and decreased the hyperlocomotor activity in the open field test in MK-801-induced schizophrenia model mice, thus providing evidence of potential antipsychotic action [55]. A short communication reported quercetin augmentation of clozapine in two treatment-resistant patients, based on the hypothesis that hyperactivity of efflux pumps in the BBB may explain treatment resistance in antipsychotic-treated patients. Quercetin at the doses of 3000 mg/day in Case 1, and 2000 mg/day in Case 2 was effective in ameliorating clinical symptoms, although the precise mechanism of action of quercetin remains to be elucidated [56].

A significant amount of disability in schizophrenia derives from cognitive deficits, which are currently viewed as core features of the illness. Besides the role of dysfunctional neurotransmitter activity, inflammatory processes may be relevant for cognitive deficits in schizophrenia, by a direct action of inflammatory markers (IL-6, TNF-α, and CRP), or via a secondary increase in proinflammatory activity induced by a high production of kynurenic acid. Both FGAs and SGAs do not address cognitive dysfunction, which remains generally stable during the course of illness. A recent, 8-week, uncontrolled study evaluated the effect of bergamot (*Citrus bergamia*) polyphenols (BPF) on cognitive functioning in a sample of schizophrenia patients treated with SGAs. Bergamot differs from other citrus fruits for flavonoids composition (neoeriocitrin, neohesperidin, melitidin, naringin, rutin, neodesmin, brutieridin, rohifolin and poncirin) and high content. BPF at the dose of 1000 mg/day showed efficacy on cognitive domains (mainly executive functions and attention), as demonstrated by changes in neuropsychological test scores [57].

Antipsychotic (AP) treatment is associated with a series of adverse effects; first-generation antipsychotics (FGAs) induce extrapyramidal symptoms caused by dopamine blockade on the nigrostriatal dopaminergic projection, and result in decrease of activity of antioxidant defense enzymes such as SOD and catalase; increased ROS and oxidative stress are further involved in the pathophysiology of tardive dyskinesia, a serious and often irreversible consequence of AP long-term treatment. Second-generation antipsychotics (SGAs) are mainly limited by drug-induced weight gain and metabolic syndrome (MetS), which is a leading cause of morbidity and mortality in patients with schizophrenia. In haloperidol-treated animals, pretreatment with quercetin dose-dependently ameliorated drug-induced catalepsy, a model for extrapyramidal side effects score [58]. In mice chronically treated with haloperidol, an FGA, administration of rutin significantly and dose-dependently attenuated drug-induced behavioral disorders, such as orofacial dyskinetic movements, stereotypic rearing, and locomotor activity. At higher doses, rutin decreased lipid peroxidation level products in the subcortical region, and induced an increase in the levels of cellular defense mechanisms (glutathione), also preventing decrease in SOD and catalase levels, but only at higher doses [59]. An in vitro study designed to evaluate possible differences between an FGA, haloperidol, and an SGA, amisulpride, on lipid peroxidation in human plasma, and to test the effects of polyphenol compounds, showed that only haloperidol caused a significant increase of lipid peroxidation that was reversed by resveratrol and quercetin; quercetin displayed a stronger antioxidant activity than resveratrol [60]. As previously mentioned, SGAs, particularly clozapine, olanzapine, and quetiapine, are associated with weight gain, glucose dysregulation, and dyslipidemia (MetS). Two studies have evaluated the efficacy and safety of BPF treatment on clinical and metabolic parameters in SGAs-treated subjects. BPF at low (500 mg/day) and higher (1000 mg/day) doses did not substantially change metabolic parameters, and resulted minimally effective for reducing body weight and BMI in the study samples [61,62].

Regarding the potential use of citrus polyphenols in schizophrenia, many questions remain unanswered; oxidative stress and the inflammatory process are involved in the pathogenesis of the illness, and APs have been shown to increase lipid peroxidation and oxidative stress, thus the route to neuroprotection should be a primary aim in the treatment of this condition. However, this research field needs more results coming from human studies, not only from preclinical evidence.

5.3 Transnosographical Dimensions in Mental Disorders: Cognition and Memory

Most mental disorders include disruption of several dimensions of cognition. Cognitive deficits may increase the vulnerability towards psychiatric disorders, may be a

core, trait feature (putative endophenotype) and an early marker of illness, and may affect the course of illness and relapses, also predicting functional and community outcomes independently of clinical symptoms. A substantial amount of evidence points at the construct of executive functioning (EF) impairment as the most consistently associated with psychiatric disorders. Executive functions, often referred to as the cognitive abilities implicated in regulatory or executive control systems, include inhibitory control and ability to organize and adaptively use information contained in working memory, cognitive flexibility, goal-oriented processes such as the initiation and maintenance of efficient strategies, hypothesis generation, temporal response sequencing, abstract reasoning, and the programming and planning of motor behavior skills [63]. Available psychopharmacological treatments generally result in no or little improvement in cognitive and executive deficits, making it clear that procognitive therapy still remains an unmet need in the field of psychiatry.

In mental disorders, decline in cognitive function has also been associated with impairment of neuronal plasticity, decreased and/or aberrant neurogenesis, and neuronal death; the neuroprotective properties (interaction with signaling pathways, antioxidant and antiinflammatory activities, and modulation of cerebral blood flow) displayed by citrus polyphenols make this class of nutraceuticals a potential candidate for addressing cognition in mental disorders.

It has been shown that hesperetin at high doses inhibited H2O2-induced apoptosis, protecting cortical neurons from oxidative damage and glutamate-induced excitotoxicity, and increased the expression of antioxidant enzymes, such as catalase, glutathione peroxidase, and glutathione reductase, in mice models [64]. Naringin exerted neuroprotective effects against the degeneration of the nigrostriatal dopaminergic projection by inducing glia-derived neurotrophic factor (GDNF), an essential neurotrophic factor for adult dopaminergic neurons; furthermore, naringin attenuated the rise of TNF-α in microglia [65]. The aglycone form of naringin, naringenin, improved the memory performance of scopolamine-treated mice in an animal model of amnesia, and ameliorated spatial learning and memory associated with several markers of oxidative stress and hippocampal damage [66]. Naringenin pretreatment also reversed streptozocin-induced cognitive, behavioral, biochemical and histopathological alterations in rat hippocampus via a direct cholinesterase inhibitory effect, besides its antioxidant action [67]. Apigenin has shown cognitive enhancing effects delaying the long-term forgetting, and stimulating neurogenesis in the hippocampal region of mice [68]. Furthermore, apigenin reversed cognitive deficits in lipopolysaccharide (LPS)-intoxicated mice [69], and exerted neuroprotective effects via the inhibition of oxidative stress, the stabilization of mitochondrial function, and the reduction of neuronal apoptosis by modulating mitochondrial pathways [70]. The antineuroinflammatory activity of nobiletin is mainly exerted by suppressing microglial activation through inhibition of NO release and expression of inducible NO synthase, similarly to mynocicline; nobiletin also induces neurite outgrowth, and modulates ERK signaling pathway to increase cAMP response element-binding protein (CREB) phosphorylation in cultures of hippocampal neurons [71]. Administered daily for four months, nobiletin reversed memory impairment in fear conditioning, and decreased hippocampal amyloid deposits in transgenic mice [72]. Furthermore, nobiletin improved age-related cognitive impairment, reducing oxidative stress and tau phosphorylation, in senescence-accelerated mouse prone 8 (SAMP8) mice, a murine model of aging characterized by the early onset of memory and learning deficits, and by hystopathological features of Alzheimer disease [73].

Among exogenous modulators of neural activity and cognitive functioning, such as physical activity, and enriched environment, diet is one of the most prominent for its effect on neurogenesis; diets high in fat and refined sugars contribute to cognitive decline both in adults and in adolescents through a decrease in hippocampal BDNF, alterations in dopamine-mediated reward signaling, and inhibitory neurotransmission controlled by GABA [74,75]. Quercetin antagonized cognitive impairment induced by feeding mice with a high fat [76] or high-cholesterol diet [77], and protected neuronal PC12 cells, a model for neural differentiation, from high-glucose-induced oxidation, nitrosative stress, and apoptosis [78].

In conclusion, several studies have highlighted a variety of mechanisms by which citrus polyphenols may benefit cognitive function, and may address those neuropathological conditions resulting from neuroinflammation and oxidative stress; nevertheless, human studies incorporating dietary, cognitive, and physiological/metabolic assessments are required.

6 CONCLUSIONS AND FUTURE DIRECTIONS

The potential benefits of neuroprotection are numerous, and the possibility of preventing mental disorders and cognitive decline, and/or inhibiting illness progression, would also be of great clinical value. Citrus polyphenols and flavonoids possess a range of bioactive properties that make them interesting as potential neuroprotective agents, as suggested by in vitro and in vivo studies, which support the role of polyphenolic compounds in modulation of mental health including brain

plasticity, mood (antidepressant-like properties), behavior, and cognition.

Research has shown that citrus polyphenols can prevent and reverse neurodegeneration by protecting neural cells from radical-induced oxidative damage and neuroinflammation, which are known to play a crucial role in mental disorders; moreover, their neuroprotective effect extends to interaction with intracellular targets involved in prosurvival signaling pathways and induction of proteins that promote cognition.

However, despite extensive research on the use of natural polyphenols in neurodegenerative disorders, their use in clinical practice has yet to be achieved, for a number of reasons. The first, and most important, concerns the translation of results obtained from in vitro and in vivo studies, and from animal models, into human conditions, also taking into account that the pharmacological effects obtained at concentrations or dosages of polyphenols are unlikely to be reproduced in clinical practice. Moreover, in humans, the oral route of polyphenols and flavonoid administration can affect bioavailability and is possibly complicated by the biotransformation of original compounds to entirely different metabolites; in order to develop polyphenols as preventive and therapeutic agents, more knowledge on the pharmacokinetics of specific polyphenols and the mechanistic basis of their bioactivities in the brain is needed. It should be also borne in mind that it is rather improbable that one class of agents may exert a substantial neuroprotective action in complex disorders whose pathophysiology, in terms of molecular mechanisms, is still elusive. Finally, none of the animal models mimics an exact state of human mental disorder.

Therefore, while the use of nutraceuticals represents a potentially useful approach to mental disorder prevention and treatment, the potential use of orally administered citrus polyphenols still requires copious amount of data from both experimental and human research. Current evidence suggests that citrus polyphenols and flavonoids have the potential to become neuroprotective agents to be included in the therapeutic equipment in the field of mental health.

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SECTION II

POLYPHENOLS IN THERAPY OF OBESITY AND DIABETES

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CHAPTER

12

Anthocyanins and Diabetes Regulation

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1 INTRODUCTION

Diabetes mellitus (DM) is an hereditary, chronic, potentially debilitating and often fatal endocrine disorder, characterized by hyperglycemia and eventual glycosuria. There are two main categories of DM: insulin-dependent DM (type 1, T1DM) and insulin-independent DM (type 2, T2DM). T1DM results from an absolute deficiency in insulin secretion caused by cell-mediated autoimmune destruction of pancreatic β-cells. T2DM accounts for more than 90% of diabetes cases and largely results from excess body weight and physical inactivity. T2DM is associated with defects in muscle, adipose tissue, and liver glucose and insulin metabolism, manifested by reduced insulin sensitivity and secretion and higher insulin resistance [1]. Oxidative stress and subclinical grade inflammation play a significant role in the T2DM development [2].

Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as thiazolidinediones, biguanides, α-glucosidase inhibitors, and glinides, which are used as monotherapy or in combination to achieve better glycemic regulation [3]. However, most of these drugs have prominent side effects and fail to significantly alter the course of diabetic complications. In this regard, tremendous effort has been made to find alternative therapeutic approaches for this metabolic disease. Alternative drugs will present a lower risk for drug interactions, simplify the therapeutic regimen, and have fewer side effects.

Recently, much attention has been focused on food factors that may have beneficial antidiabetic effects. Anthocyanins are naturally occurring polyphenolic plant pigments. They are widely distributed in fruits, vegetables, and pigmented cereals, suggesting that we ingest considerable amounts of anthocyanins daily from our plant-based diets [4]. Epidemiological investigations indicated that moderate anthocyanin consumption from pigmented plant foods is associated with reduced type 2 diabetes risk [5]. The antidiabetic effects of anthocyanins and anthocyanin-rich extracts from different plant materials have been confirmed in vivo and in vitro, and the mechanisms of these compounds' hypoglycemic and insulin-sensitizing activities are being studied. In this chapter, we describe the known antidiabetic properties of plant-derived anthocyanins and anthocyanin-rich extracts to aid in our understanding of their functional mechanisms. The chemical properties, dietary sources, metabolism, and bioavailability of anthocyanins are also summarized.

2 CHEMICAL PROPERTIES OF ANTHOCYANINS

Pigments from natural sources display a wide range of colors as secondary plant metabolites. Among the many natural pigments, anthocyanins are the most important group of water-soluble plant pigments. They are responsible for most of the red, blue, purple, and even black colors of fruits, vegetables, grains, flowers, and other plant tissues or products [4]. Early research demonstrated that the bright color of anthocyanin made plants more outstanding, thus attracting animals to spread pollens and seeds to aid in breeding [6]. In addition, anthocyanins are produced as a protective mechanism against environmental stress factors including UV light, cold temperatures, and drought [7].

Chemically, anthocyanins are a type of flavonoid and are composed of glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts, possessing a characteristic C6-C3-C6 skeleton

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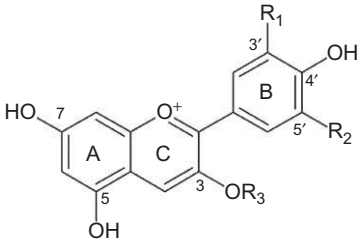

Cyanidin	R_1=OH,	R_2=H
Pelargonidin	R_1=H,	R_2=H
Peonidin	R_1=OCH_3,	R_2=H
Delphinidin	R_1=OH,	R_2=OH
Petunidin	R_1=OCH_3	R_2=OH
Malvidin	R_1=OCH_3	R_2=OCH_3

R_3 = Glucose, galactose or arabinose

FIG. 12.1 Structural classification of common anthocyanidin species.

that contains two benzoyl rings (A and B) separated by a heterocyclic (C) ring (Fig. 12.1). The molecular weights of most anthocyanins range from 400 to 1200 Da, and they have a maximum absorption between 465 and 550 nm, as well as significant absorption in the UV range between 270 and 280 nm [4]. During the past decades, over 600 anthocyanins have been identified. However, only six common anthocyanidins (aglycones of anthocyanins) are classified according to the number and position of the hydroxyl groups and are named as follows: cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) [8].

The chromophore of conjugated double bonds carrying a positive charge on the heterocyclic oxygen ring is responsible for the intense red-orange to blue-violet color produced by anthocyanins under acidic conditions [9]. Different anthocyanins have different numbers and positions of hydroxyl and methoxyl groups on the basic anthocyanidin skeleton. For example, an increase in the hydroxylation pattern results in a bathochromic shift from red to violet, including pelargonidin, cyanidin and delphinidin.

Sugar moieties such as glucose, rhamnose, galactose, xylose or arabinose as 3-glycosides or 3,5-diglycosides, are usually bound to the skeleton. These moieties may be a mono or disaccharide unit, which can be acylated with a phenolic or aliphatic acid. The glycosidic units link to the anthocyanidin by α- or β-linkage, with the sugar moiety linked to the 3-position on the C-ring or the 5,7-position on the A-ring. The linking usually occurs at the 3′-, 4′-, and 5′-positions of the B-ring. Glycosylation can also be found, although it is very rare. The saccharide residue may be acylated with a phenolic or aliphatic acid. The most common acylating agents are cinnamic, caffeic, ferulic, sinapic, acetic, malic, malonic, oxalic, and succinic acids, which are found in aliphatic acids. Thus, anthocyanins are compounds of anthocyanidin and sugar, and in the case of acylated anthocyanins, are still acids [10].

The degree of hydroxyl group methylation, the number and the position of attached and acylated sugars, and the acylation status of the sugars with aliphatic or aromatic acids significantly influence the anthocyanin's chemical qualities. Glycosylation primarily at the C-3 residue results in reduced maximum wavelength absorption and increased stability and solubility. Esterification of the sugar residues with cinnamic or aliphatic acids further improves anthocyanin stability. Generally, di-, tri-, or polyacylated anthocyanins have increased stability compared with simple and monoacylated anthocyanins [11].

Anthocyanins' characteristics depend not only on the basic structure but also on the pH, temperature, solvent type, and the presence of light, oxygen, and other factors [7]. Anthocyanin differs from other flavonoid subgroups because of a positive structural charge at acidic pH. In water, they are more stable at low pH; for example, blue wheat anthocyanins were most thermally stable at pH 1, and their degradation was slightly lower at pH 3 compared with pH 5 [12]. The anthocyanins in aqueous solution coexist in equilibrium with four main species: the flavylium cation, the quinoidal base, the hemiacetal base (carbinol pseudo-base), and chalcone (Fig. 12.2) [13]. In an aqueous solution at pH 1–3, the predominant form of the anthocyanin is the flavylium cation, which is responsible for the red color. Increasing pH is accompanied by loss of a flavylium cation proton, thereby generating a blue quinoidal base, which is a colorless carbinol pseudo-base that accumulates from hydration of the flavylium cation. This species tautomerizes through opening the C ring to generate a yellow chalcone. This special chemical structure affects the absorption, metabolism, and bioavailability of anthocyanins in vivo [14].

Apart from pH, heat is another important factor that influences anthocyanin stability [15]. Gupte et al. demonstrated that Hull blackberry cyanidin 3-glucoside stored separately under different temperature conditions for 90 days degraded to different degrees: −80°C, <2%; 2–8°C, 5%–7%; room temperature, almost 60%. This study also indicated that light had an insignificant effect [16]. The degree of Chinese purple corn extract conversion was 0.1%, 8.6%, and 73.6% in 5 min at 100°C, 130°C, and 150°C, respectively [17].

Anthocyanins are most susceptible to oxidation because they are electron deficient. The degradation of red radish anthocyanin extract in H_2O_2 was almost the same as that induced by light and heat, depending on

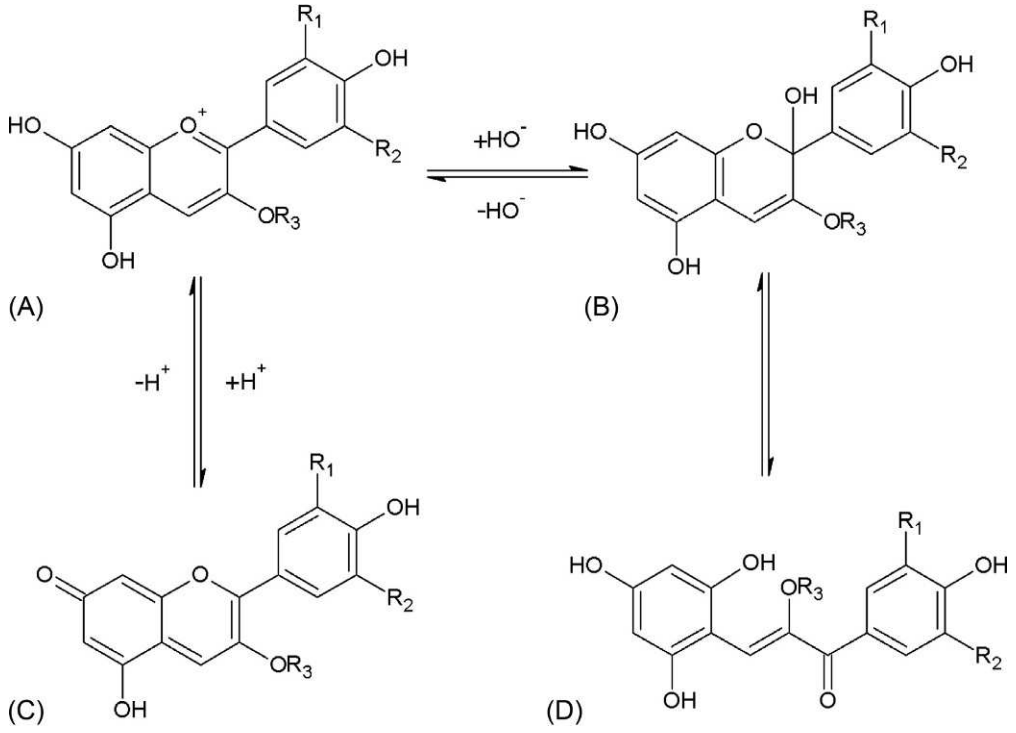

FIG. 12.2 Molecular forms of anthocyanins. (A) flavylium cation, (B) carbinol pseudo-base, (C) quinoidal base, and (D) chalcone.

the solution pH. In addition, the stability depended on H_2O_2 concentration. At pH 3 with 300 μg/mL H_2O_2, after 10 h there was approximately 20% residual anthocyanin [18].

3 DIETARY ANTHOCYANIN SOURCES

Anthocyanins are of great nutritional interest because they are very abundant in plant-derived foods. Edible anthocyanin sources include colored fruits such as berries, cherries, hawthorns, peaches, grapes, apples, and plums and some dark-colored vegetables such as red onions, red radishes, black beans, eggplants, red cabbage, and purple sweet potatoes [13]. Apart from the aforementioned fruits and vegetables, anthocyanins also accumulate in grains such as black rice, red sorghum, and purple maize [7]. The anthocyanin content of many plant-derived foods has been estimated by several groups using chromatographic or pH-differential methods, and several databases for these results have been established [19,20].

In 1976, Kuhnau reported that, in the United States, the average daily anthocyanin intake is approximately 215 mg during the summer and 180 mg during the winter [21]. However, Wu and colleagues recently estimated that the anthocyanin consumption in the United States was only 12.5 mg per day per person, using the USDA Nutrient Database of flavonoids [22]. In 10 European countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) study, the total anthocyanidin mean intake ranged from 19.83 to 64.88 mg/day [23]. In south China, the average intake of anthocyanidin was estimated at 27.6 mg/day [24], similar to those of Germany (35.1 mg/day), the United Kingdom (26.1 mg/day), Denmark (28.2 mg/day) and the Netherlands (21.9 mg/day) [23]. It is understandable that dietary habits and choices have a great influence on anthocyanin consumption. For instance, the traditional Fijian diet contains a lot of meat from sea mammals and land animals, but few fruits and vegetables, and therefore their anthocyanin intake (0.04 mg/day) is far lower than that of Europeans, who eat a mixture of food items [25]. It is also important to note that deviations may occur due to the misclassification in dietary questionnaires, as dietary habits are dynamic, frequently changing and evolving.

4 ANTHOCYANIN BIOAVAILABILITY

Bioavailability is defined in various ways. With respect to a group of bioactive substances such as anthocyanins, the commonly accepted definition of bioavailability is the proportion of the anthocyanin that is digested, absorbed, and metabolized through normal pathways [14]. Measurement of anthocyanin plasma concentration and urinary excretion has often been used to assess bioavailability. Various in vitro, animal and human studies have been conducted to investigate anthocyanin bioavailability.

Animal studies have come to a consistent conclusion that anthocyanins are absorbed mainly in their intact forms, rapidly reach the circulatory system within 0.25–2 h following consumption and are finally eliminated through the urine within 6–8 h. Experiments conducted in rats have shown that, after oral administration of *Vaccinium myrtillus* (400 mg/kg body weight), anthocyanin plasma concentration peaks after only 15 min at 2–3 μg/mL and then quickly declines within 2 h [26]. Another study confirmed that 15 min after oral supplementation of red fruit anthocyanin (a mixture of 320 mg of cyanidin-3-glucoside (C3G) and 40 mg of cyanidin-3,5-diglucoside/kg of body weight), rats had increased plasma levels with a maximum of 1563 μg C3G/L and 195 μg cyanidin-3,5-diglucoside/L. [27] Other studies measured urinary excretion to characterize anthocyanin bioavailability. For example, an experiment conducted by Nielson et al. in Watanabe heritable hyperlipidemic rabbits indicated that anthocyanin excretion from black currant juice 30 min following ingestion is 0.035% [28]. Similarly, Felgines et al. determined that, in rats adapted for 8 days to a blackberry anthocyanin-enriched diet, urinary C3G recovery in either intact or methylated forms was approximately 0.26% of the ingested amount, whereas malvidin 3-glucoside recovery was 0.67% [29]. Recent studies have also determined that intestinal microflora play a vital role in anthocyanin bioavailability. HPLC-DAS and LC-MS were employed to explore the anthocyanin metabolites fermented *in vitro* after inoculation with human fecal microflora. The results demonstrated that anthocyanin monoglucosides (3-glucosides of cyanidin, malvidin and peonidin) and diglucosides (3,5-diglucosides of cyanidin and malvidin, and cyanidin rutinoside) are de-glycosylated by colonic microflora within a period of 20 min to 2 h, followed by aglycon degradation to phenolic acids [30]. A recent study not only confirms the actions of the intestinal microflora but also demonstrates that the fermentation itself contributes to positive modulation of the intestinal bacterial population [31]. Animal experiments have also revealed that anthocyanin absorption varies greatly depending on the structure [32] itself and the food matrix [33–35].

Consistent with the animal experiments, a majority of human clinical studies also confirm that anthocyanins have rapid absorption and elimination rates. An early study reported that 15 min after administration of 25 g elderberry extract containing 1.5 g anthocyanins, anthocyanin plasma concentration peaked after 30 min at 100 μg/L. [36] Similarly, Kay et al. reported a peak plasma concentration of 96.08 nmol/L 2.8 h after oral intake of 721 mg C3G from chokeberry extracts [37]. Considerable differences exist with respect to urinary anthocyanin excretion. For example, Felgines et al. reported a high urinary excretion of anthocyanin metabolites corresponding to 1.8% of 200 g strawberries ingested, which provides 179 μmol pelargonidin-3-glucoside [33]. Conversely, in another study, C3G was established to have a minimum relative bioavailability of $12.38 \pm 1.38\%$ on the basis of the total elimination of the absorbed ^{13}C dose via urine [38]. Despite these differences, almost all of the data reports extremely small amounts of anthocyanin excretion and attributes differences in excretion to individual structures [37,39]. Both animal and human studies indicate that anthocyanins exist in plasma and urine as intact, methylated, glucuronide derivatives and sulfo-conjugated forms [40].

Overall, anthocyanins are distinct from other flavonoids in that they have their own bioavailability characteristics. First, anthocyanins are rapidly absorbed as intact glycosides, distributed into the circulatory system and excreted through the urine [40]. The maximum plasma concentration (C_{max}) is between 1.4 and 200 nmol/L for 10–720 mg anthocyanins, and can be measured from 45 min to 4 h following ingestion [7]. Second, consistent apparent bioavailability is observed across both animal and human studies and is usually much less than 0.1% of the overall anthocyanin intake. Anthocyanins are thought to be absorbed generally in the stomach and small intestine [32,41]. Furthermore, in the lower gastrointestinal tract, the role of microbial metabolism cannot be ignored [42]. Third, the amount of anthocyanin absorbed also depends on its structure, food matrices, and food processing conditions [43].

5 ANTIDIABETIC EFFECTS OF ANTHOCYANINS AND THE UNDERLYING MECHANISMS

During the past few decades, several randomized controlled trials (RCTs) have been performed with anthocyanins from different sources, and the antidiabetic properties of these bioactive compounds have included the prevention of free radical production and lipid peroxidation, reduction of blood lipids and hemoglobin A1c (HbA1c), increased insulin secretion, and improvement of insulin resistance (Table 12.1). Consistent with RCTs, cross-sectional studies also indicated dietary anthocyanin consumption was associated with decreased insulin resistance and inflammatory biomarkers in blood [59,60]. In addition, dose-response meta-analysis demonstrated nonlinear relationships of dietary intakes of anthocyanins (P for nonlinearity $= 0.006$, P for trend < 0.001) with T2DM risk [5]. The underlying mechanisms of anthocyanins that reduce the risk of T2DM are listed in the following subsections.

5.1 Amelioration of Oxidative Stress

Diabetes is associated with hyperglycemia and hyperlipidemia-induced oxidative stress [61]. Oxidative

TABLE 12.1 Antidiabetic Effects of Anthocyanins in Randomized Clinical Trials

Anthocyanin source	Anthocyanin dose (mg/day)	Duration of study	Subjects (number in group)	Effect	Reference
Blackcurrant extract	150, 300, 600	once	Healthy men and postmenopausal women consuming a high-carbohydrate meal (14M, 9W)	↓plasma insulin and glucose response	[44]
Freeze-dried wild blueberry powder	310, 724	once	Healthy young adults (4M, 13W)	↓postprandial glucose response	[45]
Freeze-dried strawberry powder	120	once	Overweight adults consumed a high-carbohydrate, moderate-fat meal [22]	↓plasma insulin ↓inflammatory markers (IL-6 and CRP)	[46]
Freeze-dried wild blueberry powder	375	6 weeks	Men with cardiovascular risk factors [17]	↓oxidized DNA bases	[47]
Purple-flesh potatoes	465	6 weeks	Healthy men [7]	↓DNA damage (8-OHdG) ↓inflammatory markers (IL-6 and CRP)	[48]
Sour cherry juice	720	6 weeks	Diabetic women [18]	↓plasma TC and LDL-C ↓plasma HbA1c	[49]
Freeze-dried strawberry powder	154	4 weeks	Women with metabolic syndrome [15]	↓plasma TC and LDL-C ↓lipid peroxidation	[50]
Aronia melanocarpa extract	60	2 months	Metabolic syndrome patients [23]	↑SOD and CAT in erythrocytes ↓serum LDL-C	[51]
Purified anthocyanin mixture	160	24 weeks	Diabetic patients [52]	↓insulin resistance index; ↑serum adiponectin and β-hydroxybutyrate	[53]
Purified anthocyanin mixture	320	12 weeks	Patients with NAFID [54]	↓insulin resistance index	[55]
Purified anthocyanin mixture	320	12 weeks	Hypercholesterolemic patients [56]	↓serum sVCAM-1 ↑serum HDL-C	[52]
Purified anthocyanin mixture	320	12 weeks	Dyslipidemic patients [57]	↓plasma LDL-C ↑plasma HDL-C	[58]
Purified anthocyanin mixture	640	4 weeks	Prehypertensive men [29]	↑plasma HDL-C	[57]

HbA1c, hemoglobin A1c; *HDL-C*, High density lipoprotein-cholesterol; *NAFLD*, Nonalcoholic fatty liver disease; *8-OHdG*, 8-hydroxydeoxyguanosine; *TC*, total cholesterol; *LDL-C*, low density lipoprotein-cholesterol; *ORAC*, oxygen radical absorbance capacity; *sVCAM-1*, soluble vascular cell adhesion molecule-1.

stress is caused by an imbalance between the production of reactive oxygen species (ROS) and their detoxification in biological systems. Oxidative stress induces the activation of major signaling pathways and transcriptional factors [i.e., activating protein (AP)-1 and Kelch-like ECH-associating protein 1-NF-E2-related factor 2 (Keap1-Nrf2)], and it accounts for many diabetes-induced complications [62]. Therefore, there is strong rationale for therapeutic antioxidant use to treat and prevent diabetic complications.

Over the past decades, considerable attention has been focused on the antioxidant properties of anthocyanins and their aglycones. Many studies have demonstrated that anthocyanins quench free radicals and terminate

chain reactions in vitro. In 1997, Wang et al. reported the antioxidant activity of five groups of available anthocyanins with a total of 14 compounds, as examined by the automated oxygen radical absorbance capacity (ORAC) assay method. Among these anthocyanins, kuromanin (cyanidin-3-glucoside, C3G) had the highest ORAC activity, which was 3.5 times stronger than the water-soluble vitamin E analog Trolox; whereas pelargonin (pelargonidin-3,5-diglucoside) had the lowest antioxidant activity but was still as potent as Trolox [63]. The antioxidant activities of anthocyanins have been linked to the compounds' chemical structure. Their antioxidant activity is specifically related to the presence of a hydroxyl group in position 3 of the C ring in positions 3′, 4′, and 5′ in ring B, enthroning delphinidin as the most potent antioxidant in the group [64,65]. The antioxidant properties of anthocyanins are further increased by acylation of the sugar residues with aromatic hydroxy acids [63].

Anthocyanin instability within the physiological pH range suggests that these compounds may not exert their full antioxidant activity within the body. However, there is emerging evidence that anthocyanins and their in vivo metabolites exert modulatory actions on antioxidant signaling molecules, enzyme activity, and gene expression in both humans and animal models. Intake of 1.16 g of a freeze-dried wild blueberry powder containing anthocyanins lowered several redox status parameters in human serum after the consumption of a high-fat meal [66]. Furthermore, a significant positive correlation was observed between serum anthocyanin content and postprandial antioxidant status. After consumption of two glasses of juice made with freeze-dried strawberry powder for four weeks, women with metabolic syndrome showed a decreasing trend of serum lipid peroxidation in the form of malondialdehyde and hydroxynonenal [50].

In a study utilizing paraquat-fed rats as an oxidative stress model, acylated anthocyanins from red cabbage efficiently attenuated the increase of hepatic paraquat-induced thiobarbituric acid-reactive substances (TBARS) and NADPH-cytochrome-P450-reductase activity [67]. In another rat study, animals were fed vitamin E-deficient diets for 12 weeks followed by supplementation with purified anthocyanin-rich extracts from the cones of *Abies koreana*. The anthocyanin-supplemented diet significantly improved plasma antioxidant capacity and decreased hepatic hydroperoxides and 8-Oxo-deoxyguanosine (8-OHdG) concentrations, indicating significant reductions of the vitamin E deficiency-induced lipid peroxidation and DNA damage, respectively [68]. Chiang et al. demonstrated that black rice anthocyanin-rich extract antioxidant effects are mediated through decreases in free-radical generation as well as increases in hepatic superoxide dismutase (SOD) and catalase (CAT) activities [69]. Purified C3G increased glutathione (GSH) synthesis in the liver of diabetic *db/db* mice through upregulation of glutamate-cysteine ligase catalytic subunit (Gclc) expression [70]. Similar results were also observed in streptozotocin (STZ)-induced diabetic rats orally treated daily with C3G (10 mg/kg) for 8 weeks [71]. These observations suggest that anthocyanins may modulate antioxidant defense by activating antioxidative enzymes and promoting GSH synthesis.

5.2 Antiinflammatory Effects

During the development of type 2 diabetes, there is chronic nonspecific inflammation, including inflammatory cytokine expression that mainly originates from adipose tissue. Thus, the reported anthocyanin antiinflammatory properties may be crucial for the treatment of this disease.

Animal studies have shown that blueberry powder supplementation (40.9 g/kg diet) reduced macrophage infiltration in white adipose tissues and decreased mRNA levels of macrophage-derived proinflammatory genes in high fat-fed mice [72]. Tsuda et al. demonstrated that high fat diet-induced adipocyte hypertrophy and adipose tissue tumor necrosis factor-alpha (TNF-α) mRNA expression was normalized by C3G-rich purple corn pigment extract administration [73]. A reduction of inflammatory adipocytokine expression (monocyte chemoattractant protein-1 and TNF-α) in white adipose tissue was also observed in KK-A^y and *db/db* diabetic mice fed a diet supplemented with purified C3G [54,56].

The transcription factor nuclear factor-κB (NF-κB) is an oxidative stress-sensitive transcription factor that controls the expression of numerous genes involved in the inflammatory response. Speciale et al. demonstrated that C3G protected human endothelial cells against TNF-α-induced alterations including the NF-κB activation, increased adhesion molecule gene expression, leukocyte adhesion to the endothelium, and intracellular H_2O_2 and lipid peroxidation byproduct accumulation [74]. In a placebo-controlled crossover design study, drinking a strawberry beverage significantly attenuated the postprandial inflammatory response as measured by high-sensitivity C-reactive protein (CRP) and interleukin-6 (IL-6) expression in the serum of overweight adults after high-carbohydrate, moderate-fat meal consumption [46]. Wang et al. demonstrated that black rice pigment fraction supplementation significantly reduce plasma levels of soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble CD40 ligand (sCD40L) and high sensitive CRP in coronary heart disease patients [75]. Several other NF-κB-related proinflammatory chemokines, cytokines, and inflammatory response mediators were decreased in the plasma of healthy or hypercholesterolemic adults after anthocyanin supplementation [76,77]. In addition,

interferon alpha (IFNα), an inducer of NF-κB activation, was also significantly decreased compared with the baseline in the anthocyanin-treated group, suggesting that NF-κB activation and translocation were inhibited by anthocyanin supplementation. In contrast with these data, no significant effects of anthocyanin supplementation on NF-κB activation-mediated inflammatory markers were observed in postmenopausal women after ingesting an elderberry extract rich in anthocyanins for 12 weeks [78]. Null or contradictory findings may be associated with more rigorous methodology, study design quality, the populations that were studied, research participant compliance, and different dose levels and distinct anthocyanin samples [79].

5.3 Pancreatic β-Cell Cytoprotection

Pancreatic β-cells are responsible for sensing and secreting the appropriate amount of insulin in response to a glucose stimulus. Because β-cells are low in free-radical quenching (antioxidant) enzymes such as CAT, SOD, and glutathione peroxidase, they are sensitive to hyperglycemia-induced oxidative stress [80]. It is known that dietary antioxidants protect pancreatic β-cells from glucose-induced oxidative stress. Multiple in vitro and in vivo studies have associated anthocyanin-mediated decreases in oxidative stress with increased pancreatic β-cell insulin production in type-2 diabetics.

In a study comparing the effects of different anthocyanins and anthocyanidins on insulin secretion from rodent pancreatic β-cells (INS-1 832/13), C3G and delphinidin-3-glucoside were the most effective insulin secretagogues among the 9 anthocyanins/anthocyanidins tested at 4 and 10 mmol/L glucose concentrations [81]. These results indicated that the number of hydroxyl groups in the anthocyanin ring B plays an important role in the β-cells' ability to secrete insulin. Sun et al. demonstrated that pretreatment of β-cells with C3G-rich bayberry fruit extract (containing 0.5 μmol/L C3G) prevented cell death, increased cellular viability, and decreased mitochondrial reactive oxygen species production and H_2O_2-induced cell necrosis [82]. The extracts dose-dependently up-regulated pancreatic duodenal homeobox 1 gene expression, which contributed to increased insulin-like growth factor II gene transcript levels and insulin protein expression in pancreatic INS-1 cells [83]. Consistently, anthocyanin enriched extract of wild Chinese blueberry could effectively protect β-cells against glucolipotoxicity and promote insulin secretion in vitro [84].

Treatment with anthocyanin extract from black soybean seed coats protected pancreatic tissue from STZ-induced apoptosis in diabetic rats by regulating apoptosis-associated proteins such as Bcl-2, Bax, and cleaved caspase-3 [85]. After 8 weeks of anthocyanin treatment from cornelian cherries (*Cornus mas*), high fat–fed mice exhibited preserved islet architecture and insulin staining [86]. Increased insulin expression was also observed in alloxan-induced diabetic Wistar rats or genetically diabetic mice that were treated with anthocyanin-rich extract [87,88]. The authors attributed large increases in plasma insulin levels to enhanced islet function and not to marked islets degranulation.

5.4 Hypoglycemic Action

Insulin resistance and glucose intolerance are two symptoms that are characteristic of prediabetes. Insulin resistance usually precedes glucose abnormalities and facilitates the progression of impaired glucose tolerance to T2DM. Continuous hyperglycemia is known to cause toxic reactions in tissues. Thus synthetic chemical agents aimed at inhibiting carbohydrate hydrolysis in the small intestine and prompting peripheral tissue glucose utilization are basic T2DM therapeutic approaches. Therefore there is a great deal of interest in natural agents that exert similar effects, and anthocyanins have been effective in this manner.

α-Glucosidase (AGH, EC 3.2.1.20), which is a membrane-bound enzyme located in the small intestinal epithelium, catalyzes the cleavage of glucose from disaccharides [89]. Thus, inhibition of AGH activity may be one of the most effective approaches to control postprandial blood glucose. Matsui et al. observed potent AGH inhibitory activity of 12 anthocyanin extracts using a free AGH assay system. In particular, acylated anthocyanins displayed strong maltase inhibition with IC_{50} values of <200 μmol/L, whereas no sucrase inhibition was observed [90]. In descending order of potency, the effect was $Pg > Pn \approx Cy$, strongly suggesting that lack of replacement at the 3′(5′)-position of the aglycon B-ring may be essential for inhibiting AGH action [91]. When diacylated anthocyanin (100 mg/kg) was administered to healthy Sprague-Dawley rats after maltose loading (2 g/kg), a reduction in blood glucose levels was observed corresponding to decreased serum insulin levels [92]. In addition, no significant changes in blood glucose were observed when sucrose or glucose was ingested, suggesting that the antihyperglycemic effect of anthocyanin was because of maltase inhibition, not because of sucrose or glucose transport inhibition at the intestinal membrane. Similarly, dietary supplementation with the chokeberry fruit extract (0.2%) decreased maltase activity in the small intestinal mucosa in a prediabetes rat model induced by a high-fructose diet and a simultaneous single STZ injection [93].

A major metabolic defect associated with T2DM is the failure of peripheral tissues in the body to properly utilize glucose, thereby resulting in chronic hyperglycemia.

Some compelling studies have reported that the hypoglycemic effect of anthocyanins is achieved by promoting glucose uptake in peripheral cells and tissues. Tsuda et al. demonstrated that adiponectin and leptin, which are two important adipocytokines that regulate metabolism, were upregulated by C3G in primary human and rat adipocytes [94,95]. In H_2O_2- or TNF-α-induced insulin-resistant 3T3-L1 adipocytes, C3G reduced intracellular ROS production and increased insulin-stimulated glucose uptake in a dose-dependent manner, indicating that anthocyanin-mediated amelioration of adipocyte dysfunction is a crucial target for insulin resistance prevention [96]. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a ligand-activated nuclear hormone receptor that controls glucose and lipid metabolism, as well as the transcription of proteins that are involved in glucose and fatty acid uptake [89]. To further investigate the underlying mechanism of insulin-like activity exerted by C3G, Scazzocchio et al. assessed PPAR-γ gene expression and transcriptional activity in human omental and murine adipocytes. The investigators found that the C3G-mediated increase in glucose uptake was associated with enhanced glucose transporter 4 (GLUT4) translocation and adiponectin secretion, which were caused by increased PPAR-γ activity. Furthermore, blueberry anthocyanin intake increased PPAR-γ activity in Zucker fatty rats adipose and skeletal muscle tissue [97].

GLUT4 is the major insulin-dependent glucose transporter, which is predominantly present in skeletal muscles, myocardium, and adipose tissue. GLUT4 plays an important regulatory role in whole-body glucose homeostasis. GLUT4 localization to the plasma membrane can be increased by PPARγ activation in the absence of insulin stimulation [98]. Black soybean anthocyanin administration in STZ-induced diabetic rats markedly enhanced GLUT4 membrane localization in heart and skeletal muscle tissues, decreased fasting blood glucose levels, and improved heart hemodynamic function [85]. Similarly, purified C3G (0.2 g/kg diet for 5 weeks) upregulated white adipose tissue GLUT4 expression and ameliorated hyperglycemia in KK-A^y diabetic mice [54]. Further, dietary anthocyanin-rich extract from mulberry significantly decreased the levels of glucose 6-phosphatase and phosphoenolpyruvate carboxykinase in the liver of diabetic C57BL/Ksj-db/db mice [99]. These observations suggest that anthocyanins may improve hyperglycemia via activation of PPARγ and promote GLUT4 translocation in peripheral tissues and inhibition of gluconeogenesis in the liver.

5.5 Regulation of Lipid Metabolism

Long-term dysregulation of lipid metabolism is strongly correlated with the development of insulin resistance and type 2 diabetes. Supplementation of diets with anthocyanins or anthocyanin extracts has been associated with decreased serum triglyceride, total cholesterol, and non-HDL cholesterol and increased serum HDL-C and apolipoprotein A-I (apo A-I) in several clinical studies (Table 12.1). This finding implicates multiple mechanisms involved in lipid metabolism regulation, including AMP-activated protein kinase activation. Microarray analysis results from Tsuda et al. revealed that adipocytokine, as well as some of lipid metabolism-related genes (uncoupling protein 2, acylCoA oxidase 1 and perilipin), were significantly induced by anthocyanin C3G in isolated rat adipocytes [95]. AMPK activation may be associated with these changes; however, the AMP:ATP ratio was significantly decreased by anthocyanin administration. Administration of anthocyanin extract, from purple sweet potatoes to mice that were fed a high-fat diet for 4 weeks reduced weight gain, hepatic triglyceride accumulation and improved serum lipid parameters. In addition, the anthocyanin fraction significantly increased AMPK and acetyl-coenzyme A carboxylase (ACC) phosphorylation, and down-regulated hepatic sterol regulatory element-binding protein 1 and its target genes ACC and fatty acid synthase (FAS) levels [100]. Consistent with this report, hepatic triglyceride content and steatosis were alleviated by purified C3G administration in high-fat fed and *db/db* diabetic mice [56]. In KK-A^y mice, C3G supplementation decreased visceral adipose tissue and liver weight, increased plasma and skeletal muscle lipoprotein lipase activity, and ameliorated hypertriglyceridemia. The authors attributed these lipid metabolism-improving effects to C3G-induced AMPK phosphorylation [101]. in vitro studies also found that anthocyanin substantially increased AMPK activity and its downstream target ACC phosphorylation, the lipolytic enzyme expressions of peroxisome proliferator activated receptor α (PPARα) and carnitinepalmitoltransferase-1 (CPT1), thus significantly increasing fatty acid oxidation in HepG2 cells [102,103]. These studies suggest the hypolipidemic effects of anthocyanin occur via phosphorylation of AMPK and inhibition of lipid biosynthesis and stimulation of lipolysis.

6 CONCLUSION AND PERSPECTIVE

A multitude of in vitro and in vivo studies have demonstrated the antidiabetic properties of anthocyanins in cell culture and experimental diabetes models. These studies provide a foundation for further clinical trials. Although several in vitro and animal studies with anthocyanins strongly suggest their beneficial effects on hyperlipidemia-associated diabetes, advanced glycation endproducts, and oxidative stress, clinical evidence for anthocyanin use as diabetes treatment is not convincing. Most of the existing literature on anthocyanin biological activity is derived from anthocyanin-rich products such

as berries or anthocyanin-rich plant extracts, not purified anthocyanins. In addition, the effect of long-term anthocyanin exposure is still largely unknown. Therefore, additional large-scale and long-term clinical trials are needed to draw a firm conclusion about the contribution of anthocyanins to the management and prevention of diabetes.

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CHAPTER

13

The Role of Direct and Indirect Polyphenolic Antioxidants in Protection Against Oxidative Stress

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Abbreviations

ARE	antioxidant responsive element
CAT	catalase
COX	cyclooxygenase
Cys	cysteine
γ-GCS	γ-glutamylcysteine synthetase
GP	glutathione peroxidase
GR	glutathione reductase
GST	glutathione *S*-transferases
HO-1	heme oxygenase-1
IL	interleukin
iNOS	inducible nitric oxide synthase
Keap1	Kelch-like ECH-associated protein 1
LPS	lipopolysaccharide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
ROS	reactive oxygen species
SOD	superoxide dismutase
***t*-BHP**	*tert*-butyl hydroperoxide
TNF-α	tumor necrosis factor-α
UGT	uridine 5′-diphospho-glucuronyl transferase

1 INTRODUCTION

Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen and are formed endogenously when, for example, the body converts food to energy [1,2]. The ROS produced in cells include hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), and free radicals such as the hydroxyl radical ($OH^\bullet$) and the superoxide free radical ($O_2^{\bullet-}$) [1–3]. The hydroxyl radical is particularly unstable and will react rapidly and nonspecifically with most biological molecules and is considered a major player in oxidative damage [4–8]. This reactive radical is produced from H_2O_2 in metal-catalyzed redox reactions such as the Fenton reaction (Fig. 13.1) [1,2]. One major mechanism by which $O_2^{\bullet-}$ is toxic to cells is the fact that it is able to liberate the iron necessary for the Fenton reaction from iron-containing proteins. This ability of $O_2^{\bullet-}$ to release iron from ferroproteins has been demonstrated for, e.g., ferritin [9,10]. Highly reactive oxidants and electrophiles such as ROS can damage cells by direct oxidation and/or starting chemical chain reactions resulting in lipid peroxidation and oxidation of DNA and/or proteins [6,11,12]. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms, while damage to lipids leads to a decline in physiological functions and cell death. Damage to proteins causes enzyme inhibition, denaturation, and protein degradation, which also causes disruptions in normal mechanisms of cellular signaling and cell death. Furthermore, oxidative changes in lipoproteins may lead to atherosclerosis. Thus the damaging of different biomolecules by ROS may lead to cardiovascular, neurodegenerative, and inflammatory diseases, diabetes, Alzheimer's and cancer, among others [12–15].

The production of ROS can be regarded as a paradox in metabolism, and consequently the body contains a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components [16]. In general, antioxidant systems either prevent ROS from being formed, or remove them before they can damage vital components of cells. ROS also play an important regulatory role in relation to cell signaling, regulating several pathways affecting

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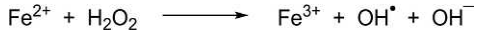


FIG. 13.1 The reactive hydroxyl radical ($OH^{\bullet}$) is produced from hydrogen peroxide (H_2O_2) via the Fenton reaction under oxidative stress.

a variety of cellular processes, such as proliferation, metabolism, differentiation, apoptosis, antioxidant, and antiinflammatory responses as well as iron homeostasis, and not least, the adaptive stress response of cells [17–21]. The function of antioxidant systems is therefore not to remove ROS entirely, but instead to keep them at the optimum level in the body [16,21,22].

An antioxidant is a molecule that inhibits the oxidation of other molecules. If we consider the human body, antioxidants are substances that, at low concentrations, prevent or retard the oxidation of biomolecules, thus preventing oxidative damage to lipids, proteins, RNA, and DNA [16,23–25]. Two major groups of antioxidants are recognized, namely enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants include phase 2 enzymes such as glutathione *S*-transferases (GSTs) and uridine 5′-diphospho-glucuronyl transferases (UGTs) (Table 13.1) [24–27,30]. Nonenzymatic antioxidants can be divided into two categories: the exogenous and the endogenous antioxidants. The exogenous antioxidants are either water-soluble (e.g., ascorbic acid [vitamin C] and polyphenolic compounds) or lipid-soluble (e.g., tocopherols [vitamin E] and carotenoids) natural products that are abundantly present in herbs, spices, fruits, and vegetables [15,31–33], whereas the endogenous antioxidants are produced in our body and consist of, e.g., glutathione, coenzyme Q (ubiquinol), α-lipoic acid, uric acid, ferritin, and thioredoxin [15,27,34–37]. Low levels of antioxidants and/or inhibition of the antioxidant enzymes, can cause an overproduction of ROS, thus leading to a condition known as oxidative stress. Oxidative stress is defined as an imbalance of anti- and prooxidative reactions in favor of the prooxidant, which leads to a condition where the production of ROS exceeds the capacity of a biological system to readily detoxify the reactive intermediates or to repair the resulting damage [38,39]. Thus, oxidative stress is an unwanted condition that may lead to a wide range of serious diseases.

The need for protection against ROS and other reactive molecules has led to the specious theory that a high intake of exogenous antioxidants protects the body from oxidative stress, which is also widely known as the antioxidant hypothesis [40,41]. This hypothesis was mainly based on the introduction of the free radical theory in 1956 by Denham Harman, who considered ROS to be one of the main reasons for the aging process, including the development of age-related diseases such as cancer, diabetes mellitus, and cardiovascular diseases [42]. The antioxidant hypothesis has been substantiated by many investigations throughout the last 30 years, which have shown that a high intake of fruit and vegetables rich in natural antioxidants such as vitamins C and E, polyphenols, carotenoids, terpenoids, and phytomicronutrients show an inverse association with the risk of cancer and the development of cardiovascular diseases [43–48]. Consequently, many initial studies have suggested that antioxidant supplements might promote health, but today we know from many clinical trials and meta-analyses of intervention studies that antioxidant supplementation has no beneficial effect on age-related diseases. In fact,

TABLE 13.1 Important Cytoprotective Proteins Induced by Activation of the Keap1-Nrf2-ARE Regulatory Pathway and Their Main Functionality [21,26–29]

Cytoprotective protein	Abbreviation[a]	Function
Catalase	CAT	Elimination of H_2O_2
5′-Diphospho-glucuronyl transferase	UGT	Phase 2 enzyme (glucuronidation)
Ferritin	–	Iron metabolism, stress response
γ-Glutamylcysteine synthetase	γ-GCS	Glutathione biosynthesis and recycling
Glutathione peroxidase	GP	Elimination of H_2O_2
Glutathione reductase	GR	Reduces glutathione disulfide to the sulfhydryl form glutathione, which is an important cellular antioxidant
Glutathione *S*-transferase	GST	Phase 2 enzyme (glutathionylation)
Heme oxygenase-1	HO-1	Heme catabolism, stress response
NAD(P)H:quinone oxidoreductase 1	NQO1	Reduction of quinones to hydroquinones
Sulfotransferase	–	Phase 2 enzyme (sulfonation)
Superoxide dismutase	SOD	Elimination of superoxide radical
Thioredoxin	–	Cysteine thiol-disulfide exchange
Thioredoxin peroxidase	–	Antioxidant enzyme whose specific function is unknown
Thioredoxin reductase	–	Reduction of oxidants and oxidized thiols

[a] *In this review.*

we now know that excess supplementation with certain putative antioxidants may be harmful and can cause severe side effects and even increase mortality [49–56]. Although the antioxidant hypothesis a priori seems attractive and logical, our present knowledge about the role of antioxidants in relation to human health certainly does not support the antioxidant hypothesis. The antioxidant hypothesis is nothing more than wishful thinking. How does this fit in with the well-documented health effects of fruits and vegetables and their relatively high concentrations of antioxidants?

The ascribed health effects of antioxidants in many studies are mainly based on in vitro studies, which do not necessarily reflect the human physiological mechanisms in vivo. These controversies regarding the effectiveness of antioxidants have stimulated research, particularly in vivo studies to focus on the bioavailability of antioxidants, to bridge the gap to the results obtained from the in vitro studies. Available epidemiological research data tend to confirm the protective effects of polyphenol antioxidants against cardiovascular diseases [57,58]. On the other hand, the reported beneficial effects of particular polyphenolic compounds against cancers and neurodegenerative ailments such as Alzheimer's and Parkinson's disease are based largely on in vitro studies [59]. The disparity between an excellent in vitro activity related to a specific disease(s) and an uncertain in vivo effect may be due to poor bioavailability coupled with low absorbability, and/or metabolism resulting in the generation of new molecules and thus the loss-of-function of the native antioxidants [15,32,60]. Another contributing factor could be that the effects observed from in vitro experiments are based on much higher doses compared to those available in a normal human diet [32,59]. Finally, the health promoting effects of food and food products could simply be due to other biomolecules with other mechanisms of action or antioxidants whose mechanisms of action are different from the traditional view of exogenous antioxidants being redox active compounds that directly quench free radicals and ROS [27,56].

There are many examples of natural products from herbs, spices, fruits, and vegetables having, for example, cytotoxic and antiinflammatory activity and thus also a potential anticancer effect [27] and/or exert their bioactivity due to the activation of endogenous antioxidant defense mechanisms [21,27,56,61–64]. One group of natural products that induce such an adaptive stress response and thereby act as indirect antioxidants are polyphenols or polyphenol-derived compounds [21,27,61–75]. Although many of these compounds exert direct antioxidant activity in vitro their bioactivity in vivo appears foremost to be related to their indirect antioxidant activity and/or related mechanisms of action, including inhibition of inflammatory proteins such as cyclooxygenases (COXs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [32]. This review primarily focuses on the role of polyphenols as inducers of cellular defense mechanisms, and in particular phase 2 enzymes, and how this defense mechanism protects against oxidative stress and the development of inflammatory diseases, diabetes, and cancer.

2 CYTOPROTECTIVE PROTEINS (PHASE 2 ENZYMES)

To counteract the damaging effects of oxidants and electrophiles, all eukaryotic organisms have evolved detoxification mechanisms that are composed of three phases: functionalization, conjugation, and excretion, which are termed phase 1, 2, and 3 detoxification, respectively. The phase 1 detoxification system is mainly composed of the cytochrome P450 supergene family of enzymes and is the first enzymatic defense against foreign compounds (xenobiotics). In the typical phase 1 reaction, the cytochrome P450 oxidases use oxygen and nicotinamide adenine dinucleotide phosphate (NADPH), as a coenzyme, introducing reactive or polar groups into xenobiotics producing inert and nontoxic compounds; however, in some cases the products of the phase 1 transformation may be highly reactive molecules that possess electrophilic or oxidant properties [27,76–79]. If such reactive molecules are not further metabolized by phase 2 detoxification (conjugation), they may cause damage to proteins, RNA, and DNA within the cell. Phase 2 reactions generally follow phase 1 activation, resulting in a xenobiotic that has been transformed into a water-soluble compound through conjugation reactions such as glucuronidation, glutathionylation, and/or sulfonation. These reactions are catalyzed by the transferase enzymes GSTs, UGTs, and sulfotransferases [21,27,76,79]. Finally, in phase 3 the conjugated xenobiotics may be further metabolized, before being recognized by efflux transporters and pumped out of the cells and excreted through the urine and/or the bile [27,76,80,81].

Several studies have shown evidence of associations between induced phase 1 and/or decreased phase 2 activities and an increased risk of disease, such as cancer and Parkinson's disease [76,82–85]. Furthermore, it has been demonstrated that selective induction of phase 2 enzymes without major effects on phase 1 enzymes has a significant reducing effect on the production of mutagenic metabolites [27,76,86,87]. These discoveries led to the widely accepted hypothesis that induction of phase 2 genes and, hence, an increased production of phase 2 enzymes, plays a central role in reducing the risk of cancer and other serious diseases [88].

The family of phase 2 enzymes includes, in addition to the classic conjugating enzymes GSTs and UGTs, many

other proteins, some of which have been traditionally known as antioxidant and/or detoxification enzymes. The latter include, e.g., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione reductase (GR), γ-glutamylcysteine synthetase (γ-GCS), NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), thioredoxin reductase, and thioredoxin peroxidase as well as other protective proteins that may not have enzymatic activities as, for example, ferritin and thioredoxin (Table 13.1) [21,24–28,30] A distinctive feature for many phase 2 enzymes is that they use substrates that are not necessarily products of phase 1 metabolism. All phase 2 enzymes share a common transcriptional regulation, are inducible, and catalyze reactions that, in most cases, collectively result in protection against damaging electrophiles and oxidants, and hence oxidative stress. Phase 2 enzymes are therefore sometimes also categorized as cytoprotective proteins, which is perhaps also a more appropriate name for these [27,28].

Inducers of phase 1 and 2 enzymes can be mono- or multifunctional. A monofunctional inducer affects more or less only one detoxification and/or antioxidant enzyme, whereas a multifunctional inducer affects multiple detoxification and/or antioxidant enzymes. The enhancement of phase 2 enzyme activity has been proposed to explain, at least in part, the ability of fruits and vegetables to protect against many cancers and other diseases due to their relatively high content of phase 2 enzyme multifunctional inducers, of which many are polyphenols or polyphenol-derived compounds [21,27,28,79,89,90].

3 INDUCTION OF CYTOPROTECTIVE PROTEINS

The induction of cytoprotective proteins in response to oxidative stress is regulated primarily at the transcriptional level by three cellular components, which are the antioxidant responsive element (ARE), the nuclear factor erythroid 2-related factor 2 (Nrf2), and the Kelch-like ECH-associated protein 1 (Keap1) that constitute the so-called Keap1-Nrf2-ARE regulatory pathway. This regulatory pathway controls the expression of genes whose protein products are involved in the detoxification and elimination of reactive oxidants and electrophilic agents through conjugative reactions and by enhancing cellular antioxidant capacity [21,27,28,91–102].

ARE mediates the transcriptional induction of a battery of genes including phase 2 genes. ARE is activated by chemical compounds with the capacity to either undergo redox cycling or be metabolically transformed to a reactive or electrophilic intermediate. Activation of gene transcription through the ARE is mediated primarily by Nrf2, a basic leucine zipper transcription factor responsible for both basal and inducible expression of cytoprotective phase 2 genes. Nrf2 activity is regulated in part by Keap1 containing highly reactive cysteine (Cys) residues and is the sensor and chemical target for inducers [21,27,28,92,95–102]. In uninduced conditions, Keap1 binds Nrf2 tightly, and subsequently undergo proteasomal degradation thus repressing its ability to enter the nucleus. However, covalent modification of the reactive Cys residues of Keap1 by small molecules (inducers) leads to dissociation of Keap1 from Nrf2, which then accumulates in the nucleus and activates in particular genes coding for phase 2 detoxifying enzymes having an ARE in their promotor sequence. Thus reactivity toward specific Cys residues triggers the induction of a battery of genes, and Keap1 therefore serves specifically as a "Cys-reactive sensor." Potent inducers of the Keap1-Nrf2-ARE regulatory pathway are therefore compounds that have the propensity to react with thiol groups in Cys such as compounds with an α,β-unsaturated carbonyl group or other electrophilic groups resulting in *S*-alkylation of Keap1. However, ROS, in particular H_2O_2, also seem to be able to activate Nrf2 via Keap1 oxidation/modification and therefore compounds that increase intracellular ROS may also be considered as inducers of the Keap1-Nrf2-ARE regulatory pathway [103,104]. Both types of inducers are found among polyphenols and polyphenol-derived compounds and/or their metabolized products (see Section 5) as well as in many other natural products such as sesquiterpene lactones, triterpenoids, polyacetylenes and glucosinolates. The latter compounds are not described in this review. A simplified view of the Keap1-Nrf2-ARE regulatory pathway is shown in Fig. 13.2.

4 DIRECT AND INDIRECT ANTIOXIDANTS AND THEIR ROLE IN PROTECTION AGAINST OXIDATIVE STRESS

The fact that induction of the endogenous antioxidant defense plays a vital role in prevention of oxidative stress has resulted in a distinction between different types of antioxidants, depending on their mechanisms of action. The traditional view of antioxidants as compounds that directly quench free radicals and ROS is too simplistic and inadequate to explain cellular protection against oxidative stress. Consequently, the concept of direct and indirect antioxidants has been introduced [21,27,29,101,105]. Direct antioxidants are low molecular-weight compounds that are redox active and able to scavenge ROS and other reactive molecules. The most effective direct antioxidants are the exogenous antioxidants ascorbate and

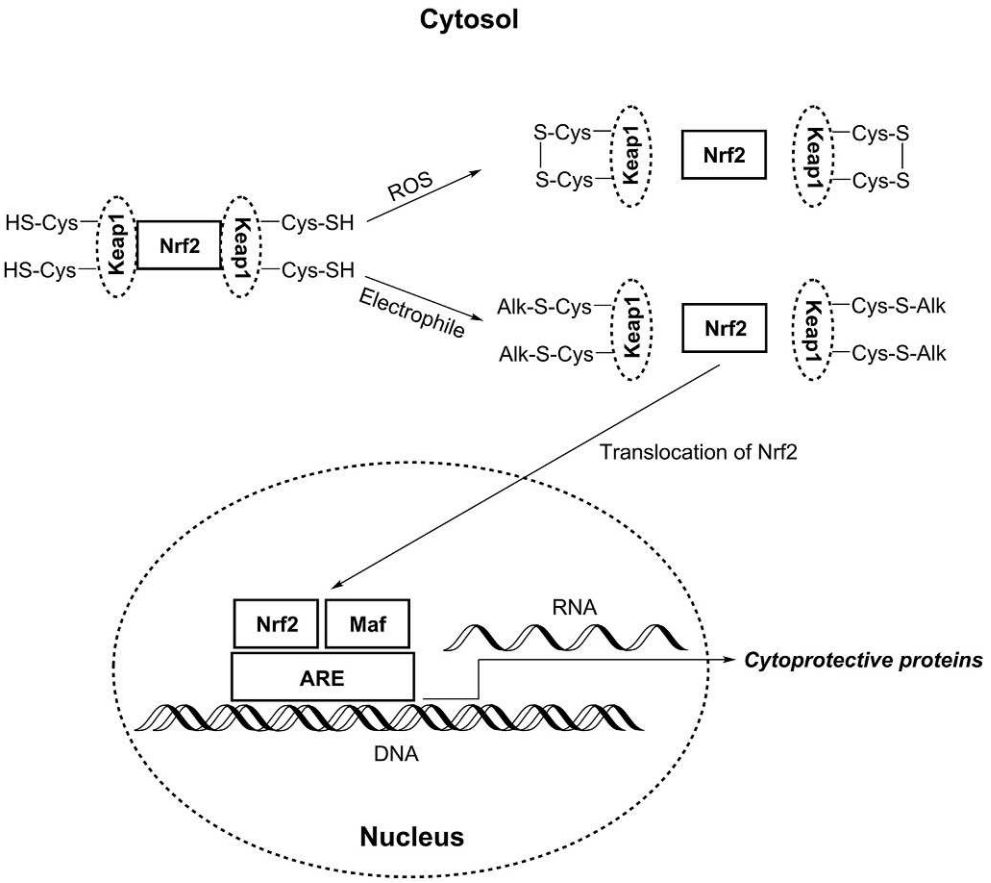


FIG. 13.2 A simplified view of the Keap1-Nrf2-ARE regulatory pathway. Nuclear factor erythroid 2-related factor 2 (Nrf2) is rapidly degraded under normal conditions, i.e., a normal redox balance. Since Kelch-like ECH-associated protein 1 (Keap1) is a cysteine-rich protein, chemical modifications induce conformational changes and the release of Nrf2. After its translocation into the nucleus, Nrf2 binds to antioxidant response elements (AREs) through the small protein transcription factor (Maf), thereby affecting the expression of cytoprotective proteins (phase 2 enzymes). *Cys*, cysteine; *Alk*, alkylation.

tocopherols as well as the endogenous antioxidants glutathione, α-lipoic acid, and ubiquinols. Direct antioxidants are short-lived and either consumed or chemically modified in the process of their antioxidant action and therefore need to be replenished or regenerated, which may evoke prooxidant effects [21,27,28]. Many polyphenol antioxidants with phenol groups in *ortho* or *para* positions, that is, compounds containing a 1,2-diphenol or a 1,4-diphenol moiety, respectively, as found in for example flavonoids, diterpenoids, and phenolic acids, are compounds that both have direct antioxidant and prooxidant properties; however, they can also act as indirect antioxidants due to the strong electrophilic nature of their oxidized forms, which can participate in electrophilic conjugation reactions (see Fig. 13.3 and Section 5) [21,28].

Indirect antioxidants are not necessarily redox active, as this is not their primary mechanism of action. A characteristic feature of indirect antioxidants, which makes them efficient antioxidants, is their ability to activate the Keap1-Nrf2-ARE regulatory pathway, resulting in transcriptional induction of various cytoprotective proteins such as GSTs, γ-GSTs, GR, GP, HO-1 and NQO1 that play a major role in the production and regeneration of glutathione, the most abundant cellular small-molecule endogenous direct antioxidant [21,27]. The redox proteins thioredoxins as well as thioredoxin peroxidase and thioredoxin reductase are also elevated through the Keap1-Nrf2-ARE regulatory pathway [105–107]. The thioredoxins act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange but have also direct antioxidant properties, such as quenching of free radicals and as partners of thioredoxin peroxidase in the scavenging process of, for example, H_2O_2 (Table 13.1) [108–110]. Thioredoxin reductases catalyze the reduction of oxidized thioredoxins using NADPH and also play a role in the regeneration of the direct antioxidant ascorbate from its oxidized form [111]. The lipid-soluble direct antioxidants ubiquinols participate in mitochondrial electron transport, protect membrane phospholipids and serum low-density lipoprotein from peroxidation, and furthermore participate

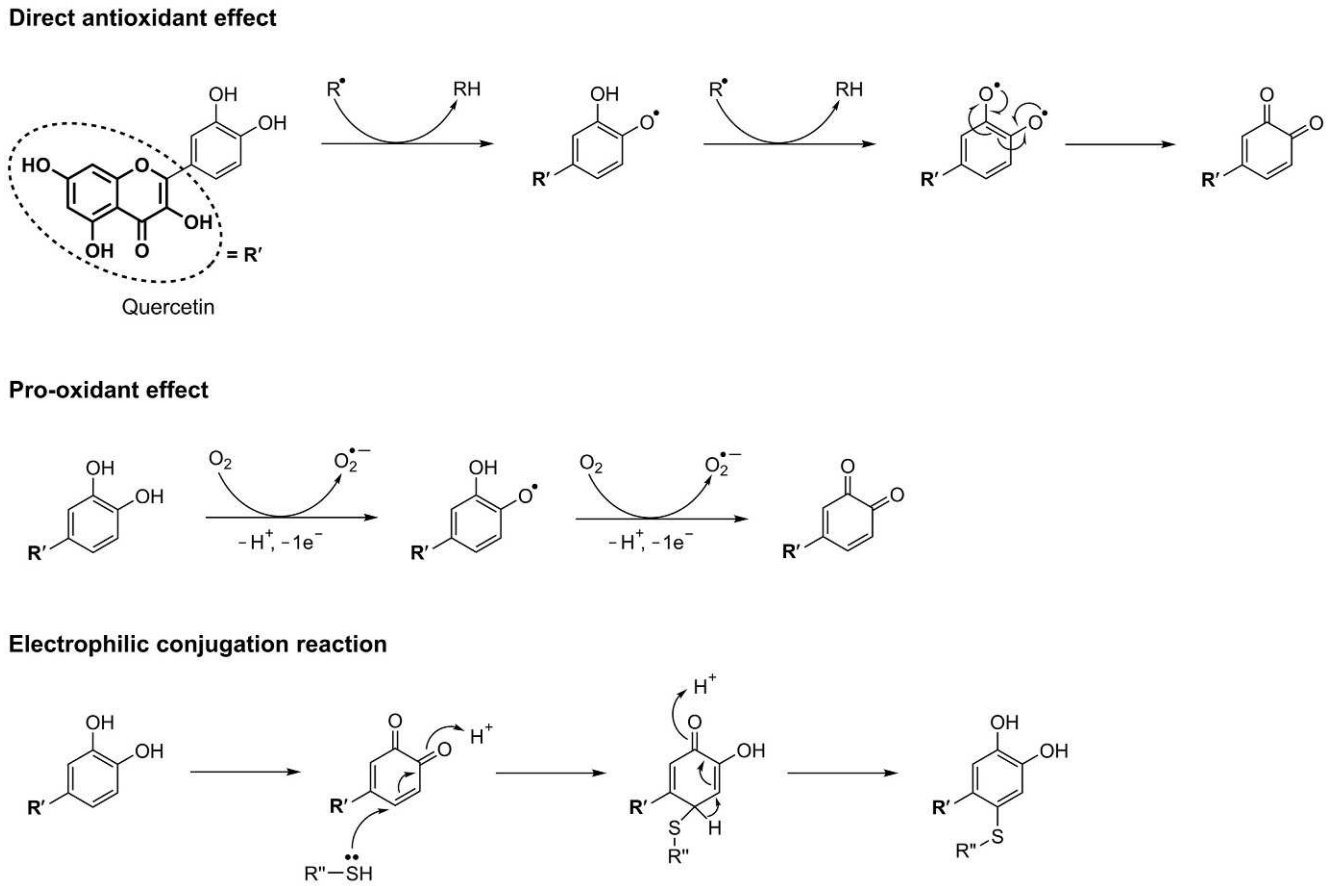


FIG. 13.3 Different types of reactions of polyphenols containing a 1,2-diphenol (catechol moiety). According to the above reactions polyphenols like quercetin both have antioxidant and prooxidant properties as well as the potential to participate in electrophilic conjugation reactions with cysteine residues of proteins (R″-SH) in a Michael reaction.

in the regeneration of other direct antioxidants such as tocopherols and ascobate [112].

Several cytoprotective proteins participate in the regeneration of ubiquinols from their oxidized forms and include thioredoxin reductase, GP, and NQO1. NQO1 is part of the antioxidant defense system and is primarily involved in the detoxification of mutagenic and carcinogenic quinones, through their two electron reduction to hydroquinones [29]. NQO1 protects cells from quinone damages by reducing them and diverting them from potential formation of damaging semiquinone reactive intermediates, which can further lead to ROS by oxidative cycling. NQO1 also protects cells from oxidative stress by maintaining antioxidant forms of ubiquinone and vitamin E and this enzymatic activity can actually be sufficient to protect against carcinogenesis [29].

Characteristic features of cytoprotective proteins are that they act as catalysts, are not consumed in their antioxidant actions, have relatively long half-lives, and are able to catalyze a wide variety of chemical reactions that almost invariably lead to deactivation of reactive electrophiles and oxidants and thus to detoxification. Hence inducers that up-regulate genes for the production of cytoprotective proteins play a major role in protection against oxidative stress.

5 POLYPHENOL AND POLYPHENOL-DERIVED INDUCERS OF CYTOPROTECTIVE PROTEINS (INDIRECT ANTIOXIDANT EFFECT)

In vivo studies have revealed that induction of the cellular defense system represents a major cellular response that involves a battery of cytoprotective proteins, which is independent of the organ and tissue type, and results in protection against various electrophiles and oxidants [27]. One of the most important groups of inducers of cytoprotective proteins found among natural products is compounds with an α,β-unsaturated carbonyl group being highly reactive towards nucleophiles, such as reactive proteins within the cellular matrix acting as Michael acceptors in a Michael-type addition reaction (Fig. 13.3). Highly reactive proteins within the cellular matrix are found in, for example, Keap1 containing cysteine thiols

that are susceptible to thiol oxidation, disulfide exchange, and *S*-alkylation. The latter reaction occurs due to a nucleophilic attack of the thiol group on electrophiles such as α,β-unsaturated carbonyls to form a covalent bond. It has been shown that Michael acceptors react with certain highly reactive cysteine thiols of Keap1, thus activating the Keap1-Nrf2-ARE regulatory pathway [21,27,28,93,113]. Consequently, it is the Michael acceptor functionalities that make many natural products highly reactive towards nucleophilic groups in biomolecules and to a large extent can explain their role as indirect antioxidants as well as many of their other bioactivities. Michael acceptor functionalities are found in many phytochemicals, including polyphenols and polyphenol-derived compounds such as curcuminoids and related compounds, chalcones, coumarins, diterpenoids, flavonoids, isoflavonoids, and phenolic acids. Importantly, many of these are abundant in edible plants and consequently are components of the human diet. In the following paragraphs a variety of electrophiles of polyphenolic origin are described that have been shown to induce the production of cytoprotective proteins and to have a protective role against diseases, such as inflammation and cancer.

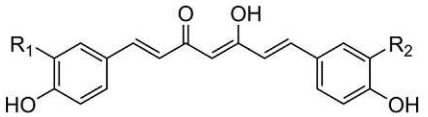
5.1 Curcuminoids, Gingerols, and Shogaols

Curcumin (**1**) (Fig. 13.4), is the main bioactive constituent in turmeric roots (*Curcuma longa* L., Zingiberaceae), which is the principal coloring and flavoring agent of curry. Curcumin possesses two Michael acceptor functionalities (β-diketone structure) in its structure, although it exists as a ketoenol rather than as a diketone. It has been shown to possess antiinflammatory, antioxidant, and antitumor properties and has furthermore been shown to induce cytoprotective proteins through the Keap1-Nrf2-ARE regulatory pathway [27,28,61,113–116]. In addition to its ability to potently induce cytoprotective proteins, curcumin is a pleiotropic agent with multiple molecular targets and much of its beneficial effect is found to be due to its inhibition of the transcription factor NF-κB [117–119]. Curcumin has for example been shown to prevent muscle damage by regulating NF-κB and Nrf2 pathways and to improve performance in vivo [119] and to inhibit NF-κB activation through thiol modification [120], as well as to inhibit the activity of thioredoxin reductase by alkylation of the reactive sites of this enzyme, which regulate the activity of NF-κB [117]. Curcumin has furthermore been shown to be beneficial in all three stages of carcinogenesis [121] and to inhibit tumor development in numerous animal models and is presently in clinical trials [122,123]. In addition, curcumin has been reported to induce HO-1 expression through Nrf2 activation in vascular endothelial cells and astrocytes [115,124]. The treatment of astrocytes with curcumin not only leads to induction of HO-1 but also other cytoprotective proteins such as NQO1 and GST, thus providing protection of neurons against oxidative stress and damage, which indicates that curcumin has neuroprotective effects and hence may be used to treat Alzheimer's and Parkinson's disease as it is capable of crossing the blood-brain barrier [124].

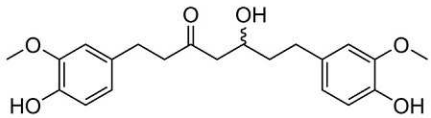
Minor polyphenolic constituents in turmeric roots are bisdemethoxycurcumin (**2**) and demethoxycurcumin (**3**) also having unsaturated ketone moieties (Fig. 13.4) and these compounds have also been shown to exhibit antiinflammatory as well as antiproliferative activity and their mode of action appears to be the same as for curcumin [125]. These curcumin derivatives are also capable of inducing Nrf2-mediated phase 2 gene expression and of interacting with Keap1 in vitro [61,114]. Likewise, the shogaols (**11**–**15**, **21**; Fig. 13.4) such as [6]-shogaol (**12**), [10]-shogaol (**14**), and [6]-dehydroshogaol (**21**) from ginger roots (*Zingiber officinale* Roscoe, Zingiberaceae), which are well-known for their antiinflammatory activity, have been shown to induce phase 2 enzymes by inducing NQO1 and Nrf2-mediated phase 2 gene expression through ARE by interaction with Keap1 in vitro [71,126–128], and in the case of [6]-shogaol also in vivo in mice [129]. Like the curcuminoids, the shogaols also contain an α,β-unsaturated carbonyl moiety, which explains their phase 2 enzyme-inducing activity as well as their bioactivity in general. The related gingerols (**16**–**20**; Fig. 13.4), however, do not contain an α,β-unsaturated carbonyl moiety, but still they appear to inhibit ROS production and proinflammatory mediators, as demonstrated in activated human leukocytes, and to be inducers of phase 2 enzymes, although their antiinflammatory and indirect antioxidant effect is considerably less compared to the shogaols [71,130]. The inducing capacity of gingerols is probably related to their *ortho*-dioxyphenol functional unit. The mechanism by which these compounds can induce cytoprotective proteins may be the same as for, e.g., many flavonoids (see Section 5.3) and phenolic acids (see Section 5.7) [71]. Although the major constituents in ginger roots are gingerols and shogaols, they also contain other related compounds such as 1-dehydrogingerdiones (**7**–**10**, Fig. 13.4) as well as diarylheptanoids (e.g., **4**–**6**, Fig. 13.4) closely related to the curcuminoids, which also have antiinflammatory activity [127,128,131–133], and most likely also are able to induce phase 2 enzymes, as demonstrated for 1-dehydro-[6]-gingerdione (**8**) [71,128,129].

5.2 Chalcones and Dihydrochalcones

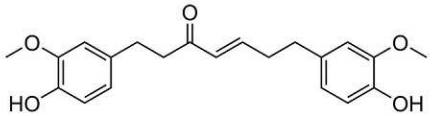
Chalcones are precursors of the flavonoids in secondary metabolism but in contrast to flavonoids they contain



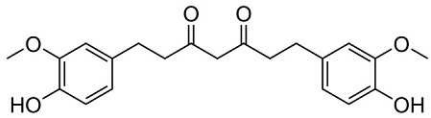
Curcumin (**1**): $R_1 = R_2 = OCH_3$
Bisdemethoxycurcumin (**2**): $R_1 = R_2 = H$
Demethoxycurcumin (**3**): $R_1 = H$, $R_2 = OCH_3$



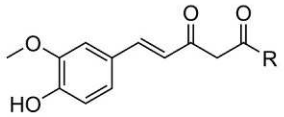
Hexahydrocurcumin (**4**)



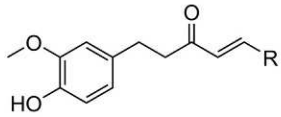
1,7-*Bis*-(4′-Hydroxy-3′-methoxyphenyl)-4-heptene-3-one (**5**)



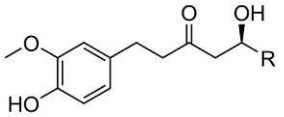
1,7-*Bis*-(4′-Hydroxy-3′-methoxyphenyl)-3,5-heptadione (**6**)



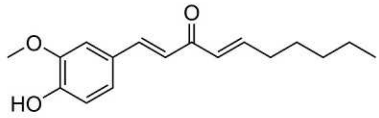
1-Dehydro-[3]-gingerdione (**7**): R =
1-Dehydro-[6]-gingerdione (**8**): R =
1-Dehydro-[8]-gingerdione (**9**): R =
1-Dehydro-[10]-gingerdione (**10**): R =



[4]-Shogaol (**11**): R =
[6]-Shogaol (**12**): R =
[8]-Shogaol (**13**): R =
[10]-Shogaol (**14**): R =
[12]-Shogaol (**15**): R =



[4]-Gingerol (**16**): R =
[6]-Gingerol (**17**): R =
[8]-Gingerol (**18**): R =
[10]-Gingerol (**19**): R =
[12]-Gingerol (**20**): R =



[6]-Dehydroshogaol (**21**)

FIG. 13.4 Chemical structures of some naturally occurring curcuminoids, gingerols, and shogaols (the dehydrated form of gingerols) and related derivatives, having one or two Michael acceptor centers, and/or can be transformed/oxidized to 1,2-diphenols and then further oxidized into electrophilic quinones. The compounds **1**–**3**, **8**, **12**, **14**, **17**, and **21** have been shown to induce the formation of cytoprotective proteins (see Section 5.1).

a reactive α,β-unsaturated carbonyl group and are therefore potential inducers of cytoprotective proteins. Isoliquiritigenin (4,2′,4′-trihydroxychalcone (**25**), Fig. 13.5) is found in various edible plants, e.g., licorice (*Glycyrrhiza glabra* L., Fabaceae), soy beans (*Glycine max* L., Fabaceae), and shallots (*Allium ascalonicum* L., Alliaceae) and has for example been found to possess antiinflammatory activity [134–136] and to exhibit significant cytotoxic and anticancer activities such as induction of cell cycle arrest [137], suppression of pulmonary metastasis of mouse renal cell carcinoma through activation of the immune system [138], and induction of apoptosis in human gastric cancer cells [139]. As a chemopreventive agent in vivo, isoliquiritigenin has been shown to prevent, for example, colon cancer in a mice carcinogenesis model [140]. One of the mechanisms by which isoliquiritigenin exerts its chemopreventive effects is by the induction of cytoprotective proteins. Isoliquiritigenin has, for example, been shown

to react with the inducer sensor Keap1 and to induce Nrf2-mediated phase 2 gene expression in vitro [127,135,136,141] and to elevate NQO1 enzyme activity [142,143]. Furthermore, isoliquiritigenin has also shown potential cancer chemopreventive activity in vivo in female Sprague-Dawley rats treated with the carcinogen 7,12-dimethylbenz(a) anthracene by showing an increased latency of mammary tumor development and to induce NQO1 in the colon as well as glutathione and GST in the liver [70,143]. The growth inhibition and apoptosis of cancer cells of the related isoliquiritigenin 2′-methyl ether (**26**) has been shown to be due to activation of Nrf2 and up-regulation of HO-1 [144]. Furthermore, it has been demonstrated that the suppressive effects of the 4-glucoside of isoliquiritigenin (isoliquiritin, **27**) and isoliquiritigenin on lipopolysaccharide (LPS)-induced inflammatory responses in murine macrophage are caused by suppressing the iNOS and COX-2 proteins and mRNA expression. This antiinflammatory effect has been shown to be associated with an induction of antioxidant and detoxification enzymes, including

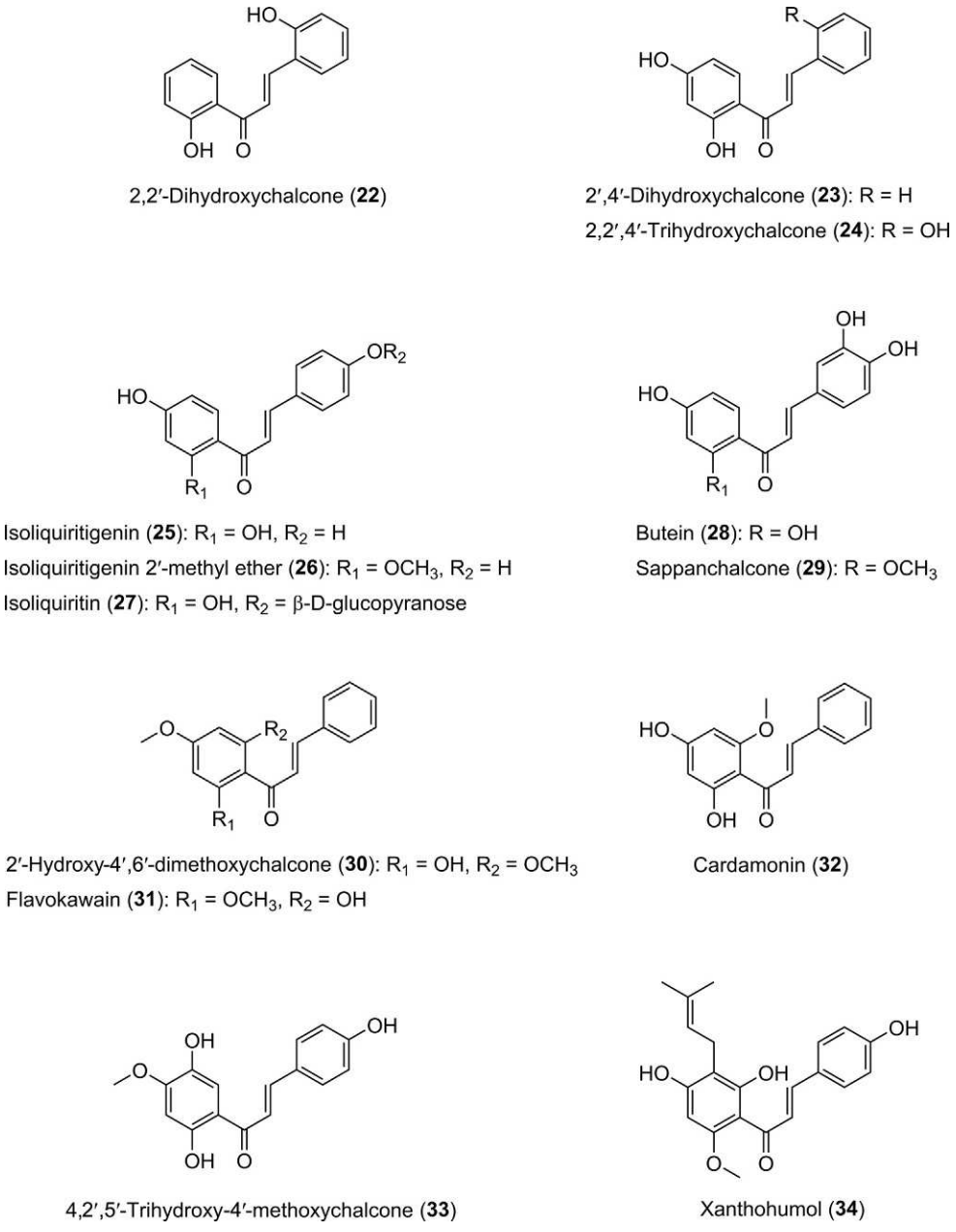


FIG. 13.5 Naturally occurring chalcones (**22**–**39**) and dihydrochalcones (**40**–**42**) that have been shown to induce the formation of cytoprotective proteins. For the chalcones this is most likely due to the presence of an α,β-unsaturated carbonyl in their structure, giving them Michael acceptor properties, whereas for the dihydrochalcones the induction of cytoprotective proteins is probably due to the presence of other electrophilic groups and/or metabolization of *ortho*-phenol groups to the corresponding reactive quinones.

(continued)

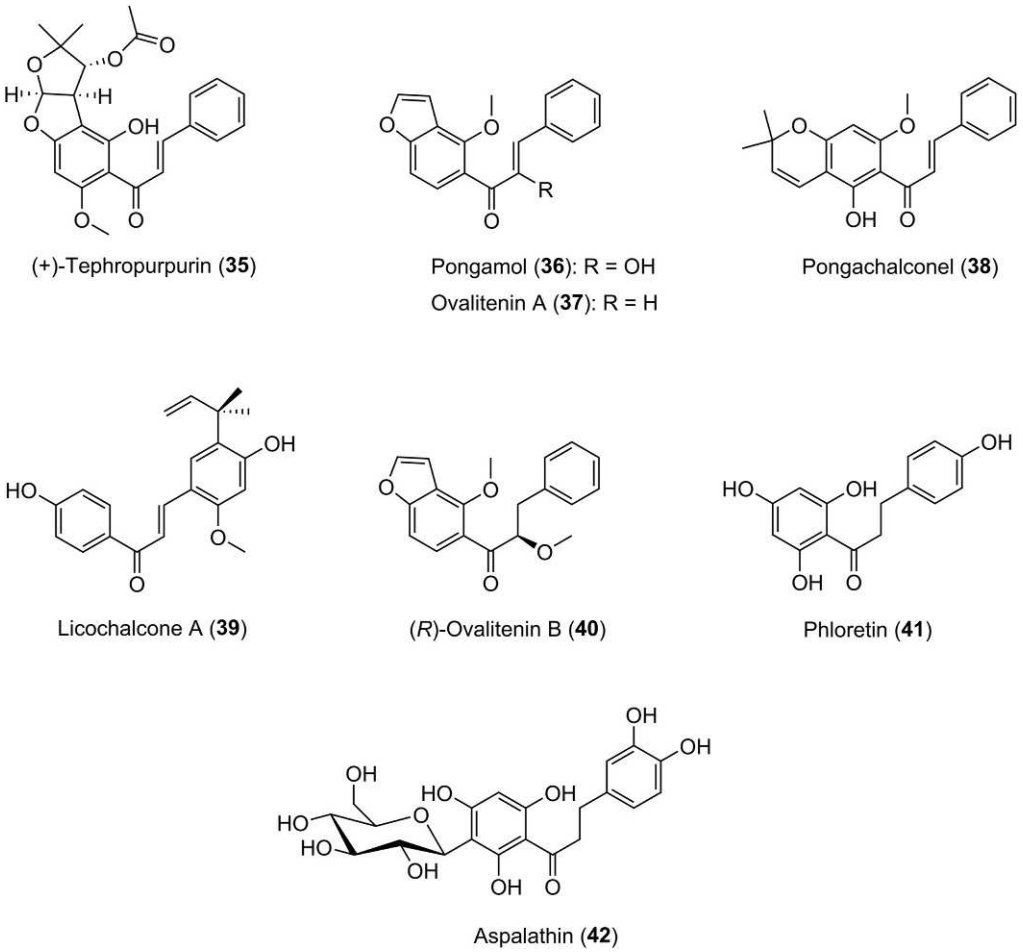


FIG. 13.5, CONT'D

UGT, NQO1, and HO-1 [135]. In relation to the antiinflammatory activity of isoliquiritigenin, it has recently also been demonstrated that isoliquiritigenin alleviates early brain injury in rats after intracerebral hemorrhage induction via suppressing ROS- and/or NF-κB-mediated NLRP3 inflammasome activation by triggering the Nrf2 antioxidant system [145]. Thus an important part of the chemopreventive and antiinflammatory effects of isoliquiritigenin and related derivatives may be ascribed to their role as indirect antioxidants.

Xanthohumol (**34**, Fig. 13.5) from hop (*Humulus lupulus* L., Cannabaceae) is a prenylated chalcone with potential anticarcinogenic activity and with a broad spectrum of inhibitory mechanisms at the initiation, promotion, and progression stages of carcinogenesis [146]. As potential antitumor-promoting mechanisms, xanthohumol has demonstrated antiinflammatory properties by inhibition of COX-1 and COX-2 activity as well as inhibition of the NLRP3 inflammasome and NF-κB signaling pathway activation [147,148], but has also been shown to prevent carcinogenesis in the progression phase, which includes inhibition of DNA synthesis and induction of cell cycle arrest in the S-phase, apoptosis, and cell differentiation [21,146,147,149–151]. Moreover, xanthohumol has been shown to induce HO-1 and NOQO1 via the Keap1-Nrf2-ARE regulatory pathway and this may account for some of the antiinflammatory and chemopreventive effects of this compound [21,127,146,147,149]. Although xanthohumol appears to be able to prevent oxidative damage by scavenging of ROS by inducing cytoprotective proteins, it is interesting to note that the ability of xanthohumol to induce apoptosis in cancer cells may be due to its ability to induce transient $O_2^{\bullet -}$ [150].

The retro-chalcone licochalcone A (**39**) present in licorice exhibits various biological properties, including antiinflammatory and antioxidant activities. It has, for example, been shown that licochalcone A protects against *tert*-butyl hydroperoxide (*t*-BHP) induced oxidative damage in RAW 264.7 cells through the inhibition of ROS generation and reduced glutathione depletion as well as a dramatic up-regulation of HO-1 and Nrf2 [152]. Almost the same effects have been observed for butein (**28**) in

rat primary hepatocytes upon induced *t*-BHP induced oxidative damage [153]. Butein has furthermore been shown to provide neuroprotective and anti-neuroinflammatory effects through Nrf2/ARE-dependent HO-1 expression by activating the PI3K/AKT pathway [154], and to induce HO-1 expression in adipocytes in vitro and in vivo [155], as well as to induce GST [156].

Other examples of polyphenolic chalcones, which have been shown to induce the production of cytoprotective proteins and thus act as indirect antioxidants as an explanation for their chemoprevention are 2,2′-dihydroxy chalcone (**22**), 2,2′,4′-tridhydroxychalcone (**24**), sappanchalcone (**29**), cardamonin (**32**) and 4,2′,5′-trihydroxy-4′-methoxychalcone (**33**), which all induce HO-1 [157–161] and the chalcones **23**, **30**, and **35**–**38** (Fig. 13.5), which have been shown to induce the production of NQO1 [114,162–165]. Furthermore, flavokawain (**31**) has shown potent apoptotic activity that may involve the formation of ROS as well as glutathione depletion [166,167]. The pleiotropic bioactivity of many chalcones are certainly closely related to their α,β-unsaturated carbonyl group, and induction of cytoprotective proteins is only one out of several mechanisms of action of these compounds.

A few dihydrochalcones have also shown an effect on oxidative stress (Fig. 13.5). Phloretin (**41**) has in both in vitro and in vivo experiments shown to increase glutathione content and to up-regulate HO-1 expression and to protect hepatocytes against oxidative stress [153], as well as exhibiting neuroprotective effects on oxidative stress injury after cerebral ischemia/reperfusion in rats, by activating the Nrf2 defense pathway [168]. Similar effects on oxidative stress have been demonstrated for aspalathin (**42**), which has been shown to protect the myocardium tissue in mice against hyperglycemia-induced oxidative stress through activation of Nrf2 and its downstream target genes [169]. Finally, (*R*)-ovalitenin B (**40**) has been shown to induce the activity of NQO1 [165]. Dihydrochalcones lack the α,β-unsaturated carbonyl moiety in their structures; however, (*R*)-ovalitenin B contain a electrophilic furan ring that is susceptible for attack from a nucleophile and aspalathin contain an *ortho*-phenol that can be metabolized to a reactive *ortho*-quinone thus explaining their ability to induce cytoprotective proteins. Phloretin on the other hand does not contain electrophilic moieties or functional groups that can easily be metabolized to reactive quinones. Consequently, the chemopreventive nature of phloretin is not easy to explain, although it cannot be excluded that it may undergo phase 1 transformation (oxidation) to a highly reactive metabolite (see Section 2).

5.3 Flavonoids and Stilbenoids

Flavonoids are structurally related to chalcones and are ubiquitously found in plants and are among the most common and widely distributed phytochemicals in fruits and vegetables. Flavonoids are, however, not as the chalcones Michael acceptors at first glance and therefore their protective effects against oxidative stress-related diseases have often been claimed to be due to their direct antioxidant activity. However, bioavailability of the different subgroups of flavonoids appears to differ greatly and the same is true for polyphenols in general, and the most abundant polyphenols in our diet are not necessarily those that have the best bioavailability profile. Furthermore, the bioactivity and mechanisms of action of flavonoids and other polyphenols, e.g., antioxidant activity, have mainly focused on the aglycones or glycosides rather than on the metabolites resulting from phase 1 and/or phase 2 metabolization and often at concentrations that cannot realistically be attained in the body [32,60]. Finally, the flavonoid conjugates resulting from phase 2 metabolization often have lower antioxidant activity compared to the original flavonoids in our diet. This clearly suggests that most of the relevant mechanisms of disease prevention by flavonoids are not related to their direct antioxidant properties but are rather due to their prooxidant action and direct interaction with target biomolecules [32,170–175]. Oxidation of flavonoids containing a 1,2-diphenol (catechol moiety), 1,4-diphenol (hydroquinone moiety), or 1,2,3-triphenol (galloyl moiety) leads to the formation of flavonoid quinones, as illustrated in Fig. 13.3, whereas 1,3-diphenols are not directly oxidized. The electrophilic properties of flavonoid quinones make them very reactive towards thiols in protein Cys residues to form cysteinyl flavonoid adducts, which may to a large extent explain their antiinflammatory and anticancer effect, among others [32,176,177]. It has, for example, been demonstrated that (−)-epigallocatechin-3-gallate forms covalent adducts with protein thiol groups through autooxidation in vitro [178]. Although covalent modification of Cys residues, including covalent modification of Keap1 has not been demonstrated in vivo for flavonoid quinones, the transformation of flavonoids to quinones is a prerequisite for induction of cytoprotective proteins [21,28,179].

Flavonoids that can readily undergo oxidation to reactive electrophilic flavonoid quinones include the flavan-3-ols **43**–**48** (Fig. 13.6), the flavonols **52**, **54**–**58**, and **60**, the flavones **65**, **67**, **68**, **70**, and **75** (Fig. 13.7), the dihydroflavonols (2*R*,3*R*)-7-methoxy-3,5,8-trihydroxyflavanone (**83**) and ampleopsin (**93**), the flavanones eriodictyol (**91**) and eriodictyol 7-*O*-glucoside (**92**) (Fig. 13.8), the aurone sulfuretin (**101**), the homoflavonoid 4-*O*-methylepisappanol (**102**), the 3-deoxyanthocyanidins (**105** and **106**), and the anthocyanins **107** and **108** (Fig. 13.9). However, sulfuretin, like other aurones, contains a reactive unsaturated carbonyl moiety (Fig. 13.9), and therefore sulfuretin does not necessarily need to be oxidized in order to become reactive towards protein

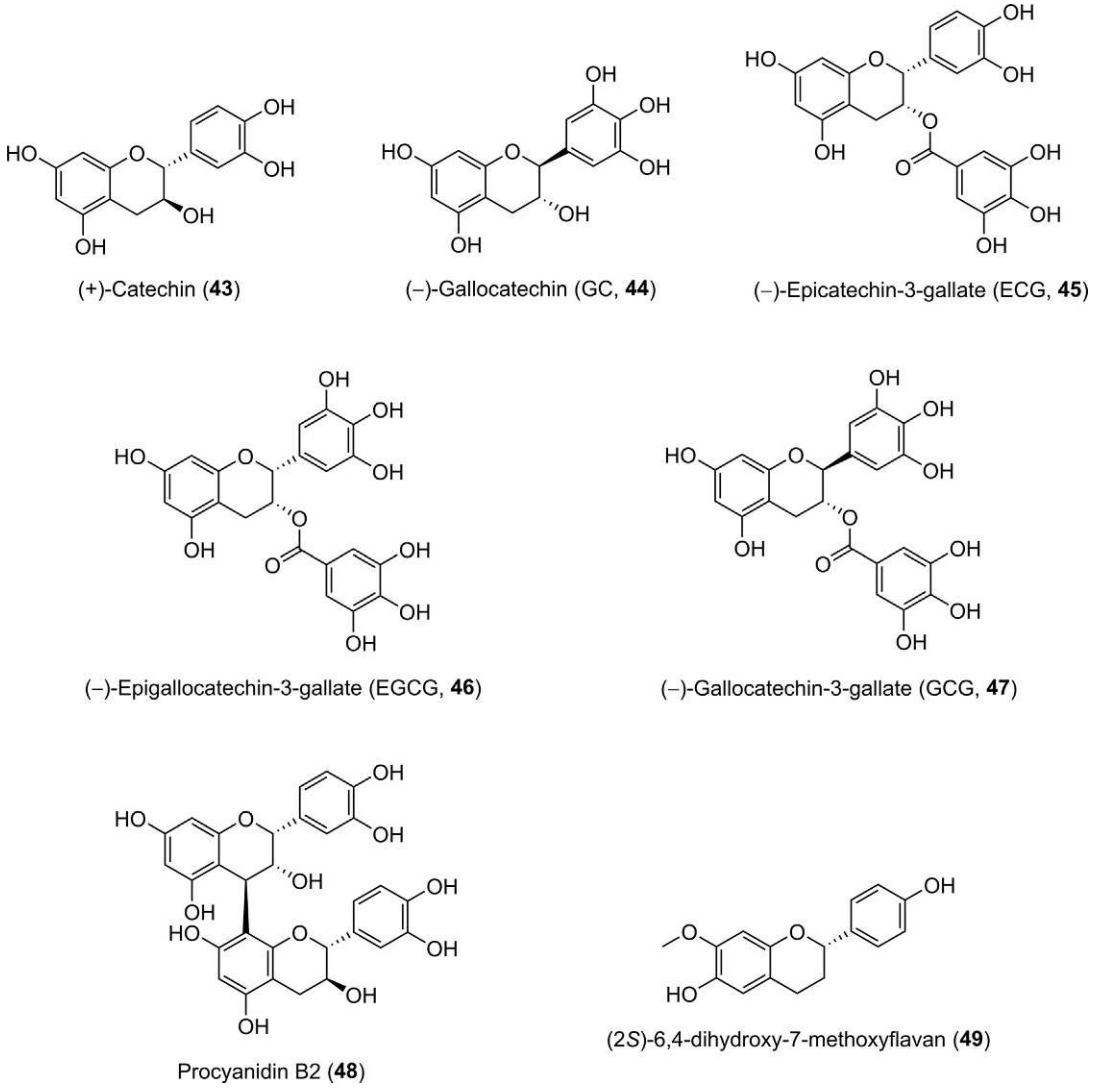
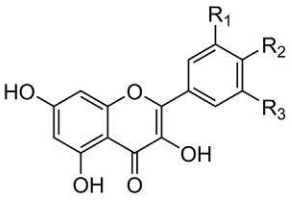


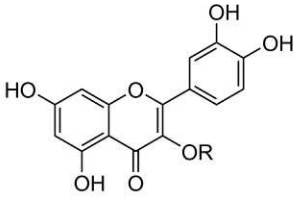
FIG. 13.6 Chemical structures of naturally occurring flavan-3-ols (**43**–**48**) and the flavan **49**, which are inducers of cytoprotective proteins.

nucleophiles. The previously mentioned natural products have all been shown to induce cytoprotective proteins such as GST, NOQ1, HO-1, CAT, among others, through the Keap1-Nrf2-ARE regulatory pathway and/or ROS formation [21,64,65,68,69,163,175,180–203].

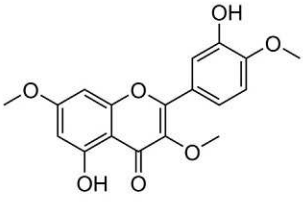
However, some flavonoids do not contain a catechol, hydroquinone, or galloyl moiety but still they are able to induce cytoprotective proteins and/or ROS formation and these include the flavonoids **49**–**51**, **53**, **59**, **61**–**64**, **66**, **69**, **71**–**74**, **76**–**82**, **84**–**90**, **94**–**100**, **103** and **104** (Figs. 13.6–13.9) [21,162,164,165,172,186,191,204–227]. These flavonoids apparently cannot readily undergo oxidation to reactive flavonoid quinone electrophiles. However, a few flavonoids such as **79**–**81** contain a reactive furano or pyrano functionality that is susceptible for attack from a nucleophile, which may explain their ability to induce cytoprotective proteins [165]. For other flavonoids the question is by which mechanism these compounds can become inducers of cytoprotective proteins. This is to the best of my knowledge not known, but may involve metabolization of the flavonoids to chalcones as it is known that chalcones can be transformed to flavonoids in vitro [146,228]. Furthermore, flavonoids may be transformed/oxidized by phase 1 enzymes to, e.g., 1,2-diphenols, and then further oxidized into their corresponding quinones. The latter may also explain the indirect antioxidant activity and the potential antiinflammatory and anticancer effect of the stilbene resveratrol (**109**, Fig. 13.9), which also does not contain any electrophilic moieties in its structure but still has been shown to induce cytoprotective proteins and ROS formation [21,229–231]. The same is true for the stilbene dimer pallidol (**111**) [232] and piceatannol (**110**) [231,233], although the latter contains a catechol moiety and



Galangin (**50**): $R_1 = R_2 = R_3 = H$
Kaempferol (**51**): $R_1 = R_3 = H$, $R_2 = OH$
Quercetin (**52**): $R_1 = R_2 = OH$, $R_3 = H$
Isorhamnetin (**53**): $R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$
Myricetin (**54**): $R_1 = R_2 = R_3 = OH$

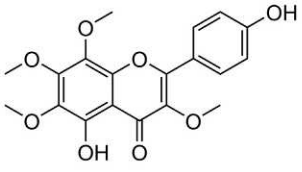


3-*O*-Methylquercetin (**55**): $R = CH_3$
Feniculin (**56**): R = α-L-arabinose
Hyperoside (**57**): R = β-D-galactopyranose
Rutin (**58**): R = rutinose

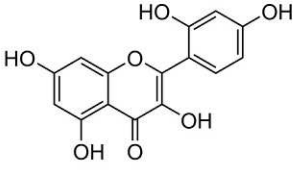


5,3′-Dihydroxy-3,7,4′-trimethoxyflavone (**59**)

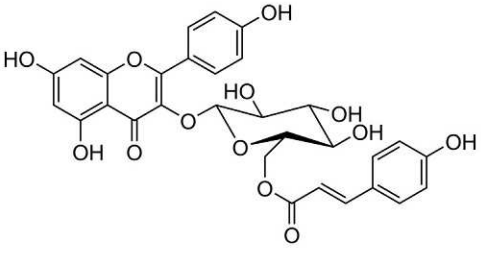
Fisetin (**60**)



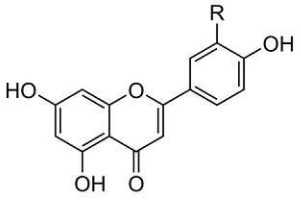
Calycopterin (**61**)



Morin (**62**)



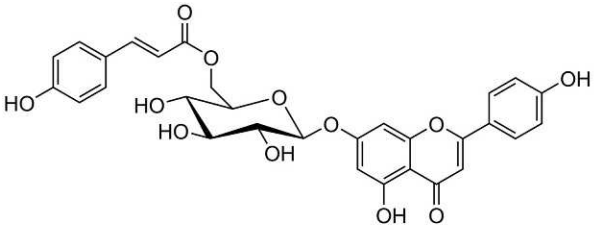
Tiliroside (**63**)



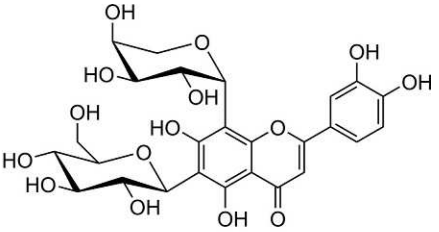
Apigenin (**64**): R = H
Luteolin (**65**): R = OH

FIG. 13.7 Chemical structures of naturally occurring flavonols (**50**–**63**) and flavones (**64**–**81**), which are all inducers of cytoprotective proteins. *(continued)*

therefore may easily be oxidized to a reactive quinone, explaining its indirect antioxidant activity. Characteristic for the induction of cytoprotective proteins by many bioactive compounds is the fact that their bioactivity and indirect antioxidant effect depend on their concentration in vivo. Resveratrol is an excellent example of a compound that in low concentrations increases intracellular ROS-inducing oxidative stress and in higher concentrations induces the Keap1-Nrf2-ARE regulatory pathway and thereby is able to trigger hormesis and an adaptive stress response, respectively [21]. Another factor that plays a role for many polyphenols is that their absorption may be high but their bioavailability is low. It is, for example. well known that stilbenes in humans and animals have a high absorption but are poorly bioavailable after oral intake. For resveratrol the absorption

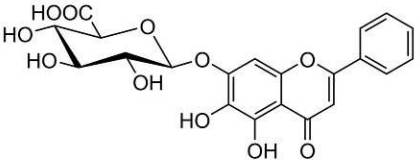


Apigenin-7-*O*-β-D-(-6″-*p*-coumaroyl)-glucopyranoside (**66**)



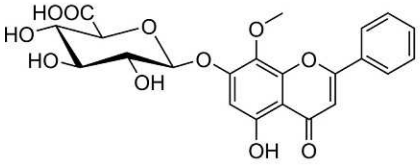
Carlinoside (**67**)

Baicalein (**68**): R = H
Oroxylin A (**69**): R = CH_3

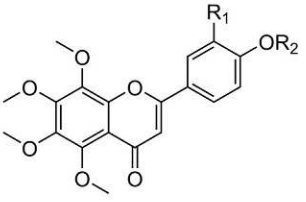


Baicalin (**70**)

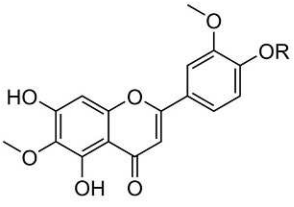
Chrysin (**71**): R = H
Wogonin (**72**): R = OCH_3



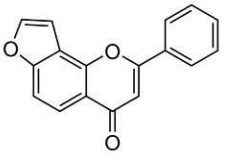
Wogonoside (**73**)



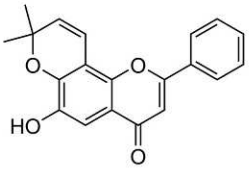
Tangeritin (**74**): R_1 = H, R_2 = CH_3
3′,4′-Didemethylnobiletin (**75**): R_1 = OH, R_2 = H
Nobiletin (**76**): R_1 = OCH_3, R_2 = CH_3



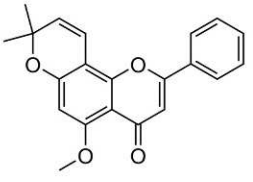
Jaceosidin (**77**): R = H
Eupatilin (**78**): R = CH_3



Lanceolatin B (**79**)



6-Hydroxy-2″,2″-dimethylpyrano
[5″,6″:7,8]flavone (**80**)



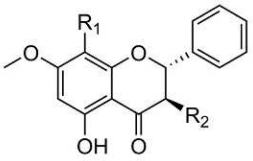
5-Methoxy-2″,2″-dimethylpyrano
[5″,6″:7,8]flavone (**81**)

FIG. 13.7, CONT'D

can be up to 75% but, due to extensive metabolism in the major sites in intestine and liver (glucuronides and sulfates are the major metabolites of resveratrol), the oral bioavailability appears to be less than 1% [231]. Thus low bioavailability, poor solubility, limited stability, high rate of metabolic breakdown, and low target specificity may play important roles for some polyphenols, such as the stilbenoids in relation to their indirect antioxidant activity and other bioactivities and may be major obstacles for their use in pharmacological applications.

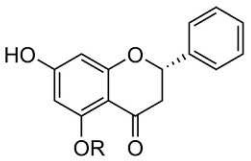
5.4 Isoflavonoids and Lignans (Phytoestrogens)

Phytoestrogens are a class of plant-derived compounds that are structurally similar to mammalian



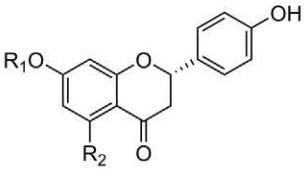
(2*S*)-5-Hydroxy-7-methoxyflavanone (**82**): $R_1 = R_2 = H$

(2*R*,3*R*)-7-Methoxy-3,5,8-trihydroxyflavanone (**83**): $R_1 = R_2 = OH$



Pinocembrin (**84**): R = H

Alpinetin (**85**): R = CH_3

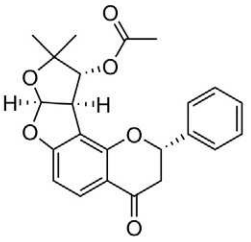


Liquiritigenin (**86**): $R_1 = R_2 = H$

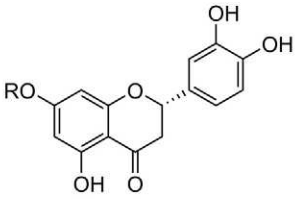
Naringenin (**87**): $R_1 = H$, $R_2 = OH$

Naringenin 7-*O*-glucoside (**88**): R_1 = β-D-glucopyranose, $R_2 = OH$

Naringin (**89**): R_1 = rutinose, $R_2 = OH$

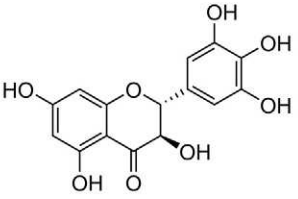


(+)-Purpurin (**90**)

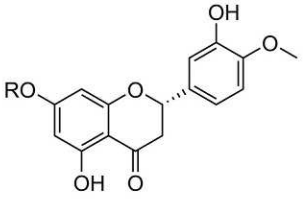


Eriodictyol (**91**): R = H

Eriodictyol 7-*O*-glucoside (**92**): R = β-D-glucopyranose

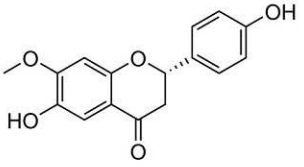


Ampleopsin (**93**)



Hesperetin (**94**): R = H

Hesperidin (**95**): R = rutinose



(2*S*)-6,4′-dihydroxy-7-methoxyflavanone (**96**)

FIG. 13.8 Chemical structures of naturally occurring flavanones (**82**, **84**–**92**, **94**, **95**, **99**, **100**) and dihydroflavonols (**83**, **93**, **97**, **98**), which are all inducers of cytoprotective proteins.

(continued)

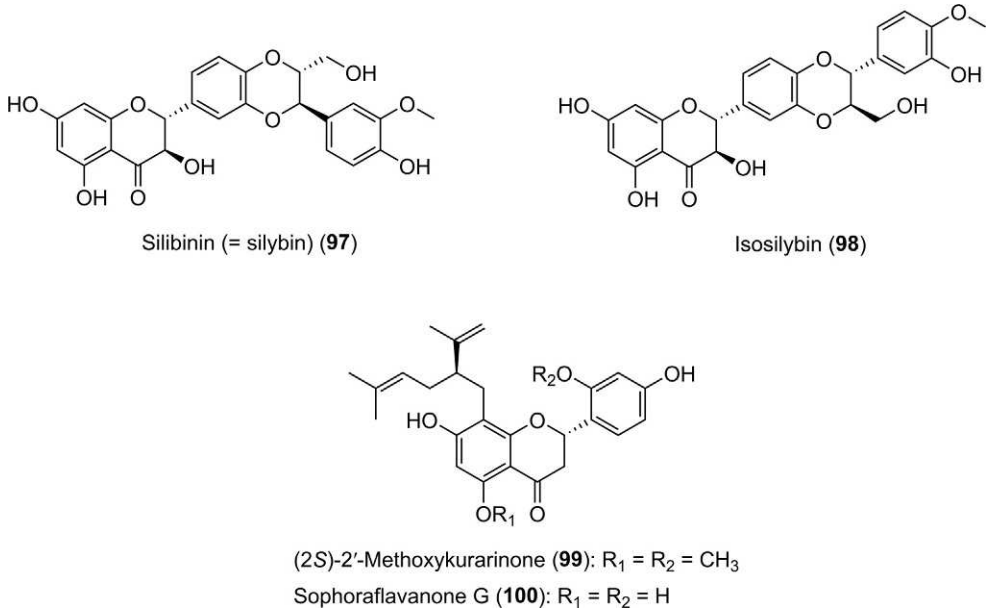


FIG. 13.8, CONT'D

estrogens. Several main classes of compounds are at present recognized as phytoestrogens and include the isoflavones and coumestans belonging to the isolavonoids, and lignans. Phytoestrogens are abundantly present in legumes, whole grains, fruits, and vegetables, being concentrated in the fruit skin, bark, and flowers of plants. A high consumption of phytoestrogen-rich foods has been linked to a reduced incidence of particularly breast, prostate, and colon cancer. The mechanisms by which phytoestrogens exert their potential anticancer effect have, for example, been linked to their ability to reduce endogenous estrogen levels and activation of the estrogen receptor (ER)-β to exert antiproliferative and prodifferentiative effects. However, the protective cellular mechanisms of the phytoestrogens against cancer still remain more or less unresolved and are most likely a combination of many factors [72,234].

One of the proposed protective cellular mechanisms by which these compounds may exert their anticancer and cellular protective effects could be through the induction of cytoprotective proteins. Several dietary phytoestrogens have been investigated for their ability to induce the cytoprotective protein NQO1, which is inducible in the cells of many target tissues of the phytoestrogens. So far, NQO1 has been shown to be induced by isoflavones (**112**–**118**) [162,163,235,236], coumestans (**119**–**122**) [72,162] and lignans (**123**, **128**, **129**) [72,237] (Fig. 13.10). The mechanisms of action by which these phytoestrogens are able to induce cytoprotective proteins is not known, but may be due to phase 1 and phase 2 metabolization resulting in reactive electrophiles, as described for some flavonoids in Section 5.3.

Sesamin (**124**) a major lignan contained in sesame seeds (*Sesamum indicum* L., Pedaliaceae) has only minor or no effect on phase 2 enzymes, whereas the catechol derivative of sesamin (1*R*,2*S*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabi-cyclo-[3,3,0]octane (**125**) (Fig. 13.10) that is produced after absorption and metabolism of sesamin has been shown to protect against H_2O_2-induced neuronal cell death through induction of HO-1 via Nrf2-ARE activation in rat pheochromocytoma PC12 cells [74,238]. Thus the metabolite **125** has the potential to reduce oxidative stress and ameliorate neurodegenerative diseases as well as other oxidative stress-related diseases. The fact that sesamin is almost inactive towards stimulation of cytoprotective proteins clearly indicates that phase 1 and phase 2 metabolization of phytoestrogens into reactive metabolites play a major role in their ability to induce the formation of cytoprotective proteins, hence reducing oxidative stress.

Furthermore, it has been indicated that the flaxseed lignan secoisolariciresinol diglucoside (**126**) and its aglycone secoisolariciresinol (**127**) (Fig. 13.10) are able to induce phase 2 enzymes, although the mechanism is unknown [73]. However, these lignans, like many other related lignans abundantly present in berries and seeds [239], are metabolized to the mammalian lignans enterodiol and enterolactone (**123**) by colonic microflora [74,240]. In fact, the intake of vegetables, fruits, berries, or whole grains is related to the enterolactone

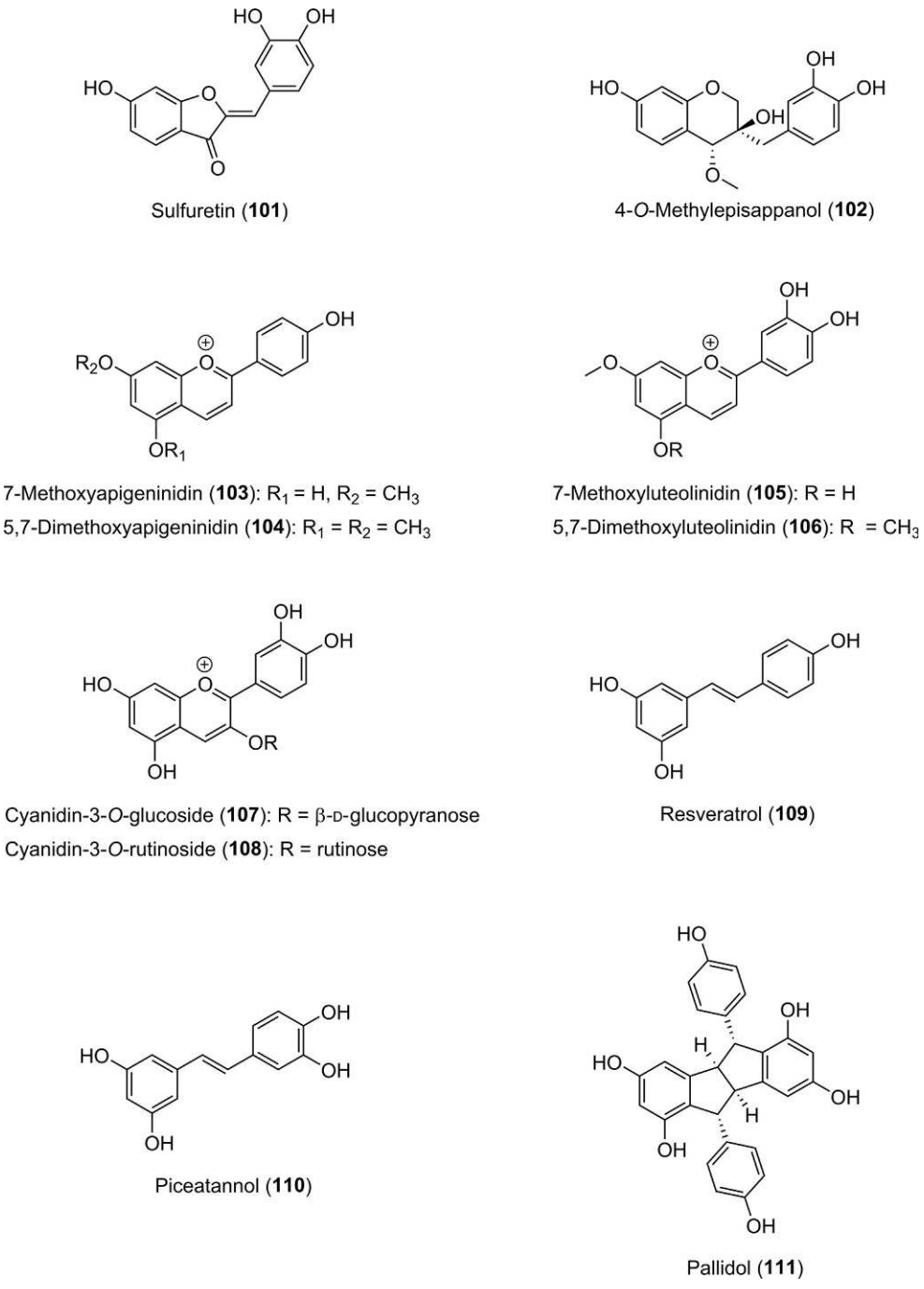
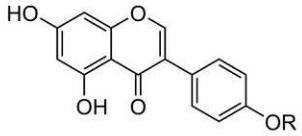


FIG. 13.9 Chemical structures of naturally occurring flavonoids, including the aurone sulfuretin (**101**), the homoflavonoid 4-*O*-methylepisappanol (**102**), deoxyanthocyanidins (**103**–**106**), and anthocyanins (**107**, **108**) as well as stilbenoids (**109**–**111**), which are all inducers of cytoprotective proteins.

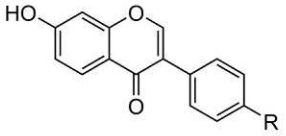
concentration in blood and excretion in urine, and therefore enterolactone may function as a biomarker of fiber-rich foods. Enterolactone has been shown to protect against oxidative stress through the production of cytoprotective proteins such as NQO1 [72] and thus it could also be interesting to investigate whether this metabolite could be a biomarker for the level of oxidative stress.

5.5 Neoflavonoids

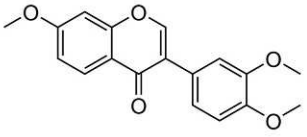
The term neoflavonoid refers to a group of C-15 naturally occurring compounds, which are structurally and biogenetically related to the flavonoids and to the isoflavonoids. In higher plants the neoflavonoids have limited distribution, being found in plant species belonging to the



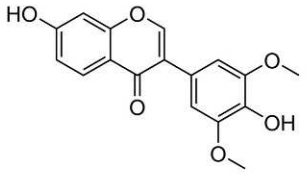
Genistein (**112**): R = H
Biochanin A (**113**): R = CH_3



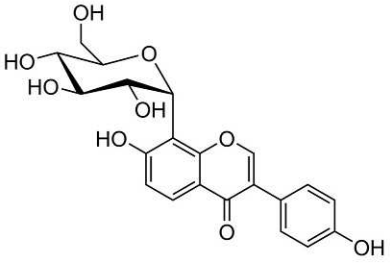
7-Hydroxyisoflavone (**114**): R = H
Daidzein (**115**): R = OH



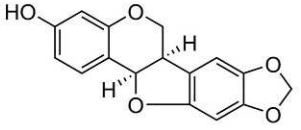
7,3′,4′-Trimethoxyisoflavone (**116**)



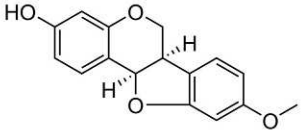
7,4′-Dihydroxy-3′,5′-dimethoxyisoflavone (**117**)



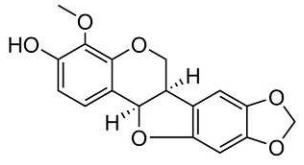
Puerarin (**118**)



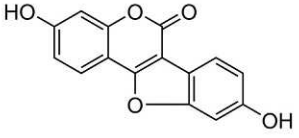
(−)-Maackianin (**119**)



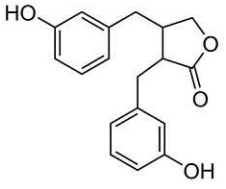
(−)-Medicarpin (**120**)



(−)-3-Hydroxy-4-methoxy-8,9-methylene-dioxypterocarpan (**121**)



Coumestrol (**122**)

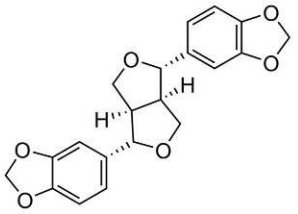


Enterolactone (**123**)

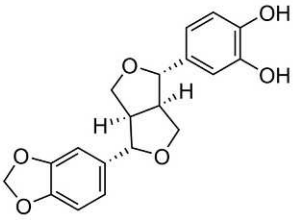
FIG. 13.10 Chemical structures of phytoestrogenic isoflavones (**112**–**118**) and coumestans (**118**–**122**) (isoflavonoids) and lignans (**123**–**129**). All compounds are inducers of cytoprotective proteins; however, enterolactone (**123**) is a mammalian lignan and is produced from the metabolization of dietary lignans by colonic microflora and the lignan **125** is a metabolized product of sesamin (**124**).

(continued)

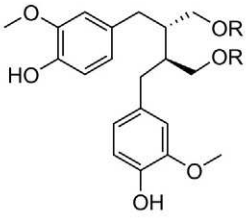
families Clusiaceae, Fabaceae, Rubiaceae, Passifloraceae, and the Polypodiaceae. From the heartwood of *Dalbergia odorifera* T. Chen (Fabaceae), which is used to treat blood disorders, ischemia, swelling, and epigastric pain, several neoflavonoids (**130**–**133**, Fig. 13.11) and flavonoids have been isolated that attenuate inflammatory responses by inhibiting NF-κB activation via Nrf2-mediated HO-1 expression, but also show other types of cytoprotective



Sesamin (**124**)

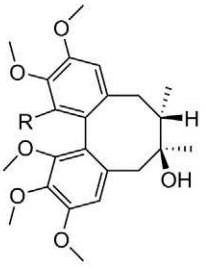


(1*R*,2*S*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane(**125**)



Secoisolariciresinol diglucoside (**126**): R = β-D-glucopyranose

Secoisolariciresinol (**127**): R = H



Angeloylgomisin H (**128**): R =

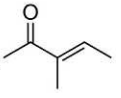
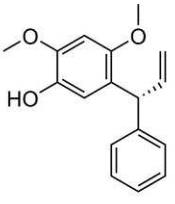
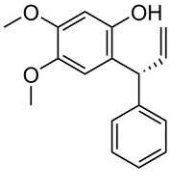
Tigoylgomisin H (**129**): R = 

FIG. 13.10, CONT'D

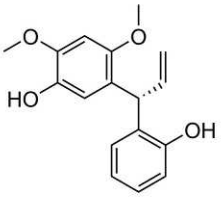
FIG. 13.11 Chemical structures of neoflavonoids (**130**–**133**), which are all inducers of cytoprotective proteins. The neoflavonoids can easily be metabolized to *ortho*- or *para*-phenols by simple demethylation in vivo, thus explaining their bioactivity. (*R*)-4-methoxydalbergione (**133**) is, however, already reactive towards endogenous nucleophiles and does not require to be further metabolized in order to activate Nrf2.



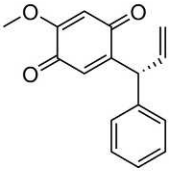
(*R*)-Dalbergiphenol (**130**)



9-Hydroxy-6,7-dimethoxydalbergiquinol (**131**)



(*R*)-Latifolin (**132**)



(*R*)-4-Methoxydalbergione (**133**)

effects towards oxidative damage [241–243]. The neoflavonoids can easily be metabolized to *ortho*- or *para*-phenols by simple demethylation in vivo, thus explaining their bioactivity. (*R*)-4-methoxydalbergione (**133**) is, however, already reactive towards endogenous nucleophiles such as Cys and does not require to be further metabolized to activate Nrf2.

5.6 Coumarins

Coumarins represent a diverse class of phytochemicals that are ubiquitous in the human diet and that possess anticarcinogenic and antiinflammatory activities, in part by inducing cytoprotective enzymes such as GST, NQO1, and HO-1. It has been shown that the induction of GST, NQO1, and/or HO-1 by the coumarins (**134**–**141**, Fig. 13.12) is caused fully or partially by an activation of the Keap1-Nrf2-ARE regulatory pathway [244–251]. Citrus coumarins such as imperatorin (**134**) and auraptene (**140**) induce the formation of GST and NQO1 by activation of Nrf2 [245], and the coumarin osthole (**138**) protects against inflammatory reactions and oxidative stress by activating the Nrf2 pathway and thus the formation of cytoprotective proteins [246]. Furthermore, it has been demonstrated that the protective effect of umbelliferone (**135**) against cyclophosphamide-induced hepatotoxicity in rats depends on coactivation of Nrf2 and the nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ) [247], whereas in another rat study it has been demonstrated that umbelliferone and the related coumarin daphnetin (**136**) ameliorate carbon tetrachloride-induced hepatotoxicity in rats via Nrf2 and HO-1 expression [248]. Another coumarin that has been shown to ameliorate induced hepatoxicity by activation of Nrf2 is, for example, glycycoumarin (**139**) [249].

The potential anticancer effect of the coumarin esculetin (**137**) appears not to be due to activation of cytoprotective proteins but instead it binds to KEAP1 and thus inhibits interaction with Nrf2, as demonstrated in pancreatic cancer cells [250]. This probably promotes nuclear accumulation of Nrf2 in the cancer cells, which induces an antiproliferative and apoptotic response possibly by attenuating NF-κB.

The mechanisms by which coumarins exert their indirect antioxidant activity is, as for many other polyphenols, through quinone electrophiles, as demonstrated for the coumarinolignoid 5′-hydroxycleomiscosin B (**141**), which has been shown to induce NQO1 through alkylation of the sulfhydryl groups of Keap1 [251]. Those coumarins that cannot readily undergo oxidation to reactive quinone electrophiles may be transformed/oxidized by phase 1

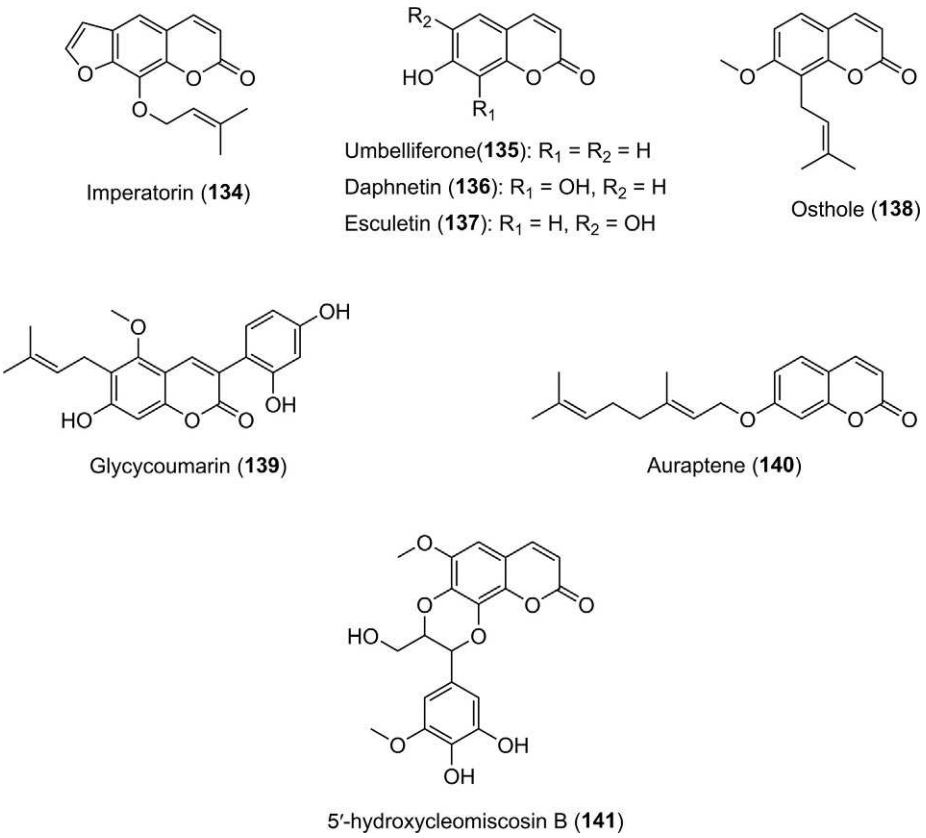


FIG. 13.12 Chemical structures of coumarins (**134**–**141**), which are all inducers of cytoprotective proteins. The coumarins that cannot readily undergo oxidation to reactive quinone electrophiles, i.e., **134**, **135**, **138**–**140**, may be transformed/oxidized by phase 1 enzymes and then further oxidized into their corresponding quinones (see Sections 5.3 and 5.6).

enzymes and then further oxidized into their corresponding quinones, as described for the flavonoids in Section 5.3.

5.7 Phenolic Acids and Miscellaneous Polyphenols

Phenolic acids are widespread in plants and their content can be very high, in particular in dried fruits. Phenolic acids and phenolic acid derivatives that have been shown to be inducers of cytoprotective proteins can be divided into three groups according to their inducer functionalities: (i) those having Michael acceptor properties and/or a potentially reactive catechol moiety (**145**–**151**) [21,115,252–260]; (ii) those only possessing a reactive catechol or galloyl moiety (**142**, **143**, **152**, **155**, **160**) [66,260–267]; and finally (iii) those who have to be demethylated in order to produce a reactive catechol or galloyl moiety (**144**) [268] (Fig. 13.13). Gallic acid (**143**) constitutes a part of the structure of some catechins (Fig. 13.6), and both types of natural products have demonstrated excellent chemopreventive effects in many target organs challenged with various carcinogens. A number of studies indicate that gallic acid is a potent inducer of phase 2 enzymes via the Keap1-Nrf2-ARE pathway and that this to a large extent may explain its chemopreventive properties and potential anticarcinogenic effect [261,262]. This could also be the case for other phenolic acids that are inducers of cytoprotective proteins, although the induction mechanisms may be different due to differences in inducer functionalities.

The arylbenzofuran derivative (2*R*,3*R*)-obtusafuran (**157**), isolated from the heartwood of *Dalbergia* species (Fabaceae) [269,270], is a flavonoid derivative that has shown therapeutic potential against neurodegenerative diseases caused by neuroinflammation [270]. The potential therapeutic effect of (2*R*,3*R*)-obtusafuran has been demonstrated in LPS-stimulated mouse BV2 microglia, resulting in the inhibition of the protein expression of inducible iNOS, iNOS-derived nitric oxide, COX-2, and COX-2-derived prostaglandin E2 (PGE2) as well as a reduction in tumor necrosis factor-α (TNF-α) and interleukin-(IL)-1β production. The inhibitory effects of (2*R*,3*R*)-obtusafuran on proinflammatory mediators and proteins is most likely associated with the induction of HO-1 expression and thus an activation of Nrf2 [270].

Surprisingly, only a few quinones of polyphenol origin have been identified and/or investigated for their indirect antioxidant activity, despite the fact that many of them are reactive to nucleophiles due to an α,β-unsaturated carbonyl in their structure. Among the quinones that have been investigated for their indirect antioxidant activity are the naphthoquinones plumbagin (**158**) and naphthazarin (**159**). The former occur regularly in the genera *Plumbago* (Plumbaginaceae) and *Diospyros* (Ebenaceae) as well as in the carnivorous plant genera *Drosera* and *Nepenthes* [271–273]. Plumbagin has been shown to protect neurons against ischemic injury through activation of Nrf2/ARE [274]. The related quinone naphthazarin, which has been isolated from the walnut Onigurumi (*Juglans mandshurica* Maxim var. *sieboldiana* Makino, Juglandaceae) [275], has been shown to be a potent activator of the Nrf2/ARE pathway and to protect neurons against glutamate-induced excitotoxicity in primary neuronal and glial cultures by up-regulating the expression of Nrf2-driven genes [276]. Mollugin (**156**), a bioactive phytochemical isolated from *Rubia cordifolia* L. (Rubiaceae), is biosynthetically related to naphthazarin, as they are both biosynthesized from the same precursor, namely 1,4-dihydroxy-2-naphthoic acid. Mollugin exhibits antimutagenic, antitumor, and antiviral activity, as well as inhibitory activity in arachidonic acid- and collagen-induced platelet aggregation [277]. In addition, mollugin has shown potent neuroprotective effects against glutamate-induced neurotoxicity and reactive oxygen species in mouse hippocampal HT22 cells as an antiinflammatory agent in LPS-induced microglial activation in BV2 cells. The antiinflammatory effects of mollugin have been demonstrated by the suppression of proinflammatory mediators, such as iNOS and COX-2 and the cytokines TNF-α and IL-6. The neuroprotective and antiinflammatory effects of mollugin appear to be linked to the up-regulation of the expression of HO-1 in HT22 and BV2 cells [277]. Thus mollugin is a promising candidate for the treatment of neurodegenerative diseases related to neuroinflammation and its bioactivity may be related to the electrophilic pyrano functionality.

Lichens are dual organisms composed of a symbiotic relationship between an alga and a fungus that live on, e.g., the bark of trees, and they produce some bioactive polyphenols such as (+)-usnic acid (**153**) and evernic acid (**154**), which have been shown to display significant protection against H_2O_2-induced cytotoxic damage in central nervous system-like cells such as the U373-MG and SH-SY5Y cell lines. This neuroprotective effect is partly due to the alteration in oxidative stress markers such as ROS and GST, indicating an involvement of the Nrf2 cytoprotective pathway [278].

5.8 Diterpenoids

The herbs rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) of the Lamiaceae family are well known for their production of phenolic diterpenoids such as carnosic acid (**161**) and carnosol (**163**) (Fig. 13.14), which are strong antioxidants possessing

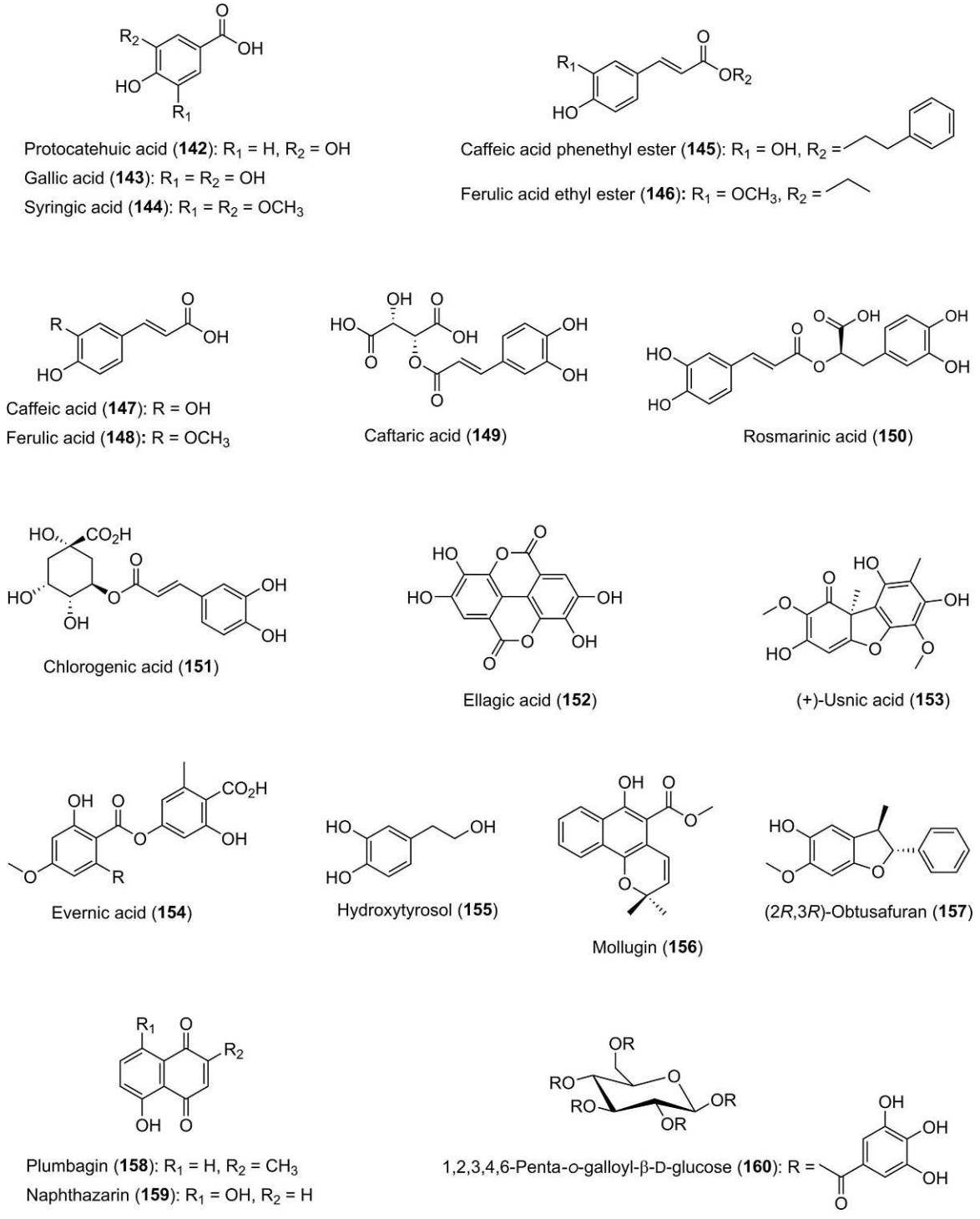


FIG. 13.13 Chemical structures of phenolic acids and phenolic acid derivatives as well as examples of miscellaneous polyphenols (**156**–**159**) that have all been shown to be inducers of cytoprotective proteins. Phenolic acid and phenolic acid derivatives can be divided into the following three groups according to their inducer functionalities: (i) those having Michael acceptor properties and/or a potentially reactive catechol moiety (**145**–**151**), (ii) those only possessing a reactive catechol or galloyl moiety (**142**, **143**, **152**, **155**, **160**), and (iii) those that have to be demethylated in order to produce a reactive catechol or galloyl moiety (**144**).

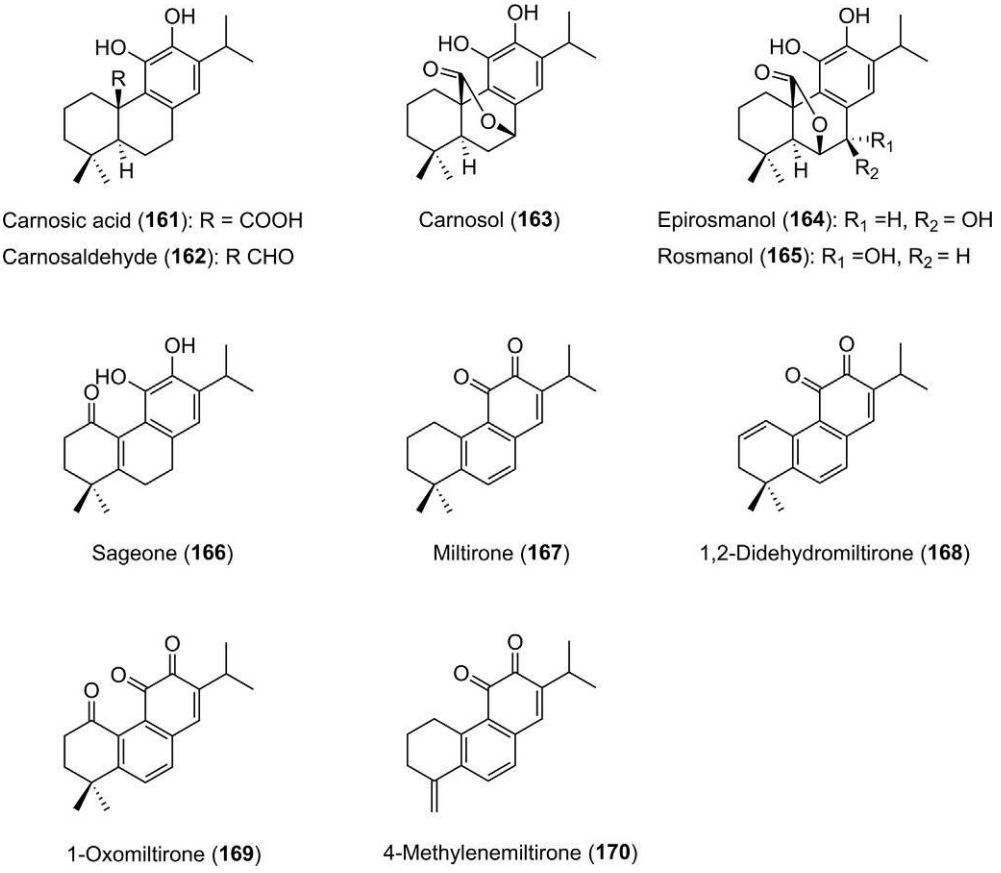


FIG. 13.14 Chemical structures of phenolic diterpenoids with phenol groups in *ortho*-position (**161**–**166**) that are easily oxidized to *ortho*-quinone-type electrophiles and electrophilic quinoid diterpenoids with a reactive α,β-unsaturated carbonyl in their structure (**167**–**170**). All diterpenoids have been shown to be inducers of cytoprotective proteins.

antiinflammatory activity as well as potential anticancer and antidiabetic effects [279–281]. Characteristic for most of the phenolic diterpenoids in rosemary and sage is the presence of phenol groups in the *ortho*-position as in the diterpenoids **161**–**166** (Fig. 13.14) that are easily oxidized to *ortho*-quinone-type electrophiles, which readily alkylates Keap1, resulting in the release of Nrf2 (Figs. 13.2 and 13.3) [21,62,75,279–283]. The ability of the diterpenoids **161**–**166** to induce cytoprotective enzymes explains their neuroprotective effects and the effect against oxidative stress. A medicinal plant closely related to sage is Chinese sage or danshen (*Salvia miltiorrhiza* Bunge), which is used in traditional Chinese medicine against various diseases. This plant is also rich in diterpenoids, the so-called tanshinones, which possess antiinflammatory and cytotoxic activity [284,285]. The tanshinones have in most cases an α,β-unsaturated carbonyl moiety in their structure and therefore these compounds are in general expected to be reactive towards biological nucleophiles and to be able to induce cytoprotective enzymes. It has, for example, been shown that the tanshinones **167**–**170** (Fig. 13.14) induce the formation of NOQ1 [286]. The indirect antioxidant activity and the reactivity of the tanshinones towards proteins in general may explain the biological activities attributed to these diterpenoids.

5.9 Alkaloids

The polyphenol-derived alkaloids capsaicin (**171**) and piperine (**172**) (Fig. 13.15) are responsible for the pungency of chili peppers of the genus *Capsicum* (Solanaceae) and black pepper (*Piper nigrum* L., Piperaceae) and long pepper (*P. longum* L., Piperaceae), respectively. These alkaloids have been shown to induce apoptosis in colon cancer and auditory cells via production of ROS and cytoprotective proteins such as HO-1 through the Keap1-Nrf2-ARE pathway [287–289]. Furthermore, it has been shown that piperine attenuates LPS-induced inflammatory responses in BV2 microglia and that this neuroinflammatory protection is due to an amplification of the expression of Nrf2 and HO-1 up-regulated by LPS [290]. In addition, the pirperidine dimer chabamide (**173**, Fig. 13.15) isolated from *P. nigrum* has been shown to induce the formation expression of HO-1 and to up-regulate the expression of other

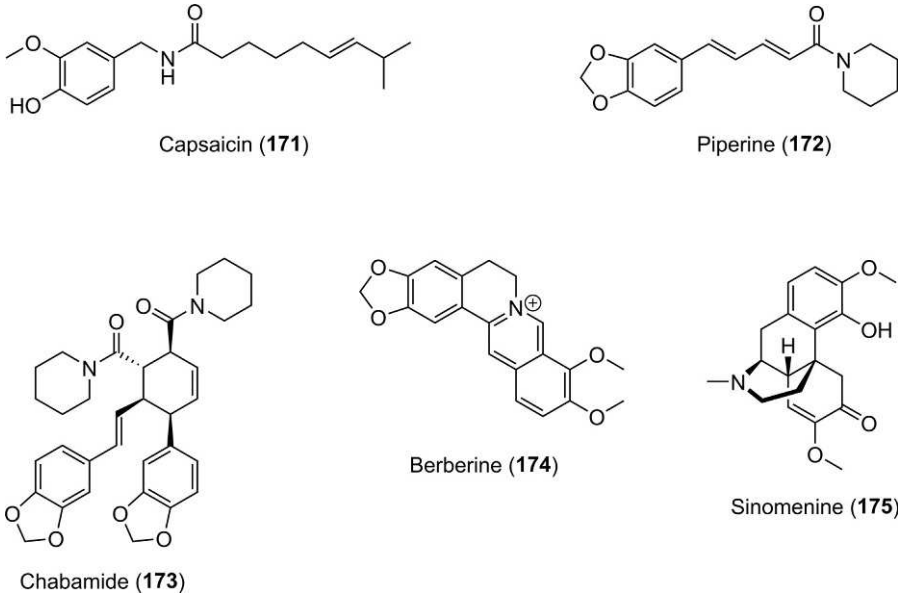


FIG. 13.15 Chemical structures of examples of alkaloids (**171**–**175**) being inducers of cytoprotective proteins. The alkaloids **171**–**174** probably need to be metabolized to their corresponding catechol derivatives followed by oxidation to their corresponding quinones in order to induce cytoprotective proteins, whereas sinomenine (**175**) already contains an electrophilic α,β-unsaturated carbonyl and is reactive per se.

Nrf2 target genes for γ-GCS and NQO1 in a concentration-dependent manner in RAW264.7 cells, which may account for the antiinflammatory effects of this alkaloid [291]. The release of Nrf2 by piperine, capsaicin and chabamide probably results from the metabolization of these alkaloids to their corresponding catechol derivatives, which then undergo oxidation to 1,2-quinone-type electrophiles, as described in some of the previous sections (see, for example, Sections 5.3 and 5.7).

Berberine (**174**, Fig. 13.15) is an isoquinoline alkaloid that is found in, for example, *Berberis* species (Berberidaceae) and known for its broad pharmacological activities against multiple diseases, including antiinflammatory diseases such as arthritis. The antiinflammatory effects of berberine have been demonstrated in several investigations and it appears that the ability of berberine to release Nrf2 plays a key role for this activity [292,293]. It has, for example, been shown that berberine suppressed the levels of proinflammatory cytokines IL-1β and TNFα and intracellular ROS in monosodium urate crystal stimulated RAW 264.7 macrophages by downregulating mRNA expression levels of, for example, IL-1β, caspase 1, and Keap1 and by up-regulating Nrf2 transcription factor and its associated antioxidant enzymes HO-1, SOD, GP, NQO1, and CAT [293]. Thus berberine may represent a potential candidate for the treatment of inflammation, such as various forms of arthritis, by suppressing inflammatory mediators and activating the Nrf2 antioxidant pathway. The mechanisms by which berberine activates the endogenous antioxidant defense system is probably as described earlier for piperine, capsaicin, and chabamide.

Finally, it has been shown that the morphinan derivative sinomenine (**175**, Fig. 13.15), an alkaloid found in the root of the climbing plant *Sinomenium acutum* (Thunb.) Rehder & E.H.Wilson (Menispermaceae), which is extensively used in the treatment of rheumatoid arthritis and various inflammatory disorders, also displays renal protective properties in animal studies [294]. It has been demonstrated that sinomenine in kidney cells and in mouse fibrotic kidney attenuates renal fibrosis through Nrf2-mediated inhibition of oxidative stress and transforming growth factor-β signaling [294]. The α,β-unsaturated carbonyl moiety in sinomenine may explain its antiinflammatory activities, including its ability to release Nrf2.

6 CONCLUSIONS

Based on epidemiologic and cohort studies, it is now widely accepted that the preventive effects of plants against various diseases such as inflammation and cancer cannot be explained by the in vitro antioxidant effect of polyphenols. On the basis of in vitro studies in cells and in vivo studies in animals and humans, it has now become clear that upregulation of a complex network of intrinsic cytoprotective phase 2 genes decreases the risk of many diseases. The mechanism of regulation of these genes comes from a series of dietary ingredients and metabolites that are able to induce an adaptive stress response, either by activation of the Keap1-Nrf2-ARE regulatory pathway and/or via the generation of ROS. Many inducers of Keap1-Nrf2-ARE are Michael acceptors being strong electrophiles capable of alkylating certain Cys residues of Keap1 and/or can induce cytosolic ROS formation, resulting in the release of Nrf2 and to the activation of gene transcription through the ARE

and thus to the formation of cytoprotective proteins. Inducers of the Keap1-Nrf2-ARE regulatory pathway are found among various types of molecules including polyphenols and polyphenol-derived compounds, many of which are present in our daily diet and/or in herbal remedies. Although many of the inducers among the polyphenols are redox active and thus can act as exogenous direct antioxidants, most of these compounds seem to exert their antioxidant actions by induction of cytoprotective phase 2 genes, acting as indirect antioxidants. The cytoprotective genes have a variety of antioxidant actions from reduction of oxidants to the production of endogenous direct antioxidants such as glutathione and thioredoxin. However, some exogenous antioxidants may exert their primary antioxidant effect as direct antioxidants and this includes the tocopherols and ascorbate. Undoubtedly, our food contains a wide variety of bioactive compounds that are able to prevent the development of diseases; however, the beneficial effects of these compounds depend on many factors such as their metabolization, reactivity towards biomolecules (in particular proteins) and pro-oxidant effects, as well as their bioavailability, i.e., the concentration in vivo in cells and not only the concentration in the systemic circulation.

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CHAPTER

14

Role of Protocatechuic Acid in Obesity-Related Pathologies: An Update

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1 INTRODUCTION

In recent years, the interest in chemicals derived from plants and their role in maintaining health and preventing diseases has been growing considerably. These chemicals are known as phytochemicals or nutraceuticals, primarily defined as a food, or part of a food, that provides medical or health benefits [1]. Natural bioactive phytochemicals, particularly those present in fruit and vegetables represent important food components, since they might offer health benefits related to the prevention of many pathologies, such as obesity.

Nowadays, obesity represents a real concern for public health throughout the world because of its clinical complications that play a key role in life expectancy. In particular, cancer and cardiovascular disease account for the greatest mortality risk linked to obesity [2]. However, conclusive evidence for the identification of natural compounds that can significantly contribute to the prevention of this disease is not yet available.

Among the phytochemicals derived from human diet, polyphenols have been widely studied because of their preventive and therapeutic properties [3,4]. Dietary polyphenols include more than 8000 compounds, and among them over 4000 flavonoids have been identified [5]. All the different groups of flavonoids share a common nucleus composed of two phenolic rings and an oxygenated heterocycle. Protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid) represents one of the main metabolites of anthocyanins, one of the most representative class belonging to the flavonoid family. Anthocyanins are usually present at high concentrations in red/purple/blue fruit and vegetables, and their daily intake is much higher than that of other polyphenols: for this reason the nutritional value of PCA is increasingly recognized.

This chapter is an update of the most recent work on the therapeutic role exerted by PCA in obesity and its related pathologies, focusing on the mechanisms of action by which PCA might exert its effects. We highlight the capability of PCA to modulate oxidative stress and inflammation that, often in combination with one another, have a major role in the pathogenesis of obesity-related pathologies. Moreover, we will also deal with the more recent and novel strategies for the treatment of obesity.

2 PROTOCATECHUIC ACID: FOOD CONTENT AND BIOAVAILABILITY

PCA is a widely distributed phenolic compound present mainly in fruits and vegetables [6,7], but also in plant-derived beverages [8] and in some herbal medicine [9]. Different factors can affect the content of PCA, among which are the type of food, the degree of ripeness, the sun exposure, and the type of culture [10,11], thus influencing its bioavailability in humans [12]. The primary dietary sources of PCA are: mushrooms (*Ramaria botrytis*), (34 mg/100 g dry weight [6]); green and red chicory, (30 mg and 25 mg/100 g fresh weight, respectively [13]); black olives, (21 mg/100 g fresh weight [14]); Tainung papayas, (9.11 mg/100 g fresh weight [15]); black raspberry, (8.35 mg/100 g dry weight [16]); Australian mango (6.83 mg/100 g fresh weight [17]).

In a very recent paper [18] the urinary excretion of 34 dietary polyphenols, and their variations according to diet and other lifestyle factors, were measured in 475

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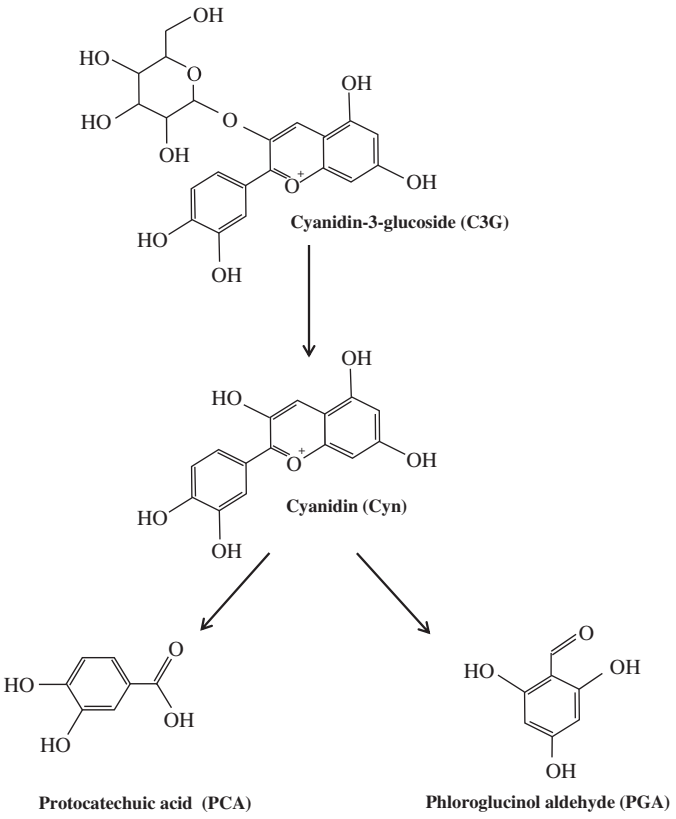

FIG. 14.1 Structural modification of the anthocyanin cyanidin-3-glucoside. The anthocyanins (C3G) undergo structural modifications, by colonic microflora or spontaneously in the bloodstream, resulting in the formation first of aglycons (Cyn) and then of phenolic acid (PCA) and aldehyde (PGA).

European adults by a novel analytical method, tandem mass spectrometry. This method allowed simultaneous quantifying of several polyphenols together with their metabolites. The significant variability found among polyphenols was most likely due to geographical variations in food consumption, as suggested by the high correlations observed between some urinary polyphenols and the intake of their main food sources. Regarding PCA, its median urinary excretion was 3.43 μmol/24 h, showing a significant positive correlation with coffee consumption.

It is important to underline that PCA concentration in vivo could be higher than the quantity ingested as such, because PCA represents one of the main metabolites of complex polyphenols, e.g., anthocyanins, widely distributed in the human diet [19].

Once absorbed, the polyphenols undergo additional structural modifications, thus generating different metabolites that, when reaching target tissues, are biologically and chemically different from the original compound [20]. For this reason, to evaluate the effectiveness of a dietary compound in disease prevention and human health improvement, it is important to be aware of both its bioavailability and its biological activity in target tissues.

Anthocyanins have always been thought to possess a low bioavailability as their recovery from biological fluids has been shown to be very low, with studies generally identifying less than 1% of the ingested dose [21,22]. This is probably due to their low stability; nevertheless, very few in vivo studies investigated several degradation products and metabolites in plasma and urine to better evaluate the bioavailability of anthocyanins. Czank et al. [23] investigated the metabolism and elimination of anthocyanins in eight men consuming 500 mg of ^{13}C-labeled cyanidin-3-glycoside (C3G). The authors detected several new metabolites and degradation products in biological samples, highlighting the complexity of anthocyanin degradation in vivo. Moreover, the total bioavailability of anthocyanins, considering also their metabolites, was estimated as 12%, substantially higher than previously perceived. Another in vivo study [24] corroborated these results, identifying large quantities of anthocyanin metabolites in human plasma and urine following elderberry consumption. The concentrations of these metabolites were considerably higher than those of their parent compounds both in plasma (+60%) and urine (+45%), thus confirming the extensive in vivo degradation of anthocyanins.

In human, PCA accounts for about 45% of the ingested C3G in the 6-h postconsumption bloodstream [25]. Moreover, it has been demonstrated that PCA reaches a concentration of 2.0 nmol/g in fecal samples collected the

day after pigmented orange juice consumption, thus supporting the key role of colonic microflora in the in vivo production of PCA [26]. It should be also pointed out that spontaneous degradation of anthocyanins to phenolic acids and aldehydes occurs under biological [25] and experimental conditions [27]. In physiological condition C3G, which represents the most abundant anthocyanin present in food, can easily be converted to cyanidin, that, in turn, rapidly and spontaneously degrades to PCA (Fig. 14.1), which can be further metabolized to glucuronide and sulfate conjugates [19].

Very recently, Muller et al. [28] examined anthocyanin bioavailability in subjects, with or without ileum, after the ingestion of a bolus of an antocyanin-rich bilberry extract. The authors showed that both anthocyanins and their degradation products were present in the biological fluids at higher amounts in healthy subjects than in ileostomy subjects (+79% and +44% in plasma and urine, respectively). The quantity of anthocyanins that goes through the intestinal gut passage corresponded to about 30%–50% of the ingested anthocyanins, but their availability in plasma was very low (0.02% in plasma and 0.03% in urine). Anthocyanins were present in plasma primarily as glucuronides, while their main degradation products were PCA, vanillic and syringic acid. The maximum plasma concentration of PCA was 40 nmol/L in ileostomy patients and 81 nmol/L in healthy subjects, much higher than that of the parent compounds. These results confirm that the formation of degradation products and metabolites occurs in significant quantities in blood and urine after anthocyanin intake [23,24].

In conclusion, all the recent research on anthocyanins has clearly explained the seeming incongruity between their low bioavailability and the high reported bioactivity. The large amount of ingested compounds undergoes structural changes in the colon, where they are hydrolyzed and degraded into simple phenolic acid that is finally absorbed, and that represents the effective bioactive components of anthocyanins.

3 OBESITY: A GLOBAL CHALLENGE YET TODAY

Obesity is defined by the World Health Organization (WHO) as "an abnormal or excessive fat accumulation that may impair health" [29]. It is a multifactorial disease caused by a complex interaction between genetic and environmental factors, which reduces longevity and quality of life [30]. The prevalence of obesity is steadily increasing worldwide, and this process has been so sudden that many researchers refer to it as an "epidemic." In the United States more than 60% of adult people are overweight or obese, but this high prevalence has been observed all over the world, in industrialized as well as in less developed countries. The WHO estimates that 39% of the world adult population is overweight and 13% is obese, and over 340 million children and adolescents aged 5–19 are overweight or obese [31].

A strong association has been established between obesity and chronic conditions such as type 2 diabetes, cardiovascular disease, nonalcoholic fatty liver disease, and several forms of cancer [32]. A very recent report has demonstrated that obesity is associated with cancer at 13 different anatomic sites; thus counteracting weight gain and excess body fat might represent an effective strategy to prevent cancer development [33].

A meta-analysis performed on the most recent published articles on medical care costs associated with obesity highlighted the high costs attributable to obesity. In fact in the United States the average annual medical cost is $1901 for an obese person [34], obesity accounting for 21% of total health care costs [35]. These data confirm that obesity has to be considered one of the major public health problems. This observation also explains why in the last few years many different approaches have been used to fight this disease, both invasive, such as bariatric surgery, and noninvasive, such as low-calorie diet, regular physical activity, and pharmacological therapy. Most of these methods are ineffective, because many individuals regain most or all of the lost weight within a few years [36]. Moreover, many drugs approved for pharmacotherapy of obesity have been withdrawn due to their severe side effects, such as heart and psychiatric diseases [37]. As a consequence, research has been prompted to develop alternative, safe, and effective methods to treat obesity and its related metabolic disorders.

It has been shown that oxidative stress has an important role in obesity [38], further confirmed by the low plasma levels of vitamins and antioxidants in obese individuals [39]. The onset of oxidative stress and the activation of inflammatory signaling are strongly connected, and together they can cause the alteration of many cellular functions regulating the balance between apoptosis and proliferation, as well as cell metabolism. Indeed the increased risk for pathologies associated with obesity is thought to be due to the chronic, low-grade inflammatory response characterizing adipose tissue in obesity [40].

Over the last few years targeting the connection between inflammation and obesity has been highlighted as a new promising therapeutic intervention, and dietary bioactive components can potentially be involved. Recent studies have demonstrated that the consumption of food-derived bioactive components, such as anthocyanins and their metabolites, is positively associated with a decreased risk of developing obesity [41]. Nutraceuticals are able to positively modulate the activity of adipocyte cell lines and further positive effects have been found on other aspects of pathogenic obesity [42]. In particular, anthocyanins have been demonstrated to be effective

against obesity [43]: in fact C3G strongly counteracts body weight gain and also decreases whole body fat composition, suggesting a key role in increasing energy metabolism, and thus supporting its use in the treatment of obesity and its related disorders.

4 MECHANISM OF ACTION OF PCA

Despite its rather simple structure, PCA can exert different relevant biological effects by acting on several molecular targets. The healthy effects of PCA are mostly associated with its antioxidant and antiinflammatory activities, but it has recently been shown that it also possesses the ability to ameliorate hyperglycemia and insulin sensitivity [44]. Moreover, PCA might have anticancer effects since it inhibits in vitro chemical carcinogenesis and exerts proapoptotic or antiproliferative effects in different tissues. In the next paragraphs we will focus on the most relevant activities of PCA, also highlighting the possible mechanisms of action.

4.1 Antioxidant Activity of PCA

Obesity is characterized by abdominal visceral accumulation of white adipose tissue, which is responsible for a state of chronic, low-grade inflammation and oxidative stress. The oxidative stress is considered a critical factor linking obesity with its associated pathologies [40]. It has been shown that oxidative stress induces preadipocyte proliferation and adipocyte differentiation, as well as favors the increase in size of mature adipocytes [45].

Several human studies have also demonstrated that oxidative stress markers are significantly increased in obese subjects [38,46]. Moreover, obesity per se induces systemic oxidative stress by several mechanisms, among which are superoxide generation from NADPH oxidases, PKC activation, oxidative phosphorylation, and glyceraldehyde autooxidation [47,48]. Several factors associated with obesity contribute to developing oxidative stress, such as high caloric and high fat diet, hyperleptinemia, hyperlipidemia and hyperglycemic insulin resistance, inflammation, endothelial dysfunction, impaired mitochondrial function, and increased muscle activity needed to carry excessive weight. It is well known that an excess of reactive oxygen species (ROS) within the cells induces oxidative modification of cellular macromolecules, which causes membrane and DNA damages and enzyme inactivation. These conditions lead to aberrant cell functions and induce, ultimately, cell death [49]. Controlling the intracellular redox state by means of efficient antioxidant machinery represents thus a crucial event in the prevention of cell damage.

This explains why humans have developed sophisticated mechanisms to cope with an excess of free radical production. Endogenous antioxidant defenses and exogenous antioxidants supplied by the diet play a pivotal role in such protective mechanisms; dietary antioxidants, such as polyphenols, have been considered pivotal in determining the risk of disease by virtue of their capability to counteract oxidative stress [50].

It has to be underlined that recent studies took into account the possible effects of cooking methods on the content of polyphenols in food. Various cooking devices were studied to understand their effect on polyphenol composition of Thai purple rice, which has a high content of anthocyanins and PCA [51]. The authors clearly demonstrated that the rice cooked under sterilization exhibited the highest content of PCA and the highest antioxidant activity, comparable to that of raw rice. Moreover, Chen et al. [52] demonstrated that edible plants rich in PCA and other phenolic acids inhibited the formation of advanced glycation end products (AGEs) in foods with high fat content cooked at a high temperature for a prolonged period of time. It must be remembered that the concentration of AGEs in foods has been considered a risk factor for many chronic diseases.

The effective in vivo antioxidant potential of dietary polyphenols depends on several factors, the main one being the chemical structure. The molecular structure of PCA suggests that it possesses strong antioxidant and antiradical activities [53], due to the position and the number of phenolic hydroxyl groups present in its molecule. It should be highlighted that PCA esters possess a higher antioxidant activity than PCA, probably due to their high lipophilicity, which represents the main factor in counteracting lipoperoxidation [54].

The effectiveness of PCA against lipoperoxidation and oxidative stress is mediated by hydrogen peroxides and oxidized lipids [55,56]. A growing body of evidence has also demonstrated a close relationship between PCA and the endogenous antioxidant defenses that could represent the main factor responsible for the antioxidant activity of PCA in biological systems. PCA increases the activity of glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase, and can directly activate mRNA transcription and activity of antioxidant/detoxifying enzymes such as GPx and glutathione reductase in murine macrophages [57]. The up-regulation of gene expression induced by PCA has been demonstrated to depend on the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates gene expression by binding the antioxidant responsive elements (ARE) present in the promoter region of phase II enzyme genes [58].

We showed that PCA increases the mRNA expression of Nrf2, which translocates into the nucleus where it binds ARE sequences present in GPx and GR promoters

[59]. These results support the concept that PCA, as well as several other polyphenols, improves antioxidant cellular defenses counteracting the oxidative stress associated with chronic-degenerative diseases [40].

Recently, Farombi et al. have demonstrated the antioxidative and antiinflammatory properties of PCA in a rat model of ulcerative colitis [60]. The administration of PCA significantly protected against colonic and hepatic oxidative damage by increasing the antioxidant potential and concomitantly by decreasing hydrogen peroxide and lipid peroxidation levels. Moreover, PCA significantly inhibited cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) protein expression in the colon of treated rats.

Neuroprotective effects of PCA, associated with the polyphenol chrysin, were observed in in vitro and in vivo models of Parkinson disease [61]. PCA improved cell viability and decreased lactate dehydrogenase release from 6-hydroxydopamine-treated PC12 cells, while attenuating neuron loss in both zebrafish and mice. Neuroprotection resulted in the combination of antioxidant and antiinflammatory cellular mechanisms. In fact, PCA and chrysin PC12 treatment (i) increased Nrf2 protein expression and transcriptional activity; (ii) upregulated the expression of antioxidant enzymes, including heme oxygenase-1, superoxide dismutase, and catalase; (iii) decreased the levels of malondialdehyde; (iv) inhibited the activation of NF-κB and the expression of iNOS. The neuroprotective effects of PCA have also been highlighted in murine cerebral cortex and hippocampus, where PCA preserved pro-/antioxidative homeostasis through improving the endogenous antioxidant system [62]. It has also been shown that PCA prevented osteoclast differentiation in RAW264.7 murine macrophage cells by counteracting oxidative stress and down-regulating the expressions of the inflammatory proteins NF-kB and COX-2 [63].

Lastly, in a human vascular cell model PCA, as well as C3G and another metabolite, vanillic acid, has been reported to possess vascular bioactivity by affecting NO and superoxide production and the expression of endothelial NO synthase, NADPH oxidase, and heme oxygenase-1 [64].

More than 500 plants contain PCA among the active constituents that impart various pharmacological properties due to their antioxidant activity. In the last years, a lot of studies were performed to characterize antioxidant content and activity of leaves, fruits, seeds, or their extracts, from plants that are normally used in local traditional medicine for antiinflammatory, antidiabetes, anticardiovascular diseases associated with obesity complications.

Various medicinal qualities have been attributed to *Helianthemum ruficomum*, an endemic species present in the northern Sahara. Its extract contains various antioxidant compounds, including PCA, showing a high antioxidant potential [65]. PCA has been also identified, among other polyphenols, as a main constituent of extracts from peel, pulp, and seed of *Prunus persica* (Tabacchiera peach) [66]. The fruits flour of *Geoffroea decorticans*, a plant of Northwestern Argentina, seems to exert preventive properties against diseases associated with oxidative stress and inflammation. In fact, the plant extract, containing PCA among other polyphenols, exhibited antioxidant activity by different mechanisms and inhibited the proinflammatory enzymes ciclooxygenase, lipoxygenase and phospholipase A2 [67]. Finally, the PCA-containing extracts of the tropical plant *Barringtonia racemose* has been shown to protect HepG2 cells against oxidative damage [68].

4.2 Antiinflammatory Activity of PCA

Inflammation is traditionally considered a defense response against infection or injury. The inflammatory process is tightly regulated by the activation of several signaling pathways that modulate the expression of both pro- and antiinflammatory mediators in resident tissue cells, such as fibroblasts, macrophages, endothelial cells, and recruit leukocytes [69].

Acute, localized inflammation is a life-saving mechanism to protect an organism from pathogens. On the other hand, a systemic chronic, low-grade, inflammatory state represents a risk factor for a wide range of pathologies, among which are metabolic syndrome, atherosclerosis, type 2 diabetes, cardiovascular disease, and cancer [70].

Inflammation can be considered as the extreme end of a spectrum ranging from a homeostasis state to an inflammatory response state. It should be kept in mind that all the systems (cell, tissue, organ) are in a homeostatic condition if the values of regulated key variables are within an acceptable range. When the homeostatic capacity is unable to maintain these values, a stress response is engaged; if this response is not able to restore homeostasis, an inflammatory response is induced [71].

The prolonged inflammatory response leads to the activation of proinflammatory mediators responsible for ongoing tissue injury and organ dysfunction. As a consequence, there is an augmented risk of developing several chronic diseases, including obesity [72].

Low-grade chronic inflammation is a peculiar characteristic of obesity, and adipose tissue releases many inflammatory mediators. Infiltrating macrophages seem to be especially important in this release, representing a major source of proinflammatory mediators, thus contributing to the sustainment and exacerbation of chronic inflammation [73]. Targeting inflammation represents an attractive and current strategy to counteract obesity.

Recently, food-derived bioactive compounds have been spotlighted as a regulator against various chronic diseases, including obesity, due to their low toxicity, as opposed to drugs that induce severe side effects [74].

Polyphenols can act as modifiers of pro/antiinflammatory signal transduction pathways to elicit their beneficial effects [75], and PCA seems to be a good candidate to target key inflammatory regulators [76]. The most important targets of PCA involved in its antiinflammatory activity are discussed in the following subsections.

4.2.1 Cytokine Production

Cytokines regulate several biological processes among which are cell growth and differentiation, immunity, inflammation, and metabolism. The production of pro- and antiinflammatory cytokines is tightly controlled by complex feedback mechanisms. Proinflammatory molecules, such as TNFα, IL-6, IL-8, and IL-1β, are normally responsible for fighting pathogens, while antiinflammatory molecules, such as adiponectin, restore physiological conditions, thus maintaining homeostasis [70].

Large families of small cytokines, called chemokines, are able to control the migration and residence of immune cells. Some chemokines are considered proinflammatory, and their release can be induced during an immune response at the site of infection, while others are considered homeostatic and are involved in controlling of cell migration during tissue development or maintenance. Thus, inflammation represents the result of sustained imbalance between pro- and antiinflammatory mediators, and might determine the outcome of the disease.

Moreover, TNFα and IL-1β act reciprocally with endothelial cells to produce adhesion molecules, such as intracellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1, that allow the progression of inflammation, exacerbating the severity of clinical disorders [77].

For all these reasons, targeting the cytokine system with plant-derived compounds could be a novel approach for the treatment of inflammatory diseases. Several studies have demonstrated that anthocyanins can affect pro- and antiinflammatory molecules in vitro at different levels.

Zhang et al. [78] have recently investigated the effects of PCA on acute lung injury induced by LPS in mice. The authors showed that the overproduction of proinflammatory mediators, such as TNFα and IL-1β, induced by LPS, were counteracted after the treatment with PCA. Therefore, they suggest that PCA could represent a useful tool for the therapy of lung inflammatory diseases.

Fat accumulation in adipose tissue produces hypertrophy of adipocytes and causes elevated levels of some proinflammatory adipocytokines, which are considered the key modulators linking obesity and inflammation.

Adzuki beans (*Vigna angularis*), one of the most widespread leguminous plants in East Asia, possesses antioxidant and antiinflammatory activities. Yook et al. [79] have recently shown the effects of black adzuki beans, containing different polyphenols among which is PCA, on colon inflammation triggered by obesity in mice. Black adzuki beans decreased the concentrations of several circulating proinflammatory cytokines such as TNFα, IL-1β, and IL-6 in obese mice, probably through the inactivation of NF-κB. These antiinflammatory effects make these beans a potential dietary supplement for the prevention and the attenuation of obesity and its related disorders.

Finally, novel data suggest that PCA and other anthocyanin metabolites are bioactive at physiological concentrations and could have the potential to modulate cardiovascular disease progression by altering the expression of inflammatory mediators, such as IL-6 and VCAM-1 [80].

4.2.2 Nitric Oxide

Nitric oxide (NO) is a key mediator, able to spread and promote the inflammatory response. Several in vivo and in vitro studies have highlighted the ability of PCA to counteract the production of NO, and very recently these data have been confirmed. In fact, under conditions of nitrosative stress, which occurs during inflammation in the central nervous system, it has been shown that PCA represents a neuroprotective factor and antiinflammatory agent [81]. Reductions in NO production suggest that PCA may be involved in the modulation of the expression of genes encoding proinflammatory mediators, such as NF-κB.

4.2.3 NF-κB

Among a huge number of proinflammatory mediators NF-κB represents the main effector that, directly or indirectly, regulates inflammatory responses [76]. It is present as an inactive form in the cytoplasm bound with its inhibitory subunit IκB. Upon activation, NF-κB translocates to the nucleus, activating a variety of target genes, among which are genes encoding proinflammatory cytokines, such as IL-1β, IL-6, IL-12, and TNFα, and proinflammatory enzymes such as iNOS and COX-2 [82].

Therefore, inhibitors of NF-κB may have an important role in novel therapies against inflammation.

It has recently been demonstrated that PCA has a protective role in LPS-induced acute lung injury in mice. In fact, PCA suppressed both p38MAPK and NF-κB signaling pathways, reducing the overproduction of TNFα and IL-1β [78]. A similar effect has been described in the brain of mice, where PCA counteracts the overexpression of COX-2, by inhibiting the protein expression of NF-κB [61], and in mouse aortic endothelial cells where PCA inhibits the TNFα-induced activation of NF-κB [77].

Furthermore, it is well known that the antiinflammatory mechanisms are crucial to maintain the homeostasis and the integrity of the bowel. Crespo et al. [83] have recently investigated the antioxidant effect of PCA in a mouse model of inflammatory bowel disease. Data obtained revealed that PCA-treated mice showed an increased expression of antioxidant enzymes and, concomitantly, a reduced expression of proinflammatory cytokines. In particular, NF-κB activation, measured by changes in the protein concentration of its subunit p65, revealed a strong increase in colitis mice that was counteracted by PCA treatment in a dose-dependent manner.

In conclusion, all these recent data confirm that PCA represents a key player in the prevention of inflammation, due to its ability in inhibiting the synthesis and/or the activity of the most important mediators involved in inflammation. The consumption of antocyanin-rich food, which increases the level of PCA in plasma, could represent an effective strategy to prevent or counteract the induced inflammation present in obesity and its related pathologies.

4.3 Antihyperglycemic Activity of PCA

The intracellular insulin signaling system plays a key role in many physiological processes including carbohydrate and fat metabolism, cell growth and survival. The primary function of insulin is to preserve glucose homeostasis and to stimulate glucose transport [84]. Defects in insulin signaling cascade, which lead to decreased glucose utilization, are believed to have a role in the pathogenesis of insulin resistance.

The disturbance of glucose metabolism is tightly connected to obesity, in particular to increased fat depot in the abdominal area, which, in turn, results in inflammation, exacerbating systemic oxidative stress with higher levels of circulating oxidized LDL (oxLDL) and malfunction of several organs including adipose tissue [85]. Insulin resistance underlies the early stages of metabolic syndrome development; thus approaches aimed at improving insulin action have been, and remain, key targets for slowing or ultimately preventing type 2 diabetes (T2D) [84].

The medical care cost associated with T2D treatment is of major concern and has prompted a search for novel and more affordable treatments, especially from natural products, because of few or no side effects compared to conventional drugs. An increasing number of epidemiological investigations have shown that consuming diets rich in food with high content of phytochemicals, namely polyphenols, decreases the risk of developing T2D. In this regard, functional food and food bioactive components have been considered a new and promising approach in the prevention and management of T2D and its clinical complications.

Among polyphenols, ACNs have been considered one of the most interesting classes of bioactive food components. The potential of polyphenols in controlling glycemia is currently under intensive study. In 3T3-L1 adipocytes it has been shown that flavonoids, such as quercetin and epigallocatechin gallate, exert beneficial effects in the regulation of glucose transport, which represents the rate-limiting step in the maintenance of normal glucose homeostasis. ACNs exert their activity mainly on AMPK and/or SIRT1 signaling pathways.

Recently, Scazzocchio et al. have demonstrated that PCA is able to directly activate the insulin signaling cascade in human visceral adipocytes [44]. Indeed, PCA stimulates IRS-1 tyrosine phosphorylation and the downstream events, i.e., the binding of PI3K to IRS-1 and the phosphorylation of Akt. Moreover, the authors highlight that PCA counteracts oxLDL-linked insulin resistance through the activation of serine phosphorylation of IRS-1, also providing evidence that PCA triggers the insulin pathway by targeting the tyrosine kinase activity of the insulin receptor. Moreover, it has been shown that PCA is able to enhance AMPK activity.

Other recent in vivo studies have been performed to examine the effects of PCA on vascular complications in STZ-induced diabetic rats [86]. It has been shown that PCA attenuates brain mitochondrial dysfunctions contributing to the prevention of brain oxidative stress in this in vivo model. Moreover, the authors support the hypothesis that PCA administration attenuates cardiac complications by reducing oxidative stress, ameliorating cardiomyocyte apoptosis, and improving the cardiac autonomic balance and mitochondrial function. Another study from the same authors demonstrated that PCA restores arterial pressure and vascular reactivity in the same rats by virtue of its antihyperglycemic and antioxidant activities [87]. Furthermore, PCA from the leaves of *Clerodendrum volubile* exerted protective effects on T2D-associated complications by an antioxidative burst activity on both polymorphonuclear neutrophils and macrophages, as well as by an antiproliferative activity on T cells [88]. These results are worthy of note since the antioxidative burst activity also reflects a modulatory effect on the activation of proinflammatory cytokines, which are the main factors responsible for insulin resistance, suggesting a possible mechanism by which PCA may modulate glucose homeostasis and suppress complications in T2D.

A therapeutic approach for counteracting hyperglycemia is to block the absorption of glucose in the digestive organs, by inhibiting the activity of carbohydrate hydrolyzing enzymes, such as α-glucosidase. Small intestinal α-glucosidases are key enzymes involved in dietary carbohydrate digestion in humans. Inhibitors of these enzymes may be effective in decreasing carbohydrate digestion and glucose absorption so as to suppress postprandial hyperglycemia.

Very recently Ablat et al. [89] have demonstrated that PCA contained in *Brucea javanica* seeds exerts an antihyperglycemic effect in T2D rats by inhibiting glycogen phosphorylase α and α-glucosidase activities. As a result, there is an increase of glycogen synthesis, thereby improving regulation of glucose metabolism. Hence, the inhibitors of these enzymes represent molecular therapeutic targets for controlling hyperglycemia associated with T2D.

All these findings indicate that PCA might exert beneficial effects by attenuating hyperglycemia, oxidative damage, cardiac mitochondrial dysfunction, and vascular complications, and could be suggested as a supplement to prevent adverse effects in diabetic patients.

4.4 Modulation of Apoptosis by PCA

Targeting adipocyte apoptosis represents an attractive and novel strategy for the treatment of obesity [90]. It is well known that obesity is characterized by the increase not only in the size but also in the number of adipocytes. Therefore, in addition to the current strategies addressed to reduce mainly adipocyte size, fat loss could also be achieved by apoptosis that decreases the total number of adipocytes [91]. However, it should be taken into account that provoking apoptosis in adipose tissue may be harmful to other adjacent tissues. Moreover, the adipocytes, killed via apoptotic pathways, may release lipids into the blood, thus promoting lipid storage in other tissues such as liver and muscle. An ideal new antiobesity drug should be able to induce apoptosis but avoid such side effects.

Apoptosis is a physiological process of cell death that has a key role in maintaining tissue homeostasis. It is mediated by two main pathways (*extrinsic*, or death receptor pathway, and *intrinsic*, or mitochondrial pathway), both leading to the death of a cell via caspase activation. All the main factors involved in the modulation of apoptosis are suitable targets for focused interventions.

Recent studies have confirmed that many dietary polyphenols, such as resveratrol and genistein, may exert antiobesity effects by specifically activating apoptotic machinery [92]. Although studies specifically addressing proapoptotic effects of PCA in adipocytes have not been available yet, PCA has been demonstrated to exert a strong proapoptotic activity, particularly in cancer cells [93]. This is probably due not only to its ability in modulating oxidative stress but also to its direct action on apoptotic regulatory proteins and signaling pathways [94].

On the other hand, it should be underlined that the antiapoptotic activity of PCA has been extensively described. Our group has demonstrated that PCA counteracts the cytotoxic activity of oxLDL in murine macrophages [95]. It is well known that oxLDL possesses atherogenic properties and can damage arterial wall cells by inducing p53-dependent apoptosis. The treatment of macrophages with PCA is able to inhibit apoptosis not only by counteracting oxidative stress occurrence but also by modulating the signaling pathways involved in caspase activation. In particular we have demonstrated that the antiapoptotic mechanism of PCA is related to the activation of the JNK-mediated survival signals, such as Nrf2. These results could have important implications in the treatment of atherosclerosis, since the occurrence of apoptosis, which mainly affects macrophages in advanced vessel lesions, is a crucial event in the onset and progression of the atherosclerotic plaque.

Wang et al. have highlighted the beneficial antiapoptotic effects of C3G and its metabolites PCA and ferulic acid in retinal cells of rabbits [96]. These compounds attenuate the photo-oxidation-induced apoptosis by reducing the expression of the proapoptotic proteins caspase-3 caspase-9 and Bax, and concomitantly increasing the antiapoptotic Bcl2, most likely through the activation of the Nrf2/HO-1 antioxidant pathway. Finally, it has also been demonstrated that PCA provides a significant protection against cardiac apoptosis. Deng et al. have shown that PCA supplementation in hypertensive rats decreases apoptotic cells by inhibiting proapototic protein expressions [97]. These results have been confirmed by other studies carried out in a rat model of myocardial ischemia/reperfusion. In these rats PCA significantly inhibits the apoptotic rate and the expression of cleaved caspase-3 in cardiomyocytes subjected to hypoxia/reoxygenation injury [98].

Based on the current evidence from these animal studies, it is possible to hypothesize that PCA could represent a therapeutic tool addressing the prevention of cardiac apoptosis in human hypertension often associated with obesity.

4.5 Effect of PCA on Gut Microbiota

Among the most studied factors that potentially affect obesity is the intestinal microbiota, which has been recently proposed to play a key role [99,100], even if the involved mechanisms have not been fully understood. However, recent studies have highlighted the potential role of the microbial families present in the human gut in controlling energy homeostasis [101].

The human gut microbiota possesses very large metabolic capabilities, and contains about 150-fold more genes than the human genome, providing the host with many, otherwise inaccessible, metabolic capabilities, thus influencing many aspects of human health [102]. In the healthy state, the gut microbiota provides nutrients and energy to the host via the fermentation of dietary components undigested in the small intestine, therefore

contributing to the "energy harvest" from dietary food [103]. This activity depends on different factors, among which is the nondigestible carbohydrate fraction contained in the diet [104], and could potentially be involved in the occurrence of obesity [105].

Evidence from animal and human studies suggested that the composition of gut microbiota could be involved in weight regulation and obesity development [99]. Specific bacterial populations, among which are *Lactobacillus* and *Bifidobacterium*, have been demonstrated to be strongly linked to obesity. As a matter of fact, the "switch" of gut microbiota composition toward a healthier "nonobese" profile might probably represent a promising new strategy to combat obesity [106].

In spite of the remarkable interindividual variation in humans, it has become increasingly clear that diet strongly influences microbiota composition; in particular, dietary polyphenols may exert antiobesity effects by specifically modulating gut microbiota populations.

Neyrinck et al. have demonstrated the effectiveness of a polyphenol-rich pomegranate peel extract in alleviating tissue inflammation and hypercholesterolaemia in high-fat diet-induced obese mice [107]. Balb/c mice were fed either a control diet or a high-fat diet with or without the enriched extract over a period of 4 weeks. Interestingly, the supplementation with the extract rich in polyphenols increased the intestinal pool of *Bifidobacteria*, without modifying body weight gain, but counteracting the high-fat-induced expression of inflammatory markers both in the colon and the visceral adipose tissue. All together, these results support the hypothesis that polyphenols contained in pomegranate may control inflammatory pathologies associated with diet-induced obesity also through the modulation of the gut microbiota.

Esposito et al. [108] have studied the preventive effect of an anthocyanin-rich black currant extract on the development of obesity and hyperglycemia in lean and diet-induced obese mice. The animals were further subdivided according to the presence of a healthy or an antibiotic-disrupted gut microbiota. The results clearly showed that the consumption of the anthocyanin-rich extract was able to decrease weight gain only in mice with healthy gut microbiota. Moreover, the feces of gut microbiota-deficient mice contained an increased level of anthocyanins and decreased level of their metabolites, such as PCA. Thus, gut microbiota seem to have a key role in the protective effect of anthocyanins against obesity and the associated insulin resistance.

The effect of different phenolic compounds, among which is PCA, contained in extra-virgin olive oil and thyme has recently been investigated in a randomized cross-over human trial [109]. Twelve hypercholesterolemic subjects consumed: (a) virgin olive oil containing 80 mg/kg of polyphenols; (b) an enriched virgin olive oil containing 500 mg/kg polyphenols from olive oil; and (c) an enriched virgin olive oil containing a mixture of 500 mg/kg polyphenols from both olive oil and thyme. The authors definitely demonstrated that the ingestion of the virgin olive oil rich in polyphenols deriving from olive and thyme had the better cardioprotective effect. Moreover, consumption of virgin olive oil containing mixed polyphenols strongly increased gut *Bifidobacteria* populations and polyphenol microbial metabolites, again supporting the hypothesis of the involvement of gut microbiota in polyphenol healthy effect.

Finally, although studies specifically addressing the modulation of intestinal microbiota by PCA have not been available to date, it has been shown that PCA exerts a strong antimicrobial activity [109]. In fact, this phenolic compound increased the production of reactive oxygen species in *E. coli*, *P. aeruginosa*, and *S. aureus*, resulting in lipid peroxidation and DNA fragmentation and, as a consequence, increased bacterial cell death [110]. Moreover, polyphenols, including PCA, extracted from almond skins are effective against different strains of bacteria, such as *S. enterica* and *S. aureus* [111].

All these results strongly suggest that the manipulation of gut microbiota by polyphenols represents a potential strategy to counteract obesity and its associated pathologies.

5 CONCLUSION

A growing number of scientific papers over the last few years has further clarified the possible mechanisms of action of PCA responsible for preventive and therapeutic activities against obesity and its related disorders. Recent experimental data seem to confirm that PCA exerts its beneficial effects through the fine regulation of biochemical pathways involved in the control of key cellular processes leading to oxidative stress, inflammation, and apoptosis. As a consequence, the role of nutraceuticals in counteracting metabolic dysfunctions associated with obesity and its related disorders still represents a subject of great interest.

However, many more randomized human trials need to be carried out in order to fully clarify the preventive and therapeutic effectiveness of PCA in limiting the development of obesity and its associated pathologies, ultimately proving its safety in humans.

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CHAPTER

15

Exposure to Polyphenolic Compounds Modulates Type 1 Diabetes: The Case of Genistein

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1 INTRODUCTION

Autoimmune disease is a category of diseases that result from a disordered immune response in which autoantibodies or autoreactive immune cells are produced against one's own tissues. It has to be distinguished from autoimmunity, which is defined as a failure in the mechanisms of immune self-tolerance [1]. It was previously reviewed that the common mechanisms for autoimmunity include, but are not limited to: defects in negative selection of T or B lymphocytes, defects in regulatory T (Treg) cells, inadequate function of inhibitory receptors, and defective activation of antigen presenting cells (APCs) [1]. Autoimmune diseases could be either systemic (such as systemic lupus erythematosus) or local/organ specific (such as type 1 diabetes [T1D] and rheumatoid arthritis), and are usually characterized by progressiveness, self-perpetuation, and great disease burden [2]. Both genetic (e.g., major histocompatibility complex [MHC] I and II) and environmental factors (e.g., infections [3]) are linked to autoimmune diseases.

T1D, also known as insulin-dependent diabetes or juvenile diabetes, is an autoimmune disease in which insulin-secreting pancreatic β cells are selectively destroyed by immune cells [4]. T1D is alarmingly increasing in the United States (1.25 million American children and adults) [5], and the incidence is higher in males, while women with T1D have a 40% excess risk of death from all causes compared to men with T1D [6]. In T1D patients, people under the age of 18 are mostly afflicted [7], although adult onsets of T1D are becoming more common [8,9]. For latent autoimmune diabetes in adults, it is unclear in its mechanism whether it is a late manifestation of T1D, an amalgamation of T1D and type 2 diabetes (T2D), or a completely distinct form of diabetes [10]. The mechanisms that cause males to be more afflicted (males 1.47 times higher than females) globally [11] might include hormones, genetic predisposition, environmental exposures, and epigenetics [12].

2 PATHOGENESIS OF T1D

A number of immune cell subsets play important roles in the pathogenesis of T1D, and these cells include T cells, B cells, natural killer (NK) cells, and APCs [13], although the roles of B cells are less clear [7]. Badami et al. [4] found that the $CD4^+CD25^+FoxP3^+$ Tregs were significantly reduced in T1D patients, and the $CD4^+CD25^+$ T cells could not be converted to Treg cells. It is widely accepted that destructive autoimmunity in T1D mostly depends on an important Th1-dominated autoimmune response [14,15], although a Th2-dominated islet autoimmunity may occur after a latent period [16]. In a recent study, it was shown that the ratio of Th17/Th1 plays a role in β cell destruction and T1D [17]. Common markers for T1D include autoantibodies for insulin, glutamic acid decarboxylase 65, islet antigen-2 and zinc transporter 8 [13], although blood glucose level (BGL) is often used as a readout.

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2.1 Genetics

The genetic aspects of T1D are well studied, and it is known that more than 20 different regions are associated with the susceptibility, while 40%–50% of human T1D risk can be accounted for by human leukocyte antigen (HLA, a cluster of genes in MHC-DR and -DQ genes) [18]. For example, HLA-DRB1*0301/0401 heterozygotes are strongly associated with T1D with an odds ratio of 35 [1]. It has also been reported that approximately 30% of patients with T1D are heterozygous for HLA-DQA1*0501–DQB1*0201/DQA1*0301–DQB1*0302 alleles [18].

2.2 Environment

For environmental factors, the immunity-diet-microbiota consortium has received great attention because the diet and microbiota together modulate the maturity of the immune system [19]. It was reviewed by Kagohashi et al. [20] that environmental exposure could destroy pancreatic β cells directly (e.g., attacking β cells) or indirectly (e.g., molecular homology or perturbation of immune system). Diet could regulate the gut microbiota (GMB) through direct (e.g., providing substrate for bacterial digestion) and indirect (e.g., regulation of intestinal and pancreatic physiology) effects [19]. As discussed previously, several cell types (e.g., APCs, B cells, T cells, and macrophages) interplay in the pathogenesis of T1D. One subset of T cells, the Tregs that are differentiated from $CD4^+$ cells into $CD4^+CD25^+Foxp3^+$ cells, can be induced by commensal GMB and is related to the short-chain fatty acids (SCFAs) production from GMB (this is discussed in depth later).

There are several epidemiological studies on the risk factors of T1D. Frederiksen et al. [21] reported that among children less than 5 years of age, early exposure to fruit and late exposure to rice/oats are associated with an increased risk of T1D, while breastfeeding at the time when wheat/barley is introduced to the diet showed a protective effect [21]. In another study with a Finnish cohort, it was found that the maternal consumption of butter, low-fat margarines, berries, and coffee was negatively associated with the development of advanced β-cell autoimmunity in the offspring [22].

3 POLYPHENOLIC COMPOUNDS AND T1D

Polyphenolics are naturally occurring compounds that can be widely found in fruits, vegetables, and beverages (such as tea) [23], and which the bitterness of food comes from. Common polyphenolic compounds include flavonoids (a family of plant metabolites that have antioxidant properties, and isoflavones are part of it), stilbenes, lignans, and such. When not ingested, all flavoids except flavanols are in the glycosylated forms, while the fate of glycosides in the GI tract is not well understood yet [23]. It is well accepted that polyphenolic compounds such as epigallocatechin gallate [24], curcumin [25], and resveratrol [26] have anticancer and antidiabetic effects.

3.1 Soy Food

The two major chemical classes of phytoestrogens found in diets are lignans (enterodiol and enterolactone) and isoflavones (daidzein, genistein [GEN], and glycitein) [27]. The contents of isoflavone GEN and daidzein in soy products are described in Table 15.1. Soy food contains a large amount of isoflavones, and it is specifically of interest since it takes up 12% of the infant formula market [28]. In addition, soy products are of particular interest since they are an intrinsic part of Asian cuisine (such as soymilk and tofu), and that they are widely used in vegetarian meals due to its high protein content (about 40%) [29]. As reported by the United Nations Food and Agriculture Organization in the beginning of the 20th century, the European and North American soy protein consumption was less than 1 g/day per capita, while the consumption is 8.7 g, 6.2–9.6 g, and 3.4 g in Japanese, Koreans, and Chinese, respectively [30]. In another study, the intake of soy largely depends on location, as it was reported that the intake among Chinese Americans (4 g/day) was nine-fold less compared with their counterparts from China (36 g/day). In addition, 25% of infants were fed on soy formula in the United States [31], which is partly due to its higher content of calcium and phosphorus than cow milk [32]. A cross-sectional study conducted in

TABLE 15.1 The Genistein, Daidzein, and Total Isoflavone Content in Common Soy Food

Food	Per serving	Genistein (mg)	Daidzein (mg)	Isoflavone (mg)
Soybeans, boiled	4 oz	6	6	12
Soybeans, dry, roasted	1 oz	15	19	37
Soy protein concentrate, aqueous washed	3.5 oz	56	43	102
Miso	4 oz	34	22	59
Tempeh	1 oz	5	7	12
Tofu	1 oz	4	3	7
Meatless hot dog	1 hotdog	6	3	11
Soymilk	8 oz	17	12	30

http://lpi.oregonstate.edu/infocenter/phytochemicals/soyiso/index.html#source

Philadelphia found that in infants of both gender consuming soy-based formula, the urinary GEN and daidzein concentration was 500 times higher than in those consuming milk-based formula [31].

There are several guidelines available for soy consumption. The U.S. Food and Drug Administration Code of Federal Regulation Title 21 suggests that the intake of 25 g soy protein per day, as part of a diet low in saturated fat and cholesterol, could reduce the risk of heart disease. The Chinese government recommends consuming a minimum of 50 g of soy foods daily to meet nutrient needs [33]. Additionally, the WHO, which does not have a threshold for soy intake, listed soybean as a good source of folic acid.

3.1.1 Isoflavone and GEN

Isoflavones in the soy exert estrogen-like effects. The content of GEN could vary a lot in soy food, based on the food processing. The highest content was found in roasted soybeans, reaching 1425 μg/g [34], and GEN accounts for about 50% of total isoflavone and is the most dominant isoflavone. Isoflavones have a relatively weak affinity to both estrogen receptors (ERs) α and β compared to 17β-estradiol; the affinity to ER-β is 20- to 30-fold higher than to ER-α [35], while to date, the affinity with nonclassical ERs remains uncertain. GEN shares some structural similarity with 17β-estradiol (shown in Fig. 15.1). Despite the low affinity, the serum isoflavone level can reach up to micromolar concentration following an intake of soy meal [36]. Combinatorial isoflavone exposure was found to inhibit the induction of interferon-γ positive $CD4^+$ T cells by lipopolysaccharide-activated dendritic cells in the mucosal immune system [37].

GEN is the major chemical discussed in this review. GEN is the aglycone and active form of genistin that exists in soy and is hydrolyzed to GEN in the intestine [27]. As a major endocrine disruptor (ED, a compound that competes with endogenous estrogen to bind to ER), GEN can either function as an ER agonist (when the estrogen concentration in the body is low) or ER antagonist (when the estrogen concentration in the body is high) [35]. It should be noticed, however, that the effect of soy food can also be attributed to other isoflavone subclasses [27]; for example, soy isoflavone glycitein exerts a protective effect on oxidative stress in *Caenorhabditis elegans* [38]. In addition, one study found that soy, not GEN, altered body weight in the pregnant mouse and puberty onset in female offspring [39].

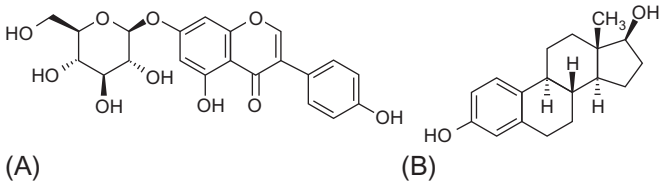

FIG. 15.1 The chemical structures of (A) genistein and (B) 17β-estradiol. Images obtained on Abcam on October 2017.

3.1.2 Epidemiological Studies

In terms of T1D and soy, among the limited epidemiological studies, one study showed that for infants aged 4–6 and 7–12 months, soy milk formula consumption was associated with a two-fold increase of T1D risk in China [40], while information on other exposure windows (e.g., prenatal, juvenile, and adult) is largely unknown. Another epidemiological study has also shown that twice as many children with T1D consumed soy-based formula in infancy as compared to control children [40,41]. In adult T1D patients, however, dietary intake of soy products showed a protective effect on renal function and lowered cholesterol [42].

The main concern of soy consumption is the effect on reproductive health with multiple health outcomes during developmental stages. In a large retrospective cohort study, soy-based formula consumption through infancy was associated with heavier menstrual bleeding and greater menstrual discomfort in young adulthood [43]. In a longitudinal study, early soy-fed girls have a median age of 12.4 years old at menarche, compared to 12.8 years old in non-soy-fed girls [44]. The same research group found that among girls aged 42 months, early-life soy consumption was associated with less female-typical play behavior, while the same trend was not found in boys [45]. In a prospective cohort study, however, the formula types were not associated with benign breast diseases in young women [46].

3.1.3 Animal Studies

In general, GEN exposure shows a protective effect in adults for diseases such as cancer and cardiovascular diseases in various animal stains [47]; however, GEN's effects on the immune related diseases varies, GEN effects being tissue specific and dependent on different factors (e.g., specific cofactors, ER levels, levels of certain intracellular kinases, and estrogen levels) [48]. To emphasize, the effect of GEN on the immune system can be largely sex-specific and age-specific, which will be the focus for this book chapter.

Adult exposure to GEN has been shown to be associated with a reduced risk of diabetes in animals. GEN is reported to have anti-T2D function due to its protective effect on pancreatic β cells [49]. There have been multiple lines of evidence showing the association between GEN intake and T1D protection [50–53]. GEN is known for its antiinflammatory functions, and has been shown to attenuate cell-mediated immune responses [54]. From a mechanistic view, GEN reduces inflammation by decreasing interleukin (IL)-6 and monocyte

chemoattractant protein (MCP)-1 levels while downregulating a number of genes involved in inflammation, as shown in an in vitro study [55]. In our study in vivo, GEN also reduced serum MCP-1 level in NOD adult males, suggesting an antiinflammatory effect [52]. Sex can also play an important role in the development of T1D [52], and it is speculated that one mechanism for GEN modulation on T1D is hormone-related.

In addition, our study found that GEN treatment in B6C3F1 males on the National Toxicology Program (NTP) standard diet, a soy-based diet, decreased BGL significantly on days 33 and 82 following STZ administration to induce diabetes, while the same trend was not observed in mice fed with a high fat diet [50]. It was also found that GEN exposure increased both $CD4^-CD8^+$ T cells and IgM^+ B cells in male fed with soy-free diet, while modulation on neutrophils by GEN in males on the soy-based NTP diet may be related to the antidiabetic or diabetogenic potentials [50]. In another study investigating renoprotective effect of GEN in C57BL/6 males, GEN reduced BGL significantly, but it showed no protective effect on plasma insulin level [56].

In adult female mice, GEN intake by oral gavage reduced the incidence and modulated the onset of T1D in NOD females fed a soy- and alfalfa-free diet, while the same effect was not observed in NOD mice on the NTP-2000 diet [51]. In contrast, with a soy-based PicoLab diet, the preventative effect of GEN on T1D was detected [52]. In addition, it was suggested that GEN can potentially prevent the onset of diabetes, elevate plasma glucose, and alter the hepatic glucose by increasing insulin secretion and preserving pancreatic β cells in NOD females [53].

In animal models, perinatal exposure to GEN was found to regulate diseases including T1D through immune-modulation. Perinatal exposure is of concern, as phytoestrogens have also been detected in amniotic fluid [57], suggesting that *in utero* exposure also occurs. Also, the metabolic and/or excretion rates of GEN are different between mother and fetus, and once GEN is transferred to the fetus, it is eliminated much more slowly from fetal tissues as compared to maternal elimination [58]. In our previous study among B6C3F1 mice, GEN led to an increased T-cell activity in the first-litter females and second-litter males, as represented by a decreased $CD4^+CD25^+$ T cells (possibly Treg cells), which indicates an increased immune response [59]. In another study, developmental GEN exposure induced alterations of total number and subpopulation ($CD4^-CD8^+$, $CD4^+CD8^-$, and $CD4^+CD8^+$) in splenic T cells, as well as number and activity of NK cells in C57BL/6 mice [60]. Similarly, perinatal exposure to GEN from gestational day 7 to postnatal day 64 induced an increase in splenic B cells, macrophages, T cells (total, helper, and cytotoxic subsets) in Sprague-Dawley rats [61].

4 MECHANISMS OF T1D MODULATION BY POLYPHENOLIC COMPOUNDS: GUT MICROBIOTA

GEN has modulatory effects on multiple diseases using different mechanisms. The potential mechanism of GEN modulation on T1D through GMB is shown in Fig. 15.2.

4.1 Development of Gut Microbiota in Human

The human intestine hosts approximately 100 trillion bacteria (at least 1000 species) [62]. GMB composition can be determined by different factors throughout life. In fetuses, early-life gut bacterial colonization plays an important role in metabolic tissue development in determining risk for autoimmune diseases [63]. This is because intestinal immune system development, such as the maturation of intestinal mucosa, Peyer's Patch, isolated lymphoid follicles, and mesenteric lymph nodes, starts from 11 weeks of gestation [19].

The bacterial DNA can transfer from mother to fetus through amniotic fluid [64], umbilical cord [63], and placenta [65]. Evidence showed that probiotic use (*Lactobacillus rhamnosus* or *Bifidobacterium lactis*) during pregnancy altered infant immune responses [66]. The mode of delivery, antibiotic use, and infant formula can all help shape the infant microbiota [19,65,67] and further modulate the immune system. For example, the composition of GMB is closely related to maternal skin, and plant-based dietary intake, compared to dairy-based food, demonstrated a distinct GMB profile [68]. Over time, food can play a major role in shaping the GMB. As reviewed by Johnson et al. [69], in some studies, an animal-based diet led to the increased abundance of *Bacteroides* and *Alistipes*, while it was found in other studies that the *Bacteroides* increased with the fiber intake. Antibiotics, which is often used to battle bacterial infection and colitis, could induce an attenuated mucus layer and reduced goblet function, suggesting a weakened mucosal barrier [70].

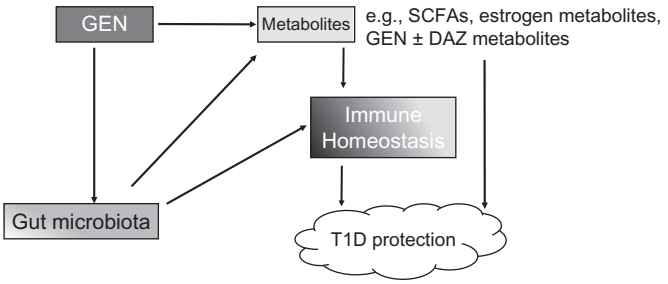

FIG. 15.2 The potential mechanism of GEN modulation on T1D through gut microbiota.

4.2 Epidemiological Evidence of Gut Microbiota and T1D

Two major phyla, *Bacteroides* and *Firmicutes*, make up most of the human GMB. After seven years of age, the ratio between *Bacteroides* and *Firmicutes* is relatively stable, while a disturbance might be contributing to metabolic syndrome such as obesity and T2D. There have been a multitude of epidemiological studies bridging microbiota and T1D. An international research group investigated the GMB among the newborn infants in Finland, and found that two species, *Bacteroides dorei* and *Bacteroides vulgatus*, were higher in infants who later developed T1D, and the abundance was in coincidence with the intake of solid food [71]. The same group looked at the metagenomic profile from stool samples from four pairs of matched case-control subjects, and observed elevated *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* and reduced *Firmicutes*, *Fusobacteria*, *Tenericutes*, and *Verrucomicrobial* in T1D cases [72]. In addition, it was found that when the subjects were approaching the toddler age, those who would be T1D-free in the future had a healthier and more stable GMB, while those would develop T1D had a less diverse and unstable GMB [73]. On the contrary, a European team analyzed the fecal samples from a European case-control study and observed an increased microbial diversity among T1D children older than 2.9 years [74]. In a Mexican case-control study, it was noticed that in T1D cases, the dispersion in the structure of GMB was higher, whereas no difference in α-diversity was found among controls, T1D at onset, and T1D under treatment [75]. The genus *Bacteroides* showed a higher abundance in cases, while *Prevotella* showed a higher abundance in control [75]. Another finding is that age might be a major determinant for certain microbial species, since children older than 2.9 years were found to have a higher fraction of *Clostridium* clusters IV and XIVa than younger children [75]. Taken together, the ratio of *Bacteroides* and *Firmicutes*, together with alteration of specific taxa, can serve as a potential biomarker for early diagnosis of T1D.

4.3 Animal Studies in Gut Microbiota and T1D

In lab animals, the research on the association between GMB and T1D largely focuses on the presence of xenobiotics, exposure window, and gut leakiness [76]. Patterson et al. [77] investigated the GMB changes in male Sprague-Dawley rats with STZ-induced T1D and found a reduced α-diversity after exposure. STZ-induced T1D also introduced β-diversity over time, while perturbing the *Bacteroidetes/Firmicutes* balance. The proportion of *Bacteroidetes* was significantly increased, while the *Firmicutes* was significantly decreased over the 4-week study period. On the other hand, a Japanese study did not find a difference in diabetes incidence between the specific pathogen-free (SPF) and germ-free (GF) NOD mice (with the susceptibility of T1D originated from defective MHC haplotype), while the insulitis was accentuated in GF mice [78]. The significant discrepancy of previous studies on how GMB could affect T1D could be attributed to genetic drift in colonies [78]. In addition, the GMB alteration could lead to the hormone level change (such as testosterone), which further contributes to the T1D incidence change [79]. Specific bacteria or bacterial products (such as segmented filamentous bacteria) would have the same effect on T1D incidence compared to GF environment [64].

By depleting the commensal bacteria, GF animals are widely used to establish an association between GMB and T1D. However, the price might be prohibitive for many labs. In that sense, antibiotics are used as a substitute since a majority of GMB can be eliminated this way. In humans, no effect has been found so far between T1D and antibiotics use [80]. With animal models, Brugman et al. [81] treated Biobreeding Diabetes-Prone (BB-DP) rats with antibiotics (sulphamethoxazole and trimethoprim, targeting both Gram-positive [G+] and Gram-negative [G−] bacteria) in drinking water, and found that the T1D was completely prevented. Similarly, Schwartz et al. [76] fed NOD mice with doxycycline (a broad-spectrum antibiotic) containing regular rodent diet and found that the incidence of T1D was significantly lower than those not fed with doxycycline. In another study treating both NOD pups and adults with vancomycin, a broad spectrum antibiotic, the principal component analysis profile showed a significant separation among the groups compared to control [82]. Importantly, vancomycin treatment was found to have a protective effect on T1D for both neonatal and adult exposure in terms of BGL and insulitis score [82]. Another study selectively depleted G− bacteria with antibiotics (neomycin, streptomycin, and polymyxin) in drinking water among NOD mice during the perinatal period, and found a similar protective effect that largely depended on the exposure window (pregnant, born, and weaned) and was transferable to the new host [83]. As reported by the same research group, the depletion of G+ bacteria with antibiotic led to an accelerated T1D in NOD mice; it was concluded that increased G+/G− ratio was associated with a reduced risk of T1D [83]. For antibiotic depletion of GMB, it should be noticed that interdependence is found for different microbial taxa; for example, G− microbials could be effectively depleted by vancomycin that mainly targets G+ microbials [84].

4.4 Gut Microbiota and Immunity

The relationship between GMB and gut integrity is described in Fig. 15.3. Besides the normal function of

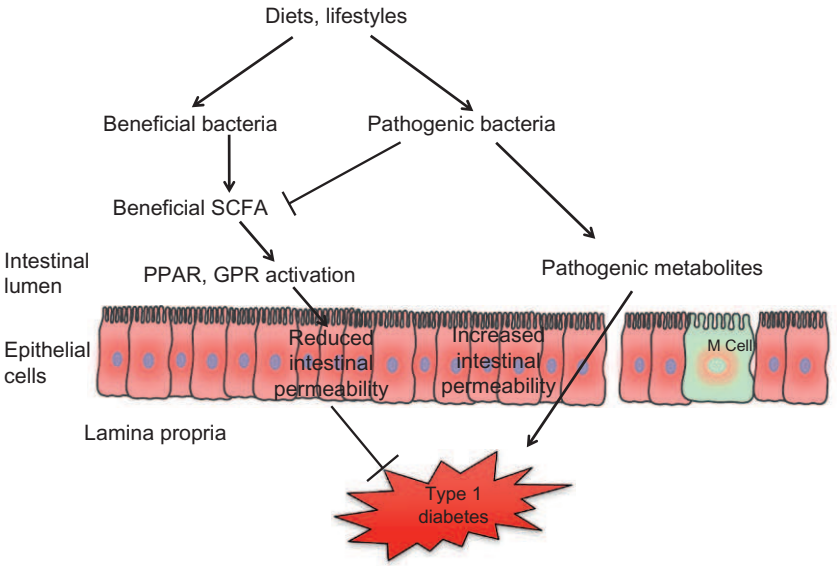

FIG. 15.3 One possible mechanism for the interaction of type 1 diabetes, gut integrity, gut microbiota, and metabolites. *Adapted from Guo T. Molecular and biochemical toxicology, 5th ed., Chapter 23, In Molecular mechanisms of immunotoxicity, 2017.*

digesting dietary nutrients such as polysaccharides, intestinal bacteria play an important role in fighting against pathogenic infections [62]. For example, the innate and adaptive immune responses against *Toxoplasma gondii* can be induced by gut commensal bacteria [85]. In addition to retaining homeostasis, the immunological barriers function to minimize the contact of intestinal bacteria with the epithelial cells, to attack pathological bacteria that tend to penetrate intestinal issues (both innate and adaptive immunity), and to minimize exposing resident bacteria to systemic immune system [62]. In terms of T1D, the GMB profile was different depending on future T1D status, and the GMB profile was further associated with changes in immune markers such as Foxp3$^+$ Tregs, CD11b$^+$ dendritic cells, and IFN-γ production [86]. On the other side, the immune system can modulate the GMB as well. It was found that the innate and adaptive immune system could coordinate antibodies (IgA) against microbiota and thus impact the microbial community [87]. IL-10, an antiinflammatory cytokine, could also modulate the structure of GMB, and the metabolism of arsenic by GMB [88].

4.5 SCFAs and T1D

SCFAs are the end products of microbial fermentation of macronutrients, in which the majority are polysaccharides not digestible by human polysaccharide lyases alone [89]. The gut bacteria *Bacteroidetes* participate in the process by providing the host with energy, manufacturing SCFAs that supply up to 10% calories to the hosts [69]. The most common SCFAs are butyrate, propionate, and acetate. It was reported that the proportion of the major SCFAs could vary greatly; however, butyrate, acetate, and proprionate make up the majority of the SCFAs in the gut [90]. SCFAs are essential to intestinal health, with butyrate being the major energy source for colonic cells and having antiinflammatory properties (e.g., immunomodulatory effects on intestinal macrophages) [72,91]. Additionally, butyrate can inhibit the expression of proinflammatory cytokines such as TNF-α [91]. Beneficial SCFAs produced by microbiota stimulate epithelial metabolism, deplete intracellular oxygen, and thus enhance the epithelial barrier function [92]. SCFAs can be directly antiinflammatory through the activation of peroxisome proliferator-activated receptor (PPAR)-γ, the suppression of NF-κB pathway, the inhibition of histone deacetylases [93], and the activation of G protein coupled receptors (e.g., GPR41, GPR43, and GPR109a) [94]. Also, Chassaing et al. [95] found that SCFAs directly inhibit intestinal atrophy and induce microbiota-mediated intestinal inflammation. More importantly, SCFAs, especially propionate and butyrate, are essential for Treg accumulation and differentiation [96]. In addition to the immune modulatory effects, SCFAs can also regulate intestinal environments by reinforcing the intestinal epithelial cell barrier [91].

The association between SCFAs produced by GMB and T1D remains largely unknown. Brown et al. [97] found that susceptibility to T1D well correlated with reduced fecal SCFAs. In an earlier study, however, the production of butyrate from beneficial bacteria was found to be associated with increased tight conjunction and thus healthy gut, while in another study, SCFAs including propionate, acetate, and succinate were linked with less mucin synthesis, increased leaky gut, and increased autoimmune diseases such as T1D [72]. Certain

strains of commensal bacteria (e.g., *Bifidobacteria*) can ward off pathogens through production of acetate and thus help maintain intestinal health [98]. To date, no studies have been conducted to investigate if oral exposure to GEN can affect SCFA content or not.

4.6 Probiotics

Probiotics are defined as "mono- or mixed cultures of live microorganisms that can beneficially affect the host by improving the properties of the indigenous microflora" [99]. Common probiotic components include *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bacillus clausii*, *Lactobacillus acidophilus*, *Lactobacillus GG*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus bulgaricus*, and *Streptococcus thermophiles* [100]. In a prospective cohort study, probiotic supplementation at the age of 0–27 days in infants was associated with a decreased risk of islet autoimmunity comparing with probiotic supplementation after 27 days [101]. One study performed in NOD mice found that the oral probiotic (VSL#3) can effectively prevent T1D while inducing antiinflammatory IL-10 production [102]. Similarly, in another study, oral administration of VSL#3 in NOD mice prevented T1D by inhibiting IL-1β expression and releasing components of inflammasome [103]. Moreover, by orally transferring *Lactobacillus johnsonii* strain N6.2 from Biobreeding diabetes-resistant rats to BB-DP rats, a study found that the probiotics conferred T1D protection in diabetes-prone rats through enhanced Th17 differentiation [104]. In addition, it was reported that the combinatorial use of probiotics and vitamin C could effectively reduce BGL more than vitamin C alone in an alloxan-induced Wistar rat model [105].

4.7 Soy Effect on Gut Microbiota

In an epidemiological study among children, it was found that *Bifidobacteria*, *Bacteroides*, and *Clostridia* were significantly reduced following soy consumption [106]. However, the change of microbial diversity was not found in this study. In addition, soy consumption could increase *Lactobacillus* [107,108], *Bifidobacterium* [107,108], and *Bacteroides* [109] in human, and the changes were comparable to lab animals [110]. It was surprising that similar results were observed despite the fact that a range of dose, soy products, and animal species were used [111]. In a fermentation study using human feces, isoflavone GEN induced an increase of *Bifidobacterium spp*. and *Lactobacillus spp*. [112], and both of the species were found to have a protective effect on T1D [72]. In a recent study from our group, GEN treatment in NOD males and females exerted a protection of T1D; however, GEN perturbed the GMB toward an antiinflammatory response in males and proinflammatory response in females, showing a sex-specific effect [52].

5 MECHANISMS OF T1D MODULATION BY POLYPHENOLIC COMPOUNDS: EPIGENETICS

Epigenetics refer to genetic effects that are not coded in DNA sequence, and common epigenetic markers include DNA methylation, histone modification, and miRNA expression. Epigenetic change of specific genes (e.g., *IL2RA*) has been associated with childhood T1D onset, as was shown in an epidemiological study [113]. As environmental factors have been implicated in diabetes, polyphenolic compounds can also affect epigenetic states [114].

5.1 DNA Methylation and Histone Modification

Soy food is considered a maternal epigenetic diet as it regulates DNA methylation and histone modification during embryogenesis [115,116]. GEN was found to inhibit DNA methyltransferase (DNMT: a group of enzymes involved in de novo methylation and DNA methylation maintenance) and histone deacetylases (HDACs: enzymes involved in histone deacetylation) [116]. In another study, it was found that the ratio of demethylated:methylated DNA is the highest in pancreatic islets among pancreatic *Ins*1 gene (an insulin gene) in NOD-SCID mice; while increased demethylated DNA was associated with prediabetic status [117].

5.2 microRNA (miRNA)

miRNA is a noncoding RNA 21–26 nucleotides long that suppresses target gene expression through the inhibition of gene translation and miRNA-mRNA interaction [118]. Studies found that two miRNAs (miRNA106b and 222) can contribute to the bone marrow transplant-induced β cell regeneration in C57BL/6J mice [119]. Another study reported that a complex miRNA species (denoted as TA1-miRNA) can reset the immune system by increasing Treg:effector T cell ratio, reducing the expression of proinflammatory T cell subtypes in pancreatic lymph node, and increasing the Treg cell expression in lymphoid tissues [120]. GEN has been shown to associate with miRNA alteration in tumor studies (e.g., up-regulation of tumor suppressor miR-574-3p [121] and down-regulation of oncogenic miR-151 [122] in prostate cancer, and down-regulation of oncogenic miR-27a in ovarian cancer [123]). GEN was found to up-regulate the let-7b, -7c, -7d, -7e miRNAs in gemcitabine-resistant pancreatic cells, and up-regulate miR-200b and -200c in

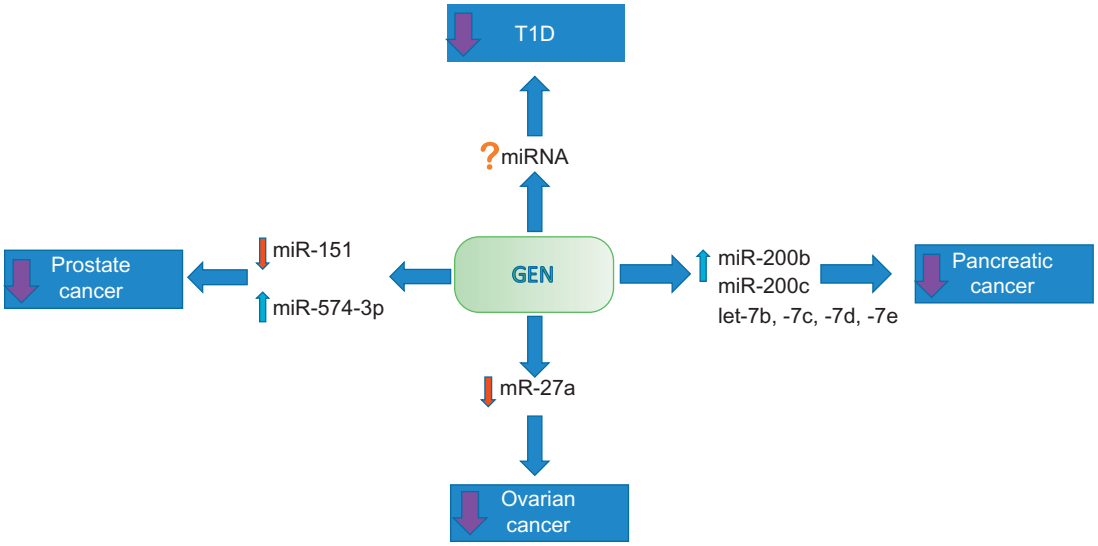

FIG. 15.4 The miRNA mechanisms of GEN modulation of diseases in human cell lines.

human pancreatic cancer cells [124]. However, the knowledge of GEN's effect on microRNA with regards to T1D has not been studied yet. Fig. 15.4 illustrates possible mechanisms of GEN modulation on different diseases by miRNA.

6 OTHER POTENTIAL MECHANISMS

Our previous study has reported that GEN can increase the production of insulin in NOD females at an oral dose of 6 mg/kg [51]. In addition, a similar study found that GEN can modulate pancreatic β-cell function via activation of the ERK1/2 signaling pathway, which is cAMP/PKA-dependent [125]. Using an STZ-induced T1D model, another study confirmed that GEN decreased β-cell loss, and improved glucose and insulin levels in adult male rats [126].

7 CONCLUSION

Starting from prenatal exposure to adult exposure, individuals can be exposed to polyphenolic compounds such as GEN, which can modulate the risk of autoimmune diseases such as T1D. Importantly, the immunological responses and consequently T1D risk largely depend on exposure windows, and evidence includes epidemiological studies and animal models; however, the mechanisms have not been fully elucidated yet. GMB and epigenetics are two widely studied areas in environmental exposures; for GMB, extensive research may prompt us to develop probiotics to prevent disease status in the future, and for epigenetics, we are able to explore functional biomarkers of effect and susceptibility for T1D. The case for the soy isoflavone GEN in modulating autoimmune diseases can serve as a model for assessing the health effect of other polyphenolic compounds.

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SECTION III

MECHANISMS OF POLYPHENOLS ANTIOXIDANT EFFECTS

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CHAPTER

16

Chocolate/Cocoa Polyphenols and Oxidative Stress

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Acronyms and Abbreviations

(COX)-2	cyclooxygenase
FMD	flow mediated dilation
GSH	glutathione
HDL	high-density lipoprotein
Hs-CRP	high sensitivity C-reactive protein
IL-1β	interleukin 1 beta
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
LDL	low-density lipoprotein
MDA	malondialdehyde
NO	nitric oxide
ROS	reactive oxygen species
SOD	superoxide dismutase
TEAC	trolox equivalent antioxidant capacity
TBARS	thiobarbituric acid reactive substances
TNF-α	tumor neurosis factor

1 INTRODUCTION

There has been a great deal of interest in recent research literature in the potential beneficial effects of cocoa due to the antioxidant polyphenol content, of which cocoa is an abundant source. It seems an attractive idea that a food that is commonly consumed for pleasure may have additional benefits to health. The beneficial effects of cocoa have been principally related to cardiovascular disease [1]; however, other data have suggested potential links to cancer [2], certain neurodegenerative disorders [3], and diabetes [4]. Cocoa polyphenols, most notably catechin and epicatechin, and their oligomers and polymers, known as the procyanidins, have strong antioxidant properties and all exert an action on oxidative stress in vitro; however, this does not always mean that this translates into positive effects in vivo. Dietary cocoa intervention studies with humans and animals have shown cardioprotective effects [5] as well as other potential health effects [6]. However, it is possible that the greatest potential for beneficial effects are seen when the subjects are subject to increased oxidative stress or a significant challenge/insult to redox homeostasis [6]. Factors such as their bioavailability, the type of cocoa and cocoa product, antioxidant status, and the type of participants (healthy, clinical) should be considered, as these might affect the efficacy of cocoa in human/clinical studies.

2 ANTIOXIDANT PROPERTIES

Flavanols (catechins), a type of flavonoid, are generally considered to be the main polyphenols of interest in cocoa and chocolate [6]. Cocoa is an abundant dietary source of such polyphenols (in particular catechin and epicatechin and their isomers); with cocoa and/or chocolate products ranking highly on tables of food antioxidant capacity [7–9] they may make a significant contribution to total dietary antioxidant intake. Factors affecting the quality and quantity of cocoa and cocoa-based products during production and manufacturing are of great importance and will consequently affect the polyphenol content and hence antioxidant capacity. Some processes such as fermentation and alkalinization lead to considerable losses of cocoa polyphenols and the content may be different depending on its origin [9]. While it is accepted that all polyphenols are amphipathic and exert potent antioxidant activity in vitro it must be acknowledged that this does not always translate to increased in vivo antioxidant defenses after oral ingestion [6]. In vitro, the antioxidant

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properties are attributable to the structural composition of these compounds, allowing them to sequester electrons and free radicals and also chelate free metal ions, which act as prooxidant catalysts [10,11].

A detailed discussion of the biochemistry and structural properties of polyphenols is beyond the scope of this particular chapter, but is described elsewhere in this book. Briefly, in food flavonoids exist primarily as 3-*O*-glycosides and polymers and numerous types of higher structures exist. Indeed, the polymers comprise a substantial fraction of dietary flavonoid intake and the procyanidins are most relevant to the human diet; these compounds consist of (+)-catechin and (−)-epicatechin monomers [10]. However, in chocolate and cocoa products/foods, (−)-epicatechin seems to be the most bioavailable flavonoid with the greatest influence on antioxidant defenses in vivo [12]. However, whether (or to what extent) the in vitro antioxidant capacity translates directly to in vivo antioxidant capacity postconsumption is dependent on many factors, such as the absorption and bioavailability (see the following). Further, the biological effects in vivo may also be influenced by "indirect effects" induced by the up- or downregulation of other components of redox balance or factors that may affect it [10]. This can be further confounded by other ingredients if consumed from foodstuffs such as chocolate (e.g., fat or carbohydrate may exert postprandial oxidative insult). Hence, there are many factors that may affect the overall antioxidant effects in vivo after consumption of such compounds from natural nutritional sources.

3 BIOAVAILABILITY

The biological activity of flavanols largely depends on the bioavailability of the compound. Factors such as absorption, intermediate metabolites, their tissue and cellular distribution after absorption and their bioactive forms should be considered [13]. Holt et al. [14] showed the presence of a dimer in the plasma 30 min postconsumption of flavanol-rich cocoa and after acute consumption, with maximal concentrations occurring at 2-h postconsumption, with their elimination normally completed after 6 h from ingestion.

There is a wide intersubject variability in the absorption rates of polyphenols and this will also depend on the molecular size of the flavanol. Typically, the smaller the compound, the higher the concentration that appears in the blood. The monomers catechin and epicatechin appear in the micromolar range. However, some monomers are absorbed more easily than others. Absorption of (−)-epicatechin in humans is relatively efficient, whereby peak catechin levels appear <10% of epicatechin concentration when delivered in a 1:1 ratio [14]. It is suggested that this flavonoid has the biggest association with health [6]. Dimers B2 and B5 have been detected in the nanomolar range in human plasma [14], whereas, larger units are less efficiently absorbed, although may have a function locally in the gut to neutralize oxidants and carcinogenic compounds or be cleaved by colonic bacteria before absorption of the resulting metabolites [6]. As a result, their bioactivity might be affective in vitro but not in vivo if they are not absorbed properly.

The food matrix in which the cocoa polyphenols are delivered appears to be an important factor in their bioavailability. For example, semisweet chocolate baking bits, cocoa powder, dark chocolate, tablets, drinks, milk chocolate and even a muffin have been used in previous polyphenols studies, which might affect the release of the polyphenols from the food, making them more or less available for absorption [6]. It has been suggested that their absorption may be decreased owing to an interaction with milk proteins, present in most cocoa-derived products [15]. However, other studies have shown no effect of milk on epicatechin antioxidant capacity or epicatechin bioavailability in healthy human subjects [16]. Furthermore, lipid and protein rich meals do not appear to affect their bioavailability; however, uptake might be enhanced if it is rich in carbohydrates, although the mechanism remains uncertain [17,18].

Factors such as their metabolic conversion in intestinal cell, liver, and other tissues, binding to proteins and accumulation in cells, and urinary elimination rate will all affect their efficacy in vivo; as a result, careful distinction between in vitro and in vivo effect of flavanols is essential [19].

4 RESEARCH ON COCOA AND OXIDATIVE STRESS

4.1 Epidemiological Studies

Epidemiological evidence has suggested the effectiveness of chocolate, or cocoa, on the prevention of cardiovascular disease and management of cardiovascular health. The Kuna Indian population of Panama has a very low incidence of hypertension and cardiovascular disease, and these natives appear to consume large amounts of cocoa in their diet. This appears to not be genetic, since migrant Kuna in urban areas report higher blood pressure [20]. In a prospective Iowa Women's Health Study [21] conducted on 34,489 postmenopausal women free of cardiovascular disease who were followed for up to 16 years, regular dietary intake of flavanols was inversely correlated with cardiovascular risk mortality. Furthermore, in the Zutphen Elderly Study [22] in 470 elderly men free from chronic diseases at baseline, cocoa intake estimated from the consumption of cocoa-containing

foods was inversely related to blood pressure and 15-year cardiovascular and all-cause mortality.

The large number of human studies of cocoa and chocolate have predominately reported beneficial effects on the vascular system; however, there is increasing evidence to suggest possible links with other diseases such as neurodegenerative disorders and cancer. Foods containing polyphenols have been associated with a lower incidence of Alzheimer's disease and dementia [3,23,24] and a potential link with diabetes [20]. Despite a link of cocoa and chocolate on risk factors for cancer a limited number of observational epidemiologic studies offer weak support for a reduction in mortality and little data relating to cancer [2].

Although previous studies suggest a trend for the benefits of flavonoids on health, the biochemical mechanisms underlying these effects remain unclear. Following a number of in vitro studies, it has been speculated that the mechanisms underlying the biological effects of flavanols may relate to antioxidants [6]. Other antioxidant mechanisms that confer protection are their ability to modulate certain cell signaling pathways and gene expression, and their ability to influence cell membrane properties and receptor function [13].

4.2 Cocoa/Dark Chocolate on Health Outcomes

As noted previously, the antioxidant properties of cocoa or cocoa-derived products in flavonoids may help protect against diseases in which oxidative stress is implicated as a casual or contributing factor. There is a currently a large body of evidence investigating the health benefits of cocoa and dark chocolate on cardiovascular disease. The measured outcomes have included endothelial dysfunction, lipoprotein oxidation, platelet function and inflammation. More recently a growing body of research has focused on factors associated with cognitive function, cancer, and diabetes.

4.2.1 Endothelial Dysfunction and Hypertension

Endothelial dysfunction is implicated as an early event in the development of atherosclerosis. Increased oxidative stress and impaired nitric oxide (NO) bioavailability are the key features of vascular dysfunction [25]. Flow mediated dilation (FMD) of the brachial artery (a measurement of the ability of the artery to relax) has emerged as a suitable physiological readout of endothelial function, which correlates with occurrence of future cardiovascular events. Studies have shown that consumption of cocoa can improve FMD. Heiss et al. [26] reported an increase in FMD reaching maximal effect 2 h after ingestion of a high flavanol cocoa drink, which was correlated with significantly higher plasma concentrations in the circulating pool of NO, RXMO. These effects are apparent in a dose-dependent manner with the dose to achieve half-maximal FMD response as 616 mg of flavanols [27,28]. However, no changes were observed in the plasma marker of oxidative stress, lipid peroxidation malondialdehyde (MDA), or antioxidant status/capacity (plasma ascorbate, urate, trolox equivalent antioxidant capacity, TEAC) in this study [27].

Short-term clinical studies have shown an effect of cocoa/dark chocolate on reducing blood pressure. In hypertensive patients after the consumption of 100 g per day (500 mg of polyphenols) for 15 days reductions in blood pressure were reported (4 mmHg) [29]. Similar findings were observed in a study in normotensive participants [4] as well as a reduction in oxidative stress markers following the consumption of 105 g of chocolate (168 mg flavanols) over 14 days [30]. It is suggested that cocoa flavanols may lower blood pressure by acting as an angiotensin 1 converting enzyme inhibitor, which also has antioxidant properties and can modulate NO production [31]. However, others have shown no effect of 2 weeks flavanol-rich (900 mg) cocoa drink on blood pressure [32]. It was concluded in a meta-analysis of five randomized controlled trials in hypertensive patients that, following cocoa administration, reductions in blood pressure (4.7 mmHg systolic and 2.8 mmHg diastolic) were observed similar to those obtained with antihypertensive drugs [33]. However, the blood pressure-reducing effects may be less pronounced in normotensive participants compared with prehypertensive patients [34]. It has been estimated that a 3-mmHg reduction in systolic blood pressure would reduce the relative risk of coronary artery disease mortality by 5%, and of all-cause mortality by 4% [35]. Therefore, introducing a moderate amount of flavanol-rich cocoa in the daily diet may be a promising strategy to improve outcomes. However, longer term studies are needed to examine if these effects are maintained with the awareness that increased consumption of cocoa-derived products/dark chocolate may be associated with weight gain, which could consequently affect blood pressure, and that commercial chocolate with its high sugar and fat content may be undesirable for those with increased cardiovascular risk. Furthermore, it should be noted that other compounds such as theobromine found in cocoa have been suggested to affect vasoactivity and be responsible for a reduction in blood pressure [36].

4.2.2 Lipoprotein Oxidation

The oxidation of low-density lipoprotein (LDL) is thought to play a major role in the initiation of atherosclerosis [37]. Oxidation of LDL lipids and apolipoproteins leads to a change in the conformation of the lipoprotein particles that facilitates their uptake by macrophages resident in the arterial wall, thereby promoting the atherosclerotic process. Evidence suggests that the antioxidant

effects of cocoa polyphenols may reduce LDL oxidation. In the animal model, Kurosawa et al. [38] observed increased antioxidant activity on LDL, reduced marker of lipid peroxidation thiobarbituric acid reactive substances (TBARS), and antiatherosclerotic activity in hypercholeterolemic rabbits following 6 months of cocoa powder treatment (10%). In healthy human volunteers, a reduction in LDL oxidative susceptibility was reported at levels of above 24 g of cocoa (1.74 g of polyphenols and 0.21 g of catechins per day) ingested over a 2-week period [39]. Furthermore, Baba et al. [40] demonstrated that consumption of ≥13 g/day of cocoa for 4 weeks decreased oxidized LDL and the effects were especially evident in subjects who had a baseline LDL concentration ≥3.23 mmol/L. These findings were coupled with a reduction in LDL cholesterol and improvements in high-density lipoprotein (HDL) cholesterol in plasma.

The effects of cocoa on LDL oxidation may differ between men and women and with age. Nanetti et al. [41] demonstrated that short-term dark chocolate consumption (3 weeks consumption of 50 g of flavonoid-rich dark chocolate daily) improved lipoprotein profile and reduced oxidative stress marker (TBARS) in lipoproteins in healthy humans, which occurred at a greater rate in women than in men. However, in middle-aged overweight/obese men and women cocoa extract (645.3 mg polyphenols) as part of ready-to-eat meals and within a hypocaloric diet over 4 weeks improved oxidative status (oxidized LDL cholesterol) with the effects most pronounced in males [42]. These findings may relate to the delicate balance between estrogen production and increased oxidative stress levels during the reproductive period in women [41], whereas men may become more oxidized than women with age [42], and as a result cocoa flavanols may become more effective in these cases of increased oxidative stress. The effects of cocoa on oxidized LDL have been attributed to the antioxidant activities in the blood after ingestion of cocoa scavenging the oxidants on the surface of the LDL, to the chelation of metal catalysts of free radical formation, or the changes in the LDL surface rendering it less susceptible to oxidation [5]. An improvement in LDL and HDL cholesterol and inhibition of oxidized LDL by cocoa polyphenols is suggested to lead to a decrease in the incidence of atherosclerotic disease [40].

4.2.3 Platelet Function

Platelets play a major role in coronary artery disease. They are found at sites of early atherosclerotic lesions. Platelets contribute to the early inflammatory events involved in the formation of plaques and also the thrombogenic process occurring after the rupture of advanced, unstable plaques [43]. A reduction in platelet reactivity and platelet-related primary hemostasis has been demonstrated in studies after a single dose of polyphenols. Rein et al. [44] administered a polyphenol-rich cocoa beverage (897 mg) or placebo to healthy volunteers in a single dose. Platelet activation, measured by expression of a platelet activation marker in response to weak agonists in vitro, and reactivity measured by platelet microparticle formation were both reduced 2 and 6 h after ingestion of the beverage. Furthermore, coagulation was inhibited by reducing the formation of hemostatically active platelet microparticles and increasing platelet-related hemostasis time. These effects appear to demonstrate a dose response [45]. Murphy et al. [46] showed a modest decrease in platelet activation and induced aggregation as well as increased epicatechin and ascorbic acid concentrations following consumption of 234 mg cocoa tablets over 28 days, but no change in oxidative stress markers (F_2-isoprostanes or TBARS). The intervention may increase the production of NO signaled by increased epicatechin, not only causing vasodilation but also inhibiting platelet aggregation, or through increased production of prostacyclin [47]. In heart transplant patients, associated with high levels of oxidative stress, dark chocolate (40 g, 70% cocoa, 624 mg polyphenol content) ingestion 2 h prior decreased platelet adhesion favoring vasodilation through the control of NO levels [25]. These effects were paralleled by a significant reduction of serum oxidative stress (8-isoprostane) and changes in serum epicatechin concentration.

4.2.4 Inflammation

Chronic inflammation has been identified in the development of chronic conditions such as obesity, degenerative diseases, and cancer. As a result, interventions that can modify the inflammatory cascade associated with disease may be regarded as potential targets in preventing such conditions. in vitro studies showed that cocoa flavanols may modulate cytokines and eicosanoids involved in the inflammatory response [48]. A significant correlation between inflammation and moderate consumption of cocoa products was found in a study comparing regular chocolate consumption with participants apparently free from any chronic disease that did not eat chocolate for at least one year [49]. A j-shaped curve was observed; consumers of up to 1 serving (20 g) of dark chocolate every 3 d had serum CRP concentrations that were lower than nonconsumers or higher consumers. Markers of oxidative stress were not measured in this study. In contrast, Mathur et al. [50] found no effect on markers of inflammation (whole blood cytokines, IL-1β, IL-6, TNF-α and P-selectin) following 651 mg/day of cocoa flavonoids ingested as dark chocolate (36.9 g) and cocoa powder drink (30.95 g) for 6 weeks in healthy volunteers; although a significant reduction in LDL oxidation susceptibility was observed. Urinary marker of oxidative stress (F_2-isoprostanes) was also unaffected by the intervention. Epicatechin was not detected in the

participant's plasma in this study and the lack of effect was attributed to the short half-life of cocoa flavonoids.

The effects of cocoa on health may be more pronounced in participants characterized by risk factors and already high levels of inflammatory and oxidative stress. In an animal model of chronic inflammation such as adjuvant arthritis, a 5% cocoa diet over 6 weeks reduced ROS production and normalized superoxide dismutase and catalase activities in female rats [51]. However, this was not enough to attenuate joint swelling as a clinical measure. Similarly, Ramos-Romero et al. [52] showed beneficial effects of a cocoa diet against collagen-induced arthritis, which is mediated by anti-collagen-autoantibody response, the inflammatory response of macrophages, and Th lymphocyte proportion, but these effects were not enough to reduce the hind-paw swelling in arthritic animals during the study period. It was reported that the study period lengths (4–6 weeks) might not have been sufficient to observe a clinically beneficial effect of the cocoa on the animal model of arthritis in the studies [52].

4.2.5 Neuroprotective Effects

Reactive oxygen species (ROS) have been implicated in the development of neurodegenerative disease and since cocoa polyphenols act as antioxidants it is suggested that they might have a neuroprotective effect. In one in vitro study [3] it was shown that cells incubated with cocoa extract reduced ROS production in a dose-dependent manner and modulated MAPK activation. It was suggested that this may constitute a source of neuroprotectants with particular relevance to neurodegenerative diseases. In young healthy volunteers, Scholey et al. [53] showed acute cognitive improvements in certain performances of a Cognitive Demand Battery indicating highly effortful cognitive processing following 520 mg and 994 mg of cocoa flavanol drinks. In healthy middle-aged volunteers following 30 days daily consumption of a chocolate drink containing 250 mg or 500 mg cocoa flavanols Camfield et al. [54] demonstrated improved brain activity measured by steady-state probe topography but there was no difference in behavioral measures of accuracy and reaction measured during a spatial memory working test. Plasma polyphenol levels or markers of oxidative stress were not analyzed in these two studies. Such improvements may be attributable to improved vascular function and/or antioxidant processes within the brain associated with cocoa consumption.

Aging is usually accompanied by deficits in several domains of cognition. Regular cocoa flavanols consumption (8 weeks, 990 mg flavanols) reduced some measures of age-related cognitive dysfunction in elderly subjects with [55] and without [56] evidence of cognitive dysfunction, possibly mediated in part through improvement in insulin sensitivity. However, the contribution of a marker of oxidative stress assessed through lipid peroxidation factors (Plasma total 8-iso-prostaglandin $F_{2\alpha}$) to the variation in cognitive performance with cocoa consumption was suggested to be quite modest and only account for 7% of improvement [55]. Collectively, these data demonstrate that cocoa might be associated with age-related cognitive preservation. Although research is limited, there is some evidence to show that cocoa polyphenols can reduce the risk of neurodegenerative diseases, particularly those associated with oxidative stress such as Alzheimer's and Parkinson's disease. In a human Alzheimer's disease in vitro model, a cocoa polyphenolic extract triggered neuroprotection by activating BDNF survival pathway, both on Aβ plaque treated cells and on Aβ oligomers treated cells, resulting in the counteraction of neurite dystrophy in a human Alzeimer's disease model. This supports the potential of cocoa powder as a preventive agent for neurogeneration disease characterized by oxidative stress [57]. However, further in vivo studies in clinical populations are warranted to ascertain if findings can support epidemiological studies [58].

4.2.6 Cancer

It is suggested that polyphenols possess anticarcinogenic activity via their actions against oxidative stress and chronic inflammation, risk factors for cancer [2]. Cocoa and its flavanols have shown potential antioxidant properties and a preventive effect against certain types of cancer. In vitro, in human lung carcinoma cells slate cocoa beans extract inhibited cell proliferation, arrested the cell cycle in different phases, and increased apoptosis in a time-dependent and dose-dependent manner [59]. In vivo, in the mouse model of azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced colitis-associated cancer a 5% cocoa diet over 8 weeks resulted in a decrease in oxidative stress marker MDA, an increase in enzymatic and nonenzymatic antioxidant activities, and a reduction in inflammatory mediators inducible nitric oxide synthase (iNOS) and cyclooxygenase, (COX)-2, risk factors for colorectal cancer [60]. Furthermore, an 8-week cocoa-enriched diet (12%) was reported to suppress the early stage of chemically induced colon carcinogenesis in rats via suppression of oxidative stress markers (carbonyl groups and MDA), increased enzymatic and nonenzymatic endogenous defenses producing antiproliferative effects, together with proapoptotic effects [61]. It has been shown that polyphenolic compounds can mitigate the expression levels of different genes in the human colon adenocarcinoma cell line [62]. These findings support the potential role of cocoa as a chemopreventive agent in cancer, in particular on risk factors; however, data in human intervention studies to show a correlation between cocoa intake and cancer prevention are lacking, although a few indicate that cocoa favorably affects

intermediary factors in cancer progression such as oxidative stress and inflammation [2]. Furthermore, caution is needed when attempting to extrapolate in vitro observations to in vivo animal tumor models and into humans. Clearly further work investigating relevant biomarkers for cancer risk with cocoa products in human intervention trials is required.

4.2.7 Diabetes

Oxidative stress plays a key role in the progression and development of type 2 diabetes mellitus and its complications. Cocoa flavonoids are thought to exert beneficial effects in type 2 diabetes mellitus due to their antioxidant effect influencing insulin resistance and secretion. In human hepatic cells, it was shown that (−)-epicatechin and a cocoa phenolic extract protects against high-glucose induced oxidative stress (increased in ROS generation and carbonyl content and decrease of glutathione, GSH concentration) [63]. In an in vivo study conducted in obese Zucker diabetic rats, a cocoa-rich diet (10% for 10 weeks) was shown to delay the loss of functional beta-cell mass and delay the progression of diabetes by enhancing the activity of endogenous antioxidant defenses, preventing markers of lipid and protein oxidation from the condition and leading to beta-cell apoptosis prevention [64]. In human clinical trials, Grassi et al. [4] reported a reduced insulin resistance in patients with hypertension and those with impaired glucose tolerance and also ameliorated insulin sensitivity and β-cell function [29] following 15 days of dark chocolate (100 g/day) consumption, which may be due to improved endothelial function and antioxidant status. However, other studies observed no effect of cocoa on insulin sensitivity after 2 weeks of 900 mg cocoa flavanols/day in participants with hypertension [32], and short term (5 days) intake of cocoa flavanols (up to 900 mg flavanol per day) did not appear to improve glucose metabolism but did affect selected marker of oxidative stress (total 8-isoprostane) in obese adults at risk for insulin resistance [65]. The contrasting findings from these human intervention studies may reflect differences in dose, duration, and health status within the studies. Although some of this evidence appears promising, data on cocoa/dark chocolate consumption and oxidative stress in diabetics is still limited at present, which might be affected by the fact that recommending high-sugar and high-calorie content foods and beverages such as cocoa or flavanoid-rich chocolate to these individuals may be controversial as a result of their diagnosis. Parsaeyan et al. [66] conducted a 6-week randomized clinical control human trial, and lipid peroxidation marker MDA and inflammation (IL-6, hs-CRP and TNF-α) were reduced in type 2 diabetic patients following cocoa consumption; however, no clinical measures of diabetes were recorded in the study.

A QUICK NOTE ON CHOCOLATE CONSUMPTION

Many of the studies that have found positive outcomes of flavanoid-rich dark chocolate on markers of health have supplemented with at least 100 g per day. This would result in an additional 550 kcal per day. It is therefore advised to reduce caloric intake elsewhere in the diet to avoid significant weight gain which, if not controlled, could lead to further health issues and burden for public health.

4.3 Cocoa/Dark Chocolate Effects Under Conditions of Increased Oxidative Stress

4.3.1 Stressors

As suggested by Cooper et al. [6] the vast majority of research on cocoa has been conducted with a population of apparently healthy and well-nourished subjects under "resting" conditions. There is a reasonable body of evidence showing an increase in markers of general antioxidant defense (such as plasma antioxidant status or capacity) with dark chocolate or cocoa supplementation (e.g., Refs. [50,67,68]). For example, Spadafranca et al. [69] showed improved antioxidant defense and reduced oxidative stress/DNA damage markers after dark chocolate consumption in apparently healthy subjects (2 weeks dark chocolate intake). Importantly, in this study diet was standardized for antioxidant intake during the supplementation periods and for 4 weeks before assessment. Similar observations have also been made by others [41,70]. It could be argued, however, that this is of little biological value under normal conditions when basal/natural antioxidant defenses are not challenged or compromised. Therefore, while some useful insights have been gained from such studies into the effects of cocoa or dark chocolate on antioxidant and oxidative stress markers, the effects are likely to be minimal in apparently healthy individuals. It is possible, however, that the greatest potential for beneficial effects is seen when the subjects are subject to increased oxidative stress or a significant challenge/insult to redox homeostasis [71]. This could be related to, for example, aging, disease and inflammatory conditions, lifestyle activities such as smoking or suboptimal nutrition [6], or experimentally induced oxidative insult such as those induced by certain drugs [51,72] or physiological stress [12].

A QUICK NOTE ON ANIMAL STUDIES

These can be very useful to demonstrate efficacy and give important mechanistic insight but should always be viewed with caution (and as preliminary) until repeated in human clinical trials. There are many examples in the literature of interventions that have shown promise in animal trials but have not translated to demonstrate the same level of benefit or efficacy in human trials.

4.3.2 Pollution and Smoking

Oxidative stress may be increased in polluted environments via induction of inflammatory processes. For example, particulate matter air pollutants, which are common in urbanized and industrialized areas, can cause systemic inflammatory responses and increased free radical production [73]. Gaseous pollutants are also a potential source of oxidants and can lead to oxidative stress. This may include systemic markers of oxidative stress (especially when combined with physical exertion, [74]) but in particular to oxidative stress and injury to the airway epithelium [75]. Oxidative injury of the respiratory epithelium is minimized by antioxidant defenses of the respiratory tract lining fluid (e.g., superoxide dismutase (SOD), catalase, metal binding proteins, glutathione, uric acid, cysteine, methionine, vitamin C and vitamin E, [75]). Air pollutants/oxidants can therefore consume and deplete these antioxidant defenses and lead to oxidative stress/injury to the epithelia [75]. A similar mechanism may also explain the oxidative stress that is induced by cigarette smoke, which also contains many oxidants and inducers of ROS and can promote or increase oxidative damage [76–78].

Loffredo et al. [79] demonstrated that acute dark chocolate ingestion (40 g of 85% cocoa bar, compared to 35% cocoa milk chocolate as placebo/control) was effective at reducing urinary isoprostanes (a marker of oxidative stress/lipid peroxidation) in smokers. The same effect was not seen in nonsmoking healthy controls, presumably because they are not subjected to the same level oxidative stress/insult. However, other studies have found no effect of sustained dark chocolate consumption (daily intake for 1 week) on other markers of oxidative stress and antioxidant capacity in smokers (plasma [MDA] and antioxidant capacity, [27]).

Villarreal-Calderon et al. [73] showed that regular dark chocolate consumption in mice was associated with reduced pollution-induced oxidative stress. In this study mice were housed in clean air (controls) or in a naturally polluted environment (Mexico City) from the age of 1 month until age 17 months (old age). Of the mice housed in the polluted environment, some received regular dark chocolate (oral consumption) throughout their life and some no treatment. Regular dark chocolate treatment was 2 g (60% cocoa, containing 9.5 mg polyphenols) 3 times per week (but not every day). Compared to the clean air controls, the polluted environment was associated with increased inflammatory and prooxidant gene expression profiles in cardiac tissue. However, this was significantly blunted and down-regulated in the dark chocolate fed mice. This was likely attributable to the significant up-regulation of key endogenous antioxidant enzymes (including glutathione peroxidase-3 and catalase) seen in the dark chocolate fed animals, suggesting that chronic dark chocolate administration is effective at ameliorating negative inflammatory and oxidative stress parameters induced by such an environmental stressor.

4.3.3 Disease/Health

Jalil et al. [80] used an animal obese-diabetic model to study the effects of cocoa supplementation on oxidative stress and antioxidant capacity (as well as measures related to glucose control). In this study rats were first fed a high fat diet for 12 weeks in order to develop obesity (compared to control animals fed a normal balanced diet). Diabetes was then induced by injection of streptozotocin (a chemical which attacks and damages pancreatic beta cells). The researchers state that they chose this high fat diet–induced obesity and then diabetes induction model to mimic human diabetes syndrome in which poor diet and obesity often precede, and contribute to, the development of type 2 diabetes. Cocoa extract, or normal diet control, was then administered for a 4-week period. Focusing only on the antioxidant and oxidative stress outcomes (for the purpose of this chapter), the obese diabetic rats displayed a reduced SOD activity, compared to control, but this reduction was blunted in those fed cocoa. There were minimal effects on catalase activity in the obese diabetic rats and cocoa actually decreased the activity of this antioxidant enzyme. Overall, there did seem to be a beneficial effect on antioxidant defense and protection with cocoa, as shown by a significant increase in the marker of oxidative stress (lipid peroxidation: plasma 8-isoprostane concentration) in the obese-diabetic animals that was significantly reduced in those supplemented with cocoa. Overall, this study suggested that cocoa can enhance key endogenous antioxidant defenses and protect against oxidative stress in an obese-diabetic (proinflammatory) model. These findings have been supported in a recent human study with obese individuals at risk of insulin resistance [65]. Stote et al. [65] recruited a group of obese adults considered at risk of developing insulin resistance (i.e., a group likely to have increased markers of chronic low-grade inflammation compared to healthy controls). Diet was controlled for 5 days and subjects were provided with different doses of cocoa (ranging from 30 to 900 mg flavanol content per day) in a randomized and crossover design. They did not observe any effects on parameters related to glucose control but there was a dose-dependent lowering of some inflammatory markers and the oxidative stress marker total 8-isoprostane concentration.

Arthritis is a chronic inflammatory disease in which oxidative stress has been implicated in the pathogenesis [51]. Ramos-Romero et al. [51] used an animal model in which rats were treated with an adjuvant (*Mycobacterium butyricum*) to induce this inflammatory condition. The rats were fed with a standard diet or experimental diets supplemented with 5% or 10% cocoa, for 2 weeks before and 6 weeks after arthritis induction. The arthritic rats exhibited

increased inflammatory markers (as expected) compared to the controls. Arthritis was also associated with enhanced ROS production by macrophages (believed to be a key source of oxidative stress in arthritis) and modulation of endogenous antioxidant enzymes (decreased catalase activity and increased SOD activity, reflecting the complex interplay between oxidative stressors and antioxidant defenses in such conditions). However, the rats on the 5% cocoa supplemented diet showed significantly lower macrophage ROS (oxidative stressor), with catalase and SOD activities more closely resembling the control animals. However, the rats on the 10% cocoa supplemented diet exhibited ROS production, catalase activity, and SOD activity profiles similar to the arthritic rats without cocoa. It is possible, therefore, that there is an optimal dose and excessively high intakes may even be pro- rather than antioxidant [51], although this requires further investigation in humans. However, the authors were able to show that cocoa can modulate the oxidative stress and antioxidant perturbations associated with adjuvant-induced arthritis. Similarly, Al-Malki & Moselhy [72] have demonstrated that feeding an isolated/purified extract of epicatechin significantly blunts the chemical/drug-induced (cyclosporine A) perturbations in oxidative stress and antioxidant status in kidney tissues, suggesting that this is the primary bioactive ingredient in cocoa and also a likely mechanism by which such effects are observed with cocoa.

4.3.4 Malnutrition and/or Low Dietary Antioxidant Intake

Suboptimal nutrition can contribute to oxidative stress by leading to an imbalance between the production of ROS and antioxidant defenses. Thus malnutrition can be used as an effective model of oxidative stress with which to study the effects of antioxidant compounds. Barragán Mejía et al. [81] used a rat model of malnutrition to study the effects of cocoa powder on brain and liver tissue antioxidant markers and brain oxidative stress/lipid peroxidation. Rats were fed a reduced energy (60% of normal) diet for 30 days, to simulate malnutrition, followed by a 30-day period with a normal diet or a diet supplemented with cocoa powder. Malnutrition led to decreased GSH in liver and brain tissue as well as increased markers of lipid peroxidation/oxidative injury (TBARS) in brain tissue. However, cocoa supplementation resulted in significantly higher concentrations of liver and brain GSH and significantly lower brain TBARS compared to control rats that were not subjected to malnutrition and also malnourished rats fed a normal-energy recovery diet (after the initial 30-day period). This study showed that malnutrition can decrease key antioxidant defenses in the brain (and other tissues) and lead to oxidative injury but this can be blunted with cocoa supplementation. The authors speculate that these results are attributable to the cocoa polyphenols but these were not measured directly in this study. Ramiro-Puig et al. [3] have shown that a cocoa extract and purified (−)-epicatechin are able to reduce oxidative stress and potentially protect neuronal cells against oxidative injury in an in vitro cell culture model suggesting that it may indeed be the (−)-epicatechin within cocoa that is responsible for such effects. While there are some suggestions, and epidemiological evidence, that a high intake of dietary polyphenols is associated with lower incidence of neurological conditions such as Alzheimer's disease there is currently no direct evidence of this. Furthermore, intervention studies with apparently healthy adults show limited benefits to cognitive and neurological-related parameters, so much further work is still required before we can fully determine the real benefit, and translational significance, of such animal and in vitro findings.

4.3.5 Heat Stress

Rozan et al. [82] used an anesthetization and heat stress model to induce an increase in ROS production and oxidative stress in laboratory rats. Compared to control the administration of a cocoa polyphenol extract reduced oxidative stress and protected the rats against cognitive impairment associated with the stressor.

4.3.6 Physical Stress/Exercise

We have successfully used exercise as a model of oxidative stress in human trials. The reason for using exercise is because it is an excellent, controllable, quantifiable, reproducible, and safe in vivo model with which to study the effects of possible nutritional interventions on oxidative stress and related parameters in humans.

A QUICK NOTE ON EXERCISE AS A MODEL OF OXIDATIVE STRESS

Before discussing such studies, and given the potential links discussed previously (e.g,. health factors mentioned elsewhere in chapter) between oxidative stress and various conditions/health, we feel it important to point out that the use of exercise as an acute prooxidant model should by no means be interpreted to suggest that exercise is negatively associated with health in this way. On the contrary, regular exercise (training) has many beneficial effects, including an enhancement of antioxidant defenses and reduced oxidative stress (probably as an adaptive response to the moderate, acute, and short-lived increases in free radicals and reactive oxygen species with acute exercise). Chronic exercise (training) is actually associated with enhanced antioxidant defense and reduced (susceptibility to) oxidative stress in both healthy [83,84] and diseased populations (e.g., reference [85]. Thus, it is important to make clear the fact that we simply use exercise as an in vivo oxidative stress model, often to test the efficacy of antioxidant compounds.

Why is exercise such as good model? Because it is very easy to control, quantify, and reproduce the imposed "stressor" accurately.

- Electron "leak" at the level of the mitochondrial electron transport chain, leukocyte oxidative burst, ischemia-reperfusion, xanthine oxidase activation, and autooxidation of catecholamines have been identified as major sources of free radicals and other ROS during exercise [86–89].

Fraga et al. [30] investigated the effects of a daily supplement high in cocoa/chocolate flavanols (versus a low flavanol control) on markers of antioxidant capacity and oxidative stress. In this study the subjects were fit, healthy young men who participated regularly in sport (football/soccer: training at least twice and playing a minimum of one 90-min match per week). It is possible that this population was selected for this study with the assumption that, as sportsmen involved in regular strenuous exercise, they would be exposed to more oxidative stress and present a better model to test the efficacy of the intervention against oxidative stress. Indeed, they observed an increase in resting plasma [Malondialdehyde] (a marker of oxidative stress: lipid peroxidation) over the 2-week study period in the control arm, whereas there was a significant decrease in this measure in the flavanol-rich arm. However, there were minimal changes in markers of antioxidant capacity or plasma levels of flavanols, making these results difficult to explain. The authors speculate that this can be explained by the timing of the blood samples (i.e., in a fasted state at least 8 h after the last supplement consumption), which is feasible as epicatechin and catechin are cleared quickly, and may show that the benefits (specifically for oxidative stress markers like lipid peroxidation) may persist after this time (although the kinetics of clearance of these markers, and/or incorporation into tissues, must also be considered). In support of this, a more recent study by González-Garrido et al. [90] examined the effects of cocoa supplementation on markers of oxidative tissue damage in professional soccer players in response to an acute exercise stressor. In this study they observed a reduction in markers of oxidative damage to protein and lipid molecules (carbonyl and thiol groups, and MDA). Together these studies demonstrate the variability in results that can be observed due to differences in study protocols such as timing of cocoa consumption relative to acute exercise or if samples are obtained at rest or after exercise when longer-term/more chronic supplementation periods are employed. It is important to also note that regular training actually improves endogenous antioxidant defenses, as discussed earlier [83,84], leaving less scope for the experimental food to be effective/beneficial in this group under these resting conditions, so training status and background dietary antioxidant intake are key potential confounding factors that are often overlooked. Such potential limitations were addressed in the study of Wiswedel et al. [71] who used exercise as an acute prooxidant stressor. They showed that consumption of 100 mL of high polyphenol content (187 mg cocoa flavanols) cocoa drink significantly blunted the exercise-induced increase in oxidative stress compared to a low polyphenol (14 mg flavanols) control cocoa drink. An important aspect of this study was the careful dietary control procedures implemented to ensure that subjects were in the same state before each arm of this double-blind crossover study. The high flavanol drink increased the plasma concentration of (−)-epicatechin and tended to reduce the exercise-induced increase in plasma F_2-isoprostane concentration in their exercising subjects (incremental cycling exercise test of approximately 30 min duration). However, other markers of oxidative stress and antioxidant capacity (plasma [malondialdehyde], [alpha-tocopherol], [ascorbate] and total antioxidant capacity) were unaffected by exercise or drink consumption. The authors suggest these findings show that the consumption of cocoa polyphenols can effectively protect against oxidative stress. However, it is difficult to explain the lack of effect on the other markers. One obvious possibility is that the prooxidant challenge imposed experimentally in this study (i.e., 30 min of incremental exercise with only 10 min at the highest intensity: 150 W, which is actually a relatively modest exercise intensity) presented a relatively mild oxidative challenge, leaving little scope for any beneficial effects to be observed for the other markers. We attempted to address this in two later studies with a more strenuous and prolonged exercise stress model, one with acute dark chocolate consumption [12] and another with a more prolonged period (2 weeks) of daily dark chocolate intake [91].

In the acute intake study [12] we demonstrated that the consumption of 100 g of dark chocolate (containing ~250 mg cocoa polyphenols) significantly increased plasma (−)-epicatechin concentration by ~8-fold by 2 h after chocolate consumption, which was when the exercise was commenced. Plasma (−)-epicatechin levels remained elevated a further 2.5 h later (after a prolonged exercise bout) and after a further 1 h of recovery (~4- to 5-fold), whereas there were no changes throughout in control (fasting) and PLA (macronutrient matched but cocoa-free bar) conditions. The dark chocolate consumption was associated with a moderate increase in antioxidant defense (plasma total antioxidant capacity) before the exercise bout (i.e., 2 h postprandial). The consumption of dark chocolate was able to modulate the exercise-induced increase in some markers of lipid peroxidation (F_2-isoprostanes) while the responses of some other markers were not altered. A more prolonged period of daily dark chocolate consumption (2 weeks, [91]) seemed

to have greater effects (an important consideration in this study was to ensure that the daily chocolate intake replaced existing snacks, etc., in the diet to maintain overall dietary energy intake and hence energy balance). In this study exercise was prolonged (90 min duration) with short high-intensity "bursts" spread periodically throughout, then followed by a high-intensity period of exercise to volitional exhaustion. This poses a significant oxidative stressor. In this study, daily dark chocolate consumption, for 2 weeks, did not result in changes in markers of antioxidant capacity/status (a similar finding to the study by Fraga et al. [30]). However, the oxidative stress markers were improved in the dark chocolate arm, especially after exercise. Resting plasma [F_2-isoprostanes] was similar in dark chocolate and control conditions. However, there was a significant postexercise increase in the control condition that was almost completely blunted in the dark chocolate condition. Exercise induced increases in plasma (oxidized LDL) in both the control and dark chocolate arms, but the overall levels were lower in the dark chocolate arm, with lower concentrations at rest (preexercise), postexercise, after exhaustive exercise (but not after 1 h of recovery, at which point there was no difference between control and dark chocolate conditions). More recently, Decroix et al [92] found no effect of a beverage containing 904 mg of cocoa flavanols consumed 100 min before two strenuous 30 min cycling time trials (the exercise bouts were separated by a 100 min rest period). The intervention successfully increased plasma epicatechin concentration and antioxidant capacity but their oxidative stress marker (plasma MDA) was similar between cocoa flavanols and placebo. However, a number of studies have found no effect in this particular marker (which has been criticized for lack of specificity), whereas previous studies had shown reductions for other markers (such as F_2-isoprostanes or oxidized LDL). Furthermore, there was a slightly higher power output observed in parts of the first exercise bout in the cocoa flavanols trial of the Decroix et al. [92] study (suggesting a greater oxidative insult), yet there was no difference in oxidative stress. This could actually suggest that the ability to protect against exercise-induced oxidative stress was enhanced. Sanguigni et al. [93] also studied the effects of acute intake of cocoa polyphenols. The polyphenols were delivered in a handmade ice cream product with cocoa powder added (although other sources of polyphenols: hazelnut and green tea extracts were also added). The total polyphenol content was approximately 180 mg (although the relative contributions from cocoa powder, hazelnuts and green tea extracts were not reported) compared to about 10 mg in the placebo (milk chocolate) ice cream. In this study resting markers of antioxidant status and oxidative stress were assessed at baseline (before ice cream consumption) and 2 h postconsumption. At this point an incremental exhaustive exercise stress test was undertaken. The polyphenol condition increased plasma antioxidant capacity and reduced their markers of "oxidative stress" (reactive oxygen metabolites and hydrogen peroxide) at rest (2 h postingestion, i.e., preexercise) but unfortunately these measurements were not repeated postexercise (although there was a reduction in heart rate × systolic blood pressure [product] response to the exercise stress test).

To summarize the exercise studies, increases in flavanols levels and associated antioxidant capacity are consistently reported. This is often associated with a reduction of oxidative stress markers but these findings are less consistent. The variation is likely explained by differences in oxidative insult (e.g., specific duration and intensity of exercise), type of participant, and the marker(s) of oxidative stress selected. It could be that some markers are not specific or sensitive enough and do not give a true representation of oxidative stress in this context, or that different markers reflect different pathways or tissues with different susceptibility to oxidative damage, all of which should be taken into consideration when interpreting the findings from these studies.

5 SUMMARY

There is a robust, and increasing, body of evidence to support the positive effects of cocoa polyphenol consumption on oxidative stress and markers of health. In particular, short duration human intervention studies show evidence of an influence on the cardiovascular system through a reduction in LDL oxidation, an enhancement of flow mediated dilation and a lowering of blood pressure, which are most likely mediated through redox-sensitive pathways. Furthermore, there is growing evidence to support cocoa polyphenol consumption effects on oxidative stress in disorders relating to cognitive function, diabetes, and cancer. It is probable that the antioxidant effects of cocoa polyphenols are more pronounced in those subjected to increased oxidative stress or a significant challenge/insult to redox homeostasis, most notably in diseased patients, those with antioxidant deficiencies, or individuals subjected to physical and/or other external stressors. However, the precise mechanism on how cocoa polyphenols exert their action needs further attention. Although short-term studies show some beneficial effects on oxidative stress and/or markers of health, future studies should target populations under significant oxidative stress (and include a broad range of oxidative stress markers to indicate damage in different tissues and molecules, e.g., lipids, proteins, DNA). Furthermore, more long-term, randomized clinical trials to support epidemiological studies on disease prevention are required.

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CHAPTER

17

An Overview of Dietary Polyphenols and Their Therapeutic Effects

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1 INTRODUCTION

Polyphenols are a diverse class of naturally occurring secondary metabolites found mainly in various fruits, vegetables, cereals, and beverages such as red wine [1, 2]. These secondary metabolites have been observed to play a significant role in the prevention of various classes of diseases such as cancer and cardiovascular diseases [3, 4]. Some of the other benefits of polyphenols include defense against ultraviolet radiation and pathogens [5].

Polyphenols in food acquire characteristic features such as bitterness, astringency, color, flavor, and odor. A significant characteristic that plays a substantial role in diseased conditions is oxidative stability against oxidative stress. There has been extensive research conducted on food polyphenols due to the numerous health benefits, and it strongly suggests that regular consumption of polyphenol-rich dietary supplements shows a reduction in the occurrence of cardiovascular diseases [6]. Polyphenols have been found to have links to various other disorders such as diabetes, osteoporosis, and neurodegenerative diseases such as Alzheimer's disease [7]. More than 8000 polyphenolic compounds have been identified from various plant species, some found in the fruits of plants such as strawberries and berries. Most of the plant phenols originate from a common intermediate such as phenylalanine or a distinct precursor like shikimic acid [8]. The most common forms are conjugated with structural alterations, such as hydroxylation, methoxylation, and glycosylation. Other structure associations include amines, carboxylic and organic acids, and lipids linked with other phenols [9].

2 STRUCTURAL CLASSIFICATION OF POLYPHENOLS

Polyphenols are divided into distinctive classes of compounds, on the arrangement and the number of phenol rings present and the addition of functional groups to the phenol rings. The primary classification of polyphenols is flavonoids, phenolic acids, stilbenes, and lignans (Fig. 17.1) [10].

2.1 Flavonoids

One of the most significant classifications of polyphenols is the lower weight compound flavonoids with subclasses anthocyanin and anthoxanthins. The subclass of anthoxanthins is further divided into flavonols, flavans, flavanols, flavones, and isoflavones with catechins (Fig. 17.2) [11]. Several plant-based dietary components contain flavonoids, and most of these compounds share an underlying structure, composed of 15 carbon atoms. The carbon atoms are arranged in a C6–C3–C6 configuration with two aromatic rings (A and B) covalently bound to three carbon atoms, thus leading to the formation of an oxygenated heterocycle ring C. The standard structure of the aromatic ring arises from the formation of ring A from a molecule of resorcinol and ring B from the shikimate pathway [12]. An estimate of more than 4000 varieties

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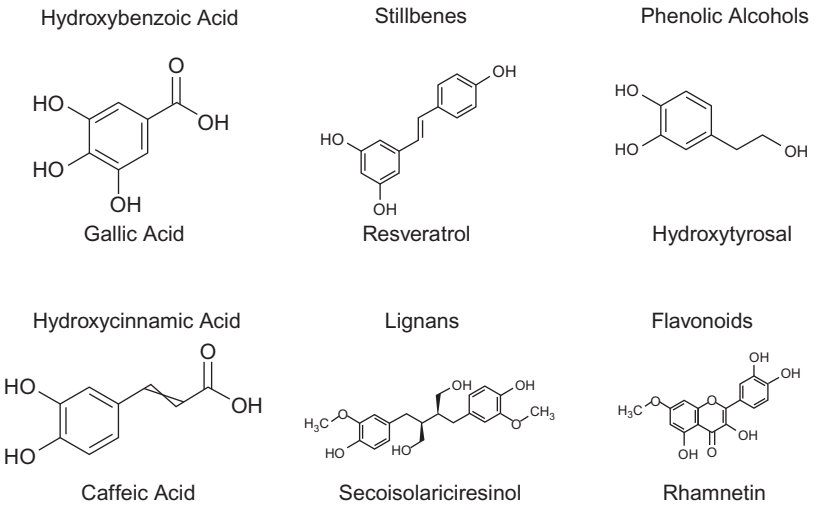

FIG. 17.1 The classification of polyphenols with structural examples.

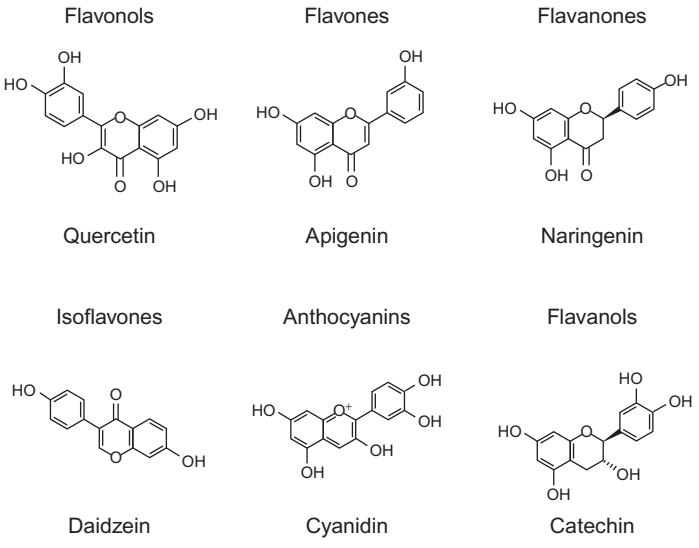

FIG. 17.2 The classification of flavonoids with structural examples.

of flavonoids has been identified, many of them responsible for the appealing color of flowers, fruits, and vegetables [13]. Structural variation in the substitution pattern of the heterocycle ring C results in the classification of flavonoids. These substitutions are the result of processes such as oxygenation, alkylation, acylation, glycosylation, and sulfonation [14].

Flavonoids exhibit extensive antioxidant properties due to their high redox potential. As a result, this enables the phenolic compounds to act as reducing agents. They play a significant role as free radical scavengers, hydrogen donors, and singlet oxygen quenchers and also possess chelating metal properties [15]. Furthermore, flavonoids are the most frequently obtained phytochemicals that possess curative properties against UV light. Moreover, these compounds have been found to be active against fungal parasites, pathogens, and oxidative cell injury [16]. The vast curative properties of flavonoids have led to great interest in understanding improved health care through a natural diet. Under regular consumption, flavonoids have been observed to reduce the occurrence of diseases such as cancer [17, 18]. Subcategories of flavonoids such as flavonols are commonly found in many fruits and vegetables. However, external factors such as growing conditions, storage, climate, and culinary methods influence their content [19]. Flavanones are characterized by the presence of a three-carbon saturated chain with an oxygen atom in C4 position. These compounds are glycosylated by a disaccharide in C7. High concentrations of flavanone are found in citrus

fruits; the compound eriodictyol is found in lemons, hesperetin in oranges, and naringenin is found in grapefruit [20].

The classes of isoflavones show structural similarities to estrogen due to similar positioning of the hydroxyl groups in C7 and C4 of the molecule. Furthermore, under some physiological effects, the compounds hold similar structures to beta-estradiol. They are also referred to as phytoestrogens. They are categorized as phytochemical compounds that are observed in many plants and plant-derived edibles in the native aglycon form or as acetyl- or malonyl-beta-glucosides. Major curative properties that can be attributed to these compounds are the prevention of diseases such as atherosclerosis or cancer [21].

Anthocyanins, a distinctive class of flavonoids that are water-soluble vacuolar pigments, appear as red, purple, or blue depending on pH. These molecules are synthesized via the phenylpropanoid pathway. These phenolic compounds are found in all plant tissues, leaves, stems, roots, flowers (forming the major component of pigment), and fruits [22]. The basic structure of anthocyanin consists of anthocyanidins. The anthocyanidins or aglycons are made up of an aromatic ring A bonded to a heterocyclic ring C, which is bonded to an oxygen molecule, and aromatic ring B via a carbon-carbon bond [23]. The glycoside form (bonded to a sugar moiety) of anthocyanidins are known as anthocyanins.

The most common forms of anthocyanidins in nature are the glycoside-derived nonmethylated anthocyanidins, such as pelargonidin-Pg, cyaniding-Cy, and delphinidin-Dp. These compounds are found in 50% of flowers, 69% of fruits, and 80% of pigmented leaves [24]. The six major anthocyanidins that are observed most frequently in plants include pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. The sugar moieties that are linked to anthocyanidins are monosaccharides (glucose, galactose, rhamnose, and arabinose). The formation of the di- and trisaccharides occurs through the combination of the four monosaccharides [25]. The sugar residues of anthocyanins are acylated with aromatic or aliphatic acids, thus leading to an integrated structure [26]. Anthocyanins, when isolated, are highly unstable and vulnerable to degradation [27]. Several factors determine the stability of these structures, and they are chemical structure, concentration, pH, storage temperature, light, oxygen, solvents, the presence of enzymes, flavonoids, metallic ions, and proteins [28].

Anthocyanins, like other phenolics, play a role as an antioxidant via donating a hydrogen atom to highly reactive radicals, thus preventing free radical formation and oxidative stress [29]. The major determinants of the compound's antioxidant properties are the number and arrangements of the hydroxyl groups and the degree of structural interaction. Furthermore, the presence of electron-donating and electron-withdrawing substituents in the ring structure contributes to its antioxidant potential. In addition to the antioxidant property, these phenols also exhibit antiinflammatory properties [30]. The use of synthetic food dyes has been prohibited in many countries due to its cellular toxicity and carcinogenicity. Recent studies have identified anthocyanin as an alternative to synthetic dyes. These phenolic compounds are considered as an alternative due to the easy extraction from fruits and vegetables and also due to the bright, attractive colors with high water solubility, thus leading to effortless integration in aqueous food components [31]. Furthermore, the administration of anthocyanin in daily dietary intake has shown antioxidant activity against neurodegenerative diseases [32, 33].

2.2 Phenolic Acids

Phenolic acids are divided into further classes of hydroxyl benzoic and hydroxyl cinnamic acids. Phenolic acids contribute to one-third of the dietary polyphenols and are present in all plants; however, they are found in abundance in acidic fruits. Major examples of phenolic compounds are caffeic acid, gallic acid, and ferulic acid [34].

2.3 Tannins

Tannins, which are relatively high molecular weight compounds, constitute the third primary classification of phenolics and are subclassified into hydrolyzable and condensed tannins [35]. Condensed tannins such as proanthocyanidins are polymeric flavonoids. The major bioprocessing pathways are defined; however, the mechanisms leading to condensation and polymerizations have not been elucidated. Major tannin compounds are conducted on flavan-3-ols (−)-epicatechin and (+)-catechin. Derivatives of gallic acid (3,4,5-trihydroxyl benzoic acid) result in t formation of hydrolyzable tannins. Esterification of gallic acid leads to the formation of core polyol. The galloyl groups are further oxidatively cross-linked or esterified to yield more complex hydrolyzable tannins [36].

The phlorotannins, the third subdivision of tannin class compounds, consist entirely of phloroglucinol, an organic compound that has been isolated from several genera of brown algae. These isolated compounds are not significant in the human diet; however, they are studied for their antispasmodic properties in a laboratory set-up [37].

The tannins follow antioxidant mechanisms such as potential metal ion chelation, accompanied by other properties such as protein precipitating agents and biological antioxidants in biological systems. Such a

variation is observed in these compounds due to large structural alterations of the compound. As a result, the development of models to study their effects in systems has been ambiguous.

2.4 Stilbenes

Another greater subclass of polyphenols is stilbenes, which consist of two phenyl bodies interlinked by a two-carbon methylene bridge. The presence of these compounds is low in the human diet, with the compound resveratrol (3,4′,5-trihydroxystilbene) being the principal class representative. These structures exist in both *cis* and *trans* isomeric forms and also frequently in glycosylated forms [38]. The production of stilbenes is a result of pathogenic or stress condition responses in plants (Bavaresco [39]). Over 70 plant species, including edibles such as grapes, berries and peanuts, have been identified to contain these compounds [40].

2.5 Lignans

Lignans are di-phenolic compounds that are products of oxidative dimerization of two phenyl-propane units. They occur in free form in nature, while their glycoside derivatives are only a minor form. These compounds and their synthetic compounds are currently examined extensively for their claimed chemotherapeutic properties and pharmacological activities [41]. Lignans, such as secoisolariciresinol, are classified as phytoestrogens. The most abundant dietary source of secoisolariciresinol is linseed, which contains up to 3.7 g/kg dry weight and low quantities of matairesinol [42].

3 DIETARY INTAKE AND CONTENT OF POLYPHENOLS

Several environmental and edaphic factors, such as the degree of ripeness, time of harvest, soil type, sun exposure, the presence of neighboring alleliopathic crops, and rainfall, contribute to the content of polyphenols. It has been observed that concentration of phenolic acids is reduced and the concentration of anthocyanin is increased during ripening [43]. Phenolic compounds play an integral role in stress response in plants, and they display curative properties by lignifications of damaged regions. They also hold antimicrobial properties leading to increased concentrations following an infection [44].

Various structural differences cause structural diversity in polyphenols; this includes the changes in positioning of the carbon in the carbon skeleton, differential oxidative states of heterocyclic and glycosylation in flavonoids, hydroxylation and acetylation in phenolic compounds, and the presence of stereoisomers in some compounds. As a result, estimating the amount of polyphenolic compounds in dietary supplements is difficult [45].

The method of administering dietary supplements also determines the content taken in. For example, consumption of apple with the presence of its peel provides 1 mg/g fresh weight of quercetin. However, consumption of the fruit without the peel results in the absence of flavonols [46]. Further, the presence of polyphenolic compounds in the aleurone cells present in the seed coat (outer layer) of wheat grain is lost during the process of refining flour [47]. The form of intake of food mainly determines the presence and form of the phenolic compounds. Compounds such as phloridzin found in apples are best recommended to be consumed as a fruit, as consumption in liquid form results in the solubilization of phenolic compounds [48]. Storage of phenolic-rich foods results in the oxidation of polyphenols, leading to changes in the color and quality of the food; some of these changes may be beneficial, such as black tea, but also can be detrimental, in the case of browning of fruits [49]. Several studies show that storage of polyphenol-rich food for the timespan of 6 months can lead to 70% reduction of the polyphenolic content [50]. In terms of cooking and boiling, fruits and vegetables such as tomatoes and onions lose about 75%–80% of their quercetin content [51].

Determining the content of polyphenols can be performed through chromatographic techniques and by using reducing reagents such as Folin-Ciocalteu [52]. Studies show that the values obtained through chromatographic methods may be comparatively lower than values obtained through Folin's assay; this is due to loss of compounds (examples: proanthocyanidin polymers and oxidized polyphenols). The total phenolic content may differ between fruits and vegetables with high and low content of polyphenols [53]. The presence of other reducing agents alters the chemical composition of polyphenols; as an example: the presence of ascorbic acid in citrus fruits reduces the Folin's reagent [54]. On an average, about 218 mg of total phenols was recorded in an average US diet through Folin assay [55].

Consumption of fruits provides greater exposure to phenolic compounds than consumption of vegetables. Compounds such as proanthocyanidins are found in high amounts in apple, plum, grape, and persimmon. Another class of flavanoids, anthocyanins, is found in cherry and other red fruits but is not commonly found in vegetables [56]. Furthermore, other sources of polyphenols include consumption of wheat-based products (consists of phenolic acids), and a small-amount consumption of chocolate contributes to the significant intake of polyphenols such as catechin and proanthocyanidin [57]. Beverages

such as tea, wine, and coffee contribute to the highest content of polyphenols. It has also been noticed that higher consumption of a type of food or beverages increases the content of the specific type of polyphenol. As an example, heavy coffee consumers have shown a higher amount of phenolic acids than flavonoids. Similarly, individuals consuming fruits and beverages such as tea or wine show increased levels of flavanols (catechins and proanthocyanidins), anthocyanins, and their oxidation products [58].

The dietary lifestyle of an individual or a specific ethnic group determines the levels and types of polyphenols present in the diet. The value related to the content of polyphenol is subjective, as the sources of these compounds are scattered. Several studies have been performed on the dietary intake of specific polyphenolic compounds in specific ethnic groups. Dutch populations have been identified to consume foods consisting of flavonols (largely quercetin) and flavones as 21 and 2 mg/day, respectively [59]. An average dietary intake of 30–40 mg/day of isoflavones was determined for the Japanese [60, 61]. However, Westerners showed reduced isoflavone content due to limited consumption of soy products [62]. Furthermore, the consumption of flavones, isoflavones, and flavonols was relatively low compared to phenolic acids. A more in-depth understanding is required elucidating the content of specific polyphenols in dietary intakes, as this will aid in understanding the mechanisms of polyphenols and disease prevention. Fig. 17.3 depicts the route of incorporation of polyphenols in particular organs [63].

4 BIOAVAILABILITY AND DISTRIBUTION OF POLYPHENOLS

Several epidemiological studies have indicated a strong correlation between polyphenol-rich dietary intake and disease prevention. The dietary intake of polyphenol-rich foods has shown curative properties in various diseases such as Parkinson's, stroke, various types of cancer, and coronary heart diseases [64, 65]. Thus this has led to understanding the biological activity and mechanism of polyphenols. Polyphenols in combination with other dietary components such as vitamin C, vitamin E. and carotenoids have shown reducing activity against oxidative stress.

The bioavailability of phenolic compounds determines the biological properties of polyphenols. It has been identified that the consumption of polyphenol-rich foods has increased the antioxidant activity and retention in plasma when absorbed through the gut barrier. Such an effect has been observed in polyphenolic edibles such as tea, red wine, blackcurrant, and apple juice [66, 67]. The bioavailability of polyphenols is established by measuring the concentration of the desired compounds in plasma and urine [68].

The structure of polyphenols governs the rate, magnitude of intestinal absorption, and the characteristics of the metabolites circulating in the plasma. The phenolic quantity for compounds such as quercetin and rutin (a glycoside of quercetin) is a relatively low value of (0.3%–1.4%) when compared with compounds such as catechins, isoflavones, flavanones, and anthocyanidins, which have

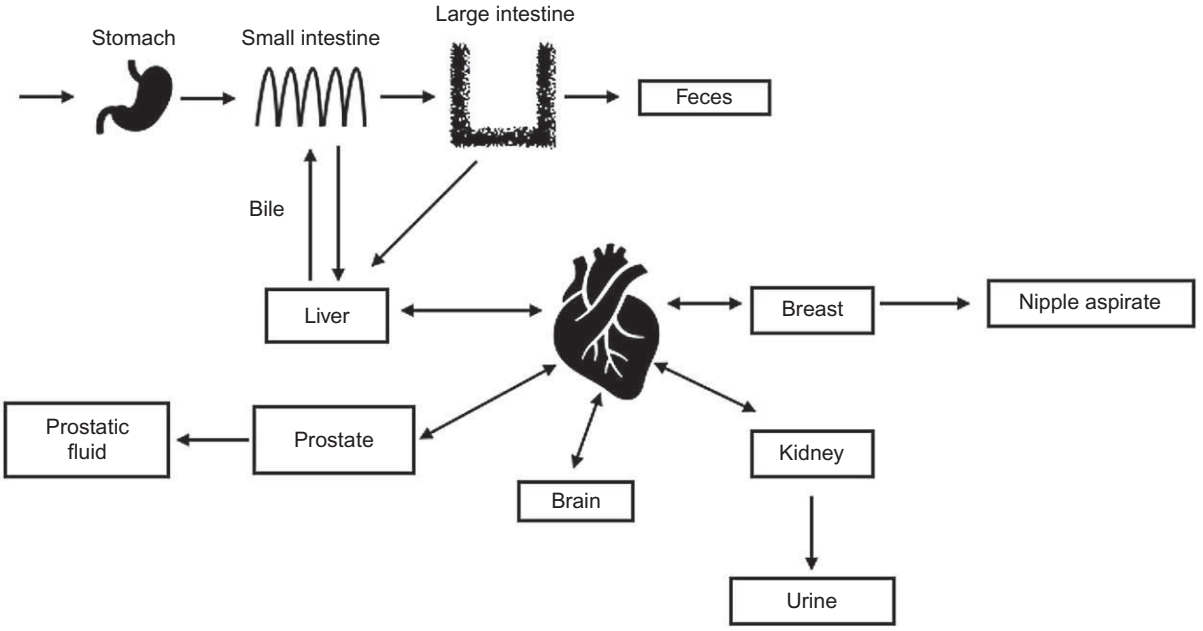

FIG. 17.3 The distribution of polyphenols throughout the body.

higher values of 3%–26% [69]. Variation in the concentration of phenolic compounds is noticed between individuals. A large sector of ingested polyphenols, absent in urine and constituting about 75%–99%, has various fates in the body; these compounds may not be absorbed through the gut barrier, may be absorbed and excreted in the bile, or may be metabolized by the colon flora and the surrounding tissues. Each class of compound is absorbed and metabolized at different loci in the body. Compounds such as quercetin glucosides found in onions were taken by ileostomized volunteers. This was thoroughly observed during the absorption in the small intestine [70]. Absorption of rutin (rhamnoglucoside of quercetin) constitutes one-half to one-third of the quercetin glucoside. Deglycosylation of these compounds is required by the intestinal microflora before absorption through the colon barrier [71].

With nominal quantities of polyphenols ingested with the diet, bioavailability studies have revealed that the concentrations of flavonoids in human plasma rarely exceed 1 mM. Maximum concentrations are most often reached 1–2 h after ingestion [72]. However, this is an exception for other polyphenols, as these metabolites are absorbed following the partial degradation by the colon microflora. For secondary metabolites such as rutin, the maximum concentration of quercetin in the plasma is achieved 9 h following ingestion [73].

Most flavonoids, after absorption by the small intestine, did not show rapid decrease in the plasma concentration (elimination half-life period of 1–2 h). The rapid degradation is facilitated by the conjugate formation of the aglycone to sulfate and glucuronide groups. The elimination half-life period for quercetin is much higher with a time frame of 24 h. Gradual elimination of quercetin occurs due to its high affinity towards plasma albumin [74].

Theoretically, polyphenols are designated as sodium-dependent glucose transporters of the small intestine. This statement holds true for polyphenols such as phlorizin (which is a well-known glucose transport inhibitor). However, in the case of C-glucosides such as puerarin, the activity of b-glucosidase in the intestinal lumen and the LPH in enterocyte initiates the hydrolysis of polyphenol-*O*-glycosides. In the absence of polyphenolic transporters, their uptake is dependent on their hydrophobic and hydrophilic property; this is essential due to the use of passive channels as the method of diffusion through the intestinal cell membrane [75].

The transport of hydrophobic polyphenols is easily feasible; however, due to lower aqueous solubility, the arrival to the intestinal wall is limited. Following the entry into the intestinal cell, polyphenols undergo metabolisms to form β-D-glucuronide and sulfonate esters [76]. As a result, very minimal amounts of aglycone diffusion occurs through the intestinal cell, which is to be excreted on the basolateral side into the bloodstream. Oral administration of polyphenols has shown significant a sector of conversion to secondary metabolites; this is due to the extensive metabolic activity of the intestinal cells [77].

Other routes of administration, such as parenterally, intramuscularly or intravenously, work antagonistically to oral administration. Studies have shown that these routes of administration have led to presence of substantial amounts of unconjugated polyphenols in the blood. Hence the mode of administration determines the biological activity of bioactive compounds. It is mainly useful in polyphenolic compounds due to their distinctive chemical composition. An example is the administration of estrogen orally and intravenously administered, where 17b-Estradiol is chemically converted to 17a-ethinyl or 3-methyl ether forms to be used in pharmacological setups; this is due to the fact that oral administration of 17b-estradiol is found to be 500 times less estrogenic in activity than administration through intravenous methods [78].

Similarly, the bioactivity of polyphenols is determined by route of administration. C-linked glycosidic polyphenols are equipped to cross over the intestinal barrier. Puerarin, a significant class of C-linked glycoside, is an 8-C-glucoside of daidzein, which is identified in extensive amounts in kudzu root of a vine (*Pueraria lobata*) found in Southeast Asia [79].

The C-glycosides are characterized as substrates for sodium-dependent glucose transporter as a result; they are not involved in hydrolyses through luminal or bacterial routes. The entrances of these substrates are rapid and are present in an unmetabolized form. A rise in the blood concentration is observed within 60 min following an oral administration of these substrates. Puerarin is found in large amounts in the urine and comparatively lesser amounts in bile as b-D-glucuronide. This compound is often observed to be carried by cells consisting of glucose transporters. Thus, trace amounts of this compound can be detected in the eye and brain [79]. This further implies that some phenolic compounds hold the ability to cross over the blood-brain barrier; such physiological activity can improve the efficacy of the therapeutic compounds.

Therefore, understanding the structural factors that influence absorption and metabolism is essential to determine the polyphenols that are best absorbed and that lead to the formation of known active metabolites.

5 EPIGENETIC MODIFICATIONS AND POLYPHENOL METABOLISM

Epigenetics, a term coined by developmental biologist Conrad H. Waddington in 1942, was defined as the reversible genetic changes in gene expression that occur due to the difference in the regulatory elements of gene

expression. DNA methylation, histone modification, and miRNA expression are the three major factors that determine the epigenome [80]. These components function as regulators of transcript stability, DNA folding and packaging, nucleosome positioning, and nuclear organization of genetic material. They play a major role as determinants of gene silencing and expression, the time of expression, and tissue-specific expression. The changes in the epigenome are mirrored in disease development [81]. Therefore, susceptibility to disease is a result of complex interactions between one's genetic inheritance and epigenetic marks imprinted on an individual's genome by endogenous and exogenous factors.

Epigenetic therapeutics is a promising field in treating various diseases; this is due to the ability to reverse epigenetic modifications (Fig. 17.4). Genes that are silenced through methylation can be demethylated. Histone complexes can be altered to be transcriptionally active by modifying acetylation and methylation mechanisms [82]. This is considered feasible via nutrient therapy, drugs, and dietary interventions. Polyphenols form a significant class of epigenetic dietary interventions, providing a perfect opportunity for designing optimal therapeutic strategies [83].

5.1 DNA Methylation

DNA methylation of cytosine structures at CpG dinucleotides is one of the most extensively studied epigenetic modifications in eukaryotic organisms. DNA methylation, in combination with histone modifications, forms the essential components of the epigenetic machinery. This regulates gene expression and chromatin architecture [84]. DNA methylation results in the structural changes of cytosine to 5-methylcytosine through the addition of methyl groups at the 50th position of the cytosine residue within CpG dinucleotides in mammalian cells [85]. Distribution of the CpG dinucleotides is not uniform throughout the genome and often results in significant quantities present in the promoter regions of genes and large repetitive sequences such as centromeric repeats, LINE and ALU retrotransposon elements. The presence of hypermethylated CpG islands within the gene promoter region can result in silencing of a gene (Fig. 17.5) [86].

DNA methylation may result in gene silencing through either preventing or promoting the activation of regulatory proteins to DNA, and this includes the inhibition of transcriptional activation by preventing transcription factors from accessing target-binding sites such as c-myc [87]. This provides the binding sites for methyl-binding/sequestering domain proteins, which can intensify gene repression by the interaction with histone modifying enzymes [88]. Thus, DNA methylation uses various mechanisms to silence genes. The direct association between DNA methylation and the phenotype of a cell can be hypothesized. Various DNA methyltransferases (DNMTs) catalyze the modifications of 5-methylcytosine complex. In fact, analogous to mutations and deletions, it is now believed that DNA methylation of genes is the most common mechanism for the transcriptional silencing of tumor suppressor genes in most human cancers [89].

Several dietary polyphenols have demonstrated the ability to be potential DNMT inhibitors. This includes their ability to reverse methylation-based silencing and restore the expression of various tumor suppressor genes. Table 17.1 depicts the phenolic compounds and their mechanisms on DNA methylation.

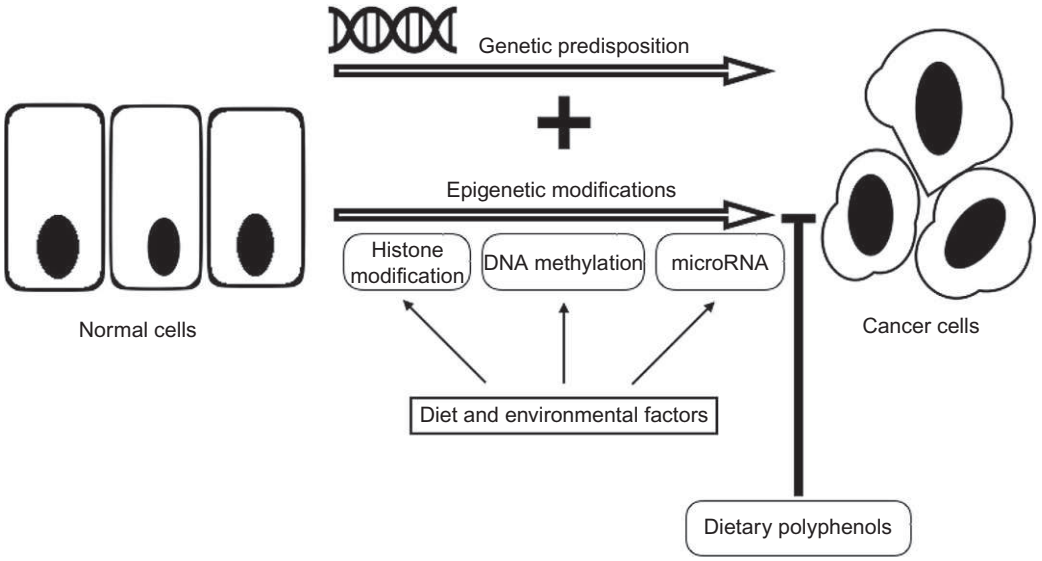

FIG. 17.4 Factors that contribute to epigenetic conditions leading to diseased condition. The use of polyphenols intervenes in these mechanisms, as promising therapeutics for diseases such as cancer.

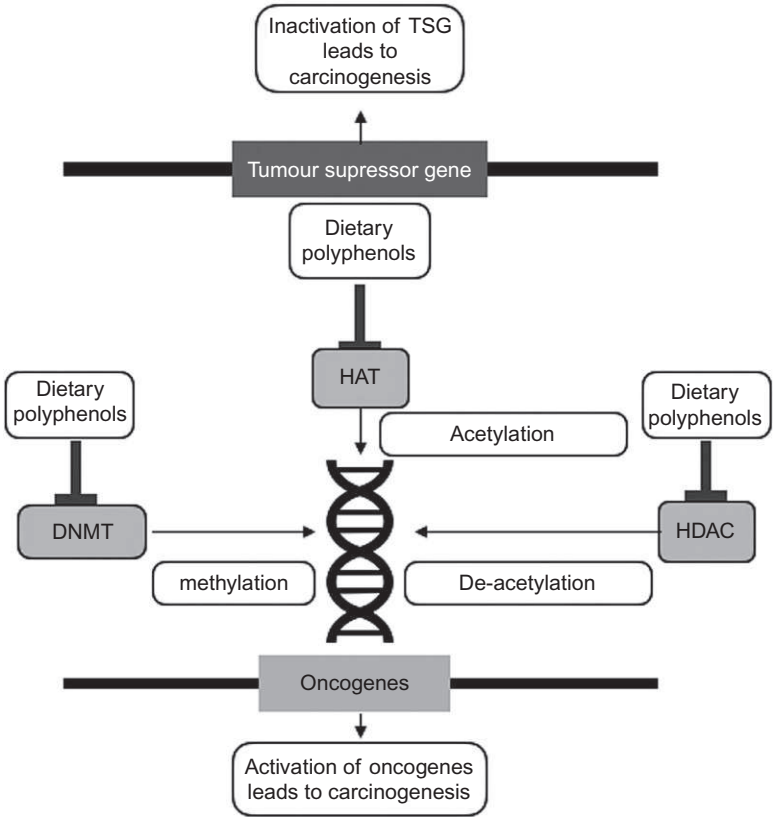

FIG. 17.5 The effect of DNA methylation, histone modification, and miRNA expression in oncogenesis. The administration of phenolic compounds provides greater therapeutic value than conventional chemotherapeutics.

5.2 Histone Modification

In addition, dietary polyphenols can regulate gene expression through changes in histones. In this regard, several polyphenols are known to possess potential HAT and HDAC inhibitory activities. Table 17.2 summarizes the current understanding of the effects of dietary polyphenols on histone modifications.

5.3 MicroRNA Expression

In recent times, microRNAs have been discovered to be major regulators of gene expression. Studies are ongoing, in order to understand miRNA gene regulation. Phytochemicals such as dietary polyphenols have been observed to control the gene expression of various oncogenic and tumor suppressive miRNAs. Table 17.3 lists some of the dietary polyphenols that play a role in controlling miRNA expression (Fig. 17.6).

Understanding epigenetic processes in diseased conditions such as cancer, neurodegenerative conditions, and aging provides immense potential as alleviation of chronic inflammatory disorders, chemoprevention, and healthy aging. This provides a platform for reversing the changes caused in the DNA as a therapeutic approach. Recent studies in epigenetics focus on the modulation of hyper- and hypomethylation. This mechanism is observed in several types of cancer, and dietary polyphenols as modulators of these changes may become a safer and healthier approach in comparison to synthetic chemotherapeutic compounds. Identification of novel epigenetic targets aids promotion of healthy aging and prevents preliminary cellular oxidative damage. Novel therapeutic approaches towards several age and lifestyle diseases can be incorporated by constructing individualized epigenetic dietary supplements. However, such treatment approaches can be performed only when all required parameters are met, such as identification of beneficial intraindividual health relationships, which are age/tissue/cell-type specific, and interindividual variation of epigenetic, genetic, and environment interactions.

6 NUTRITIONAL GENOMICS AND DIETARY POLYPHENOLS

Nutritional genomics enables optimization of human health and quality of life. This encompasses variation in the human genome and the role of nutrition in the expressed genes. The species of *Homo sapiens* displays differences in individual genomes. Significant sets of

TABLE 17.1 The Effect of Dietary Polyphenols on DNA Methylation

Dietary polyphenol	Origin of compound	Mechanism on DNA methylation	Gene targets	Reference
Caffeic acid	Catechol-containing coffee polyphenols	Inhibition of concentration-dependent manner DNA methylation catalyzed by DNMT1 Inhibition of methylation of the promoter region of RARb	RARb	[90]
Chlorogenic acid	Catechol-containing coffee polyphenols	Inhibition of concentration-dependent manner DNA methylation catalyzed by DNMT1 Inhibition of methylation of the promoter region of RARb	RARb	[91]
Curcumin	Turmeric	Inhibition of DNMT1 analog through blocking of a catalytic group in DNMT1		[92]
Epigallocatechin-3-gallate (EGCG)	Green tea	Formation of S-adenosyl-L-homocysteine (SAH) SAH—potent inhibitor of DNMT Formation of hydrogen bonds with different residues in the catalytic pocket of DNMT Inhibitor of human dihydrofolate reductase	Tumor suppressor genes: p16, RAR, MGMT, MLH1	[93]
Genistein	Phytoestrogens from soy bean	Dose-dependent inhibition of the DNMT Competitive inhibition of substrate poly(dI-dC) Noncompetitive inhibition of SAM	p16, RARb, MGMT	[94]
Isothiocyanates	Cruciferous vegetables	Demethylation and reexpression of GSTP1	GSTP1	[95]
Lycopene	Carotenoid pigment from tomatoes	Partial demethylation of promoter of GSTP1 tumor suppressor gene	GSTP1 tumor suppressor gene	[96]
Rosmarinic acid	Lamiaceae herbs	Potent inhibitor of DNMT1	RASSF1A, GSTP1, and HIN-1	[97]

variations that may or may not cause diseased conditions are known as single-nucleotide polymorphisms (SNPs). The SNPs may attribute to single-gene disorders due to deletions, additions, and distinct mutations in specific genes that lead to the absence or dysfunction of the proteins produced. Due to inheritance of one copy of one gene from the maternal end and one copy from the paternal end, the possibility of being diseased could be a resultant of two dysfunctional copies. On an average, for every 300 bases, a site-specific difference occurs throughout the genome.

Proteins are encoded by genes having about 150 to more than 7500 nucleotides. Accordingly, the presence of SNPs may be less in some genes, or greater in others. As a result, the number of SNP combinations is restricted for individuals with SNPs in several genes; such types of obstruction of SNPs are termed haplotypes. The loci and number of SNPs are determined by genetic inheritance, and individual haplotypes may be indented to those located within a particular region. In conditions such as the Y chromosome, males inherit only one copy. The concept of recombination cannot be of geographic origins, and thus an individual's patrilineal ancestor is established. SNPs are attributed to alter protein function leading to dysfunctional or loss of function. As a result, a specific ethnic group or geographical sector may possess high predominance of a particular SNP. In recent times, due to factors such as migration and mixed ethnic groups, newer SNPs can be identified in a population.

Under nutritional genomics, an example of a gene with SNP codes is that for the enzyme methyl enetetrahydro folate reductase (MTHFR). This gene comprises a polymorphism at residue 677 (C or T) leading to an alanine (677C) or valine (677T) in the protein complex. An individual may carry a CC, CT/TC, or TT genotype. The TT genotype has current prevalence in the northern regions of China (20%), the southern regions of Italy (26%), and Mexico (32%) [90]. It has a lower frequency in those of African ancestry [91]. MTHFR, the enzyme, is essential in remethylation of homocysteine to methionine. It converts 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate through catalytic mechanisms. The 677TT mutation in MTHFR reduces the enzymatic activity; as a result, individuals with this genotype acquire a mild form of hyperhomocysteinemia due to the reduced conversion of homocysteine to methionine in comparison to the standard genotype. Consumption of alcohol increases the total homocysteine concentration

TABLE 17.2 Dietary Polyphenols and Histone Modification

Dietary polyphenol	Origin of compound	Mechanism on histone modification	Histone targets	Reference
3,3′-Diindolylmethane	Broccoli and cauliflower	Inhibition of expression of HDAC1, HDAC2, and HDAC3		[98]
Anacardic acid	Cashew nuts	Potent inhibitor of p300, PCAF, and Tip60 HAT factors		[99]
Curcumin	Turmeric	HAT inhibitor Inhibition of histone deacetylase (HDAC) Downregulation of HDAC1, HDAC3, and HDAC8 proteins	H3/H4 and nonhistone proteins p53	[100]
Dihydrocoumarin	Melilotus officinalis (sweet clover)	Inhibitor of the HDAC		[101]
EGCG and green tea polyphenols	Green tea	Inhibition of acetylation of p65 and expression of NF-kB target genes Inhibition of HDAC activity	H3 and H4	[102]
Garcinol	Polyisoprenylated benzophenone derivative from garcinia fruit rinds	Inhibitor of different HATs: p300 and PCAF	H3 and H4	[103]
Genistein	Phytoestrogens from soy bean	Dose-dependent inhibition of the DNMT Competitive inhibition of substrate poly(dI-dC) Noncompetitive inhibition of SAM	p16, RARb, MGMT	[104]
Isothiocyanates	Cruciferous vegetables	Inhibition of HDAC activity	H3/H4 acetylation, p21, GSTP1	[105]
Quercetin	Citrus fruits and buckwheat	SIRT1 activator HAT inhibitor		[106]
Resveratrol	Grapes	SIRT1 activator		[107]
Sanguinarine	Bloodroot (*Sanguinaria canadensis*)	Inhibition of HAT activity Decreases H3/H4 acetylation	H3 and H4	[108]

TABLE 17.3 Dietary Polyphenols and miRNA Expression

Dietary polyphenol	Origin of compound	Mechanism on miRNA	Gene targets	Reference
3,3′-Diindolylmethane	Broccoli and cauliflower	Down regulation of miR-200 and let-7	miR-200, let-7	[109]
Curcumin	Turmeric	Upregulation of miR-22 Inhibition of miR-21	SP1 and ESR1	[110]
EGCG	Green tea	Upregulation of miR-16	miR-16	[111]
Genistein	Phytoestrogens from soy bean	Downregulation of miR-200 and let-7	miR-200, let-7	[112]

in plasma. As a result, the MTHFR 677TT genotype and alcohol mechanisms on homocysteine uptake can result in an increased intake of folate [92]. However, studies have shown that individuals with the 677TT genotype hold a low risk of colon cancer if a folate-supplemented diet is consumed [93]. This polymorphism exhibits the use of specific nutrient intake as an easy and efficient way of treating a disease.

Furthermore, another example of polymorphisms and nutritional intake is the genetic variants of genes encoding glutathione S-transferases (GSTs). This plays a major role in the metabolism of glucosinolates and in the

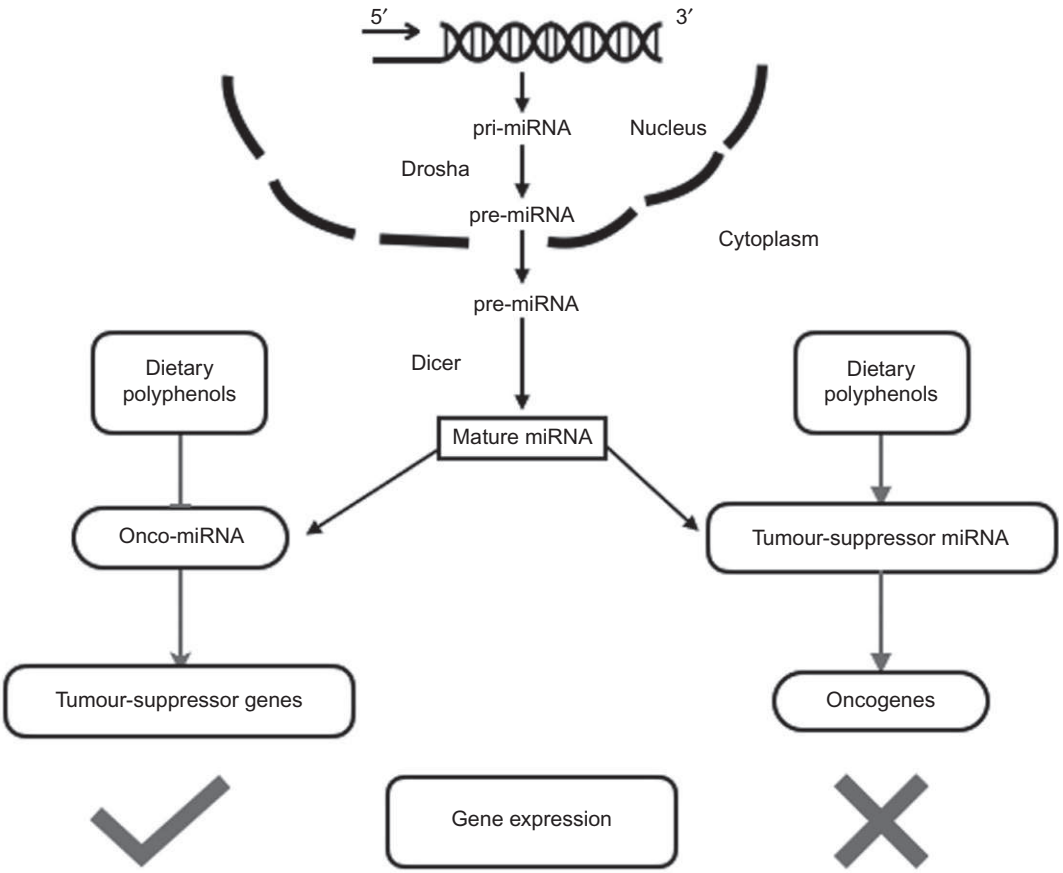

FIG. 17.6 The effect of polyphenols on miRNA expression. This is required, as this provides a deeper understanding of the role of miRNA expression in pathogenesis of disease.

bioavailability of isothiocyanates that are present in cruciferous vegetables such as broccoli. Studies show that a significant interindividual variation for LDL-cholesterol lowering response to plant sterol consumption exists, and it can be associated with a polymorphism in the ABCG8 gene. However, ambiguity remains in explaining large interindividual variations in therapeutic responses.

As per the Centers for Disease Control and Prevention [94] and the National Heart, Lung and Blood Institute, a polyphenol-rich diet contains 8 to 10 servings per day of fruits and vegetables. Isoflavones are major polyphenols found in soybeans and soy foods; proanthocyanidins are abundant in apples, grapes, and many berries; catechins (flavanols) are found in teas, mainly green tea; resveratrol is found in peanuts and grape skin [95].

Recent studies show evidence of epigenetic aberrations of essential ADME genes, which are related to drug absorption, distribution, metabolism, and excretion; furthermore, they are involved in the metabolism and disposition of phytochemicals such as polyphenols and also contribute to the interindividual variations under nutritional response. Personalized nutrition is becoming a newly recognized phenomena in nutrition research. This will result in some population subgroups receiving more advantage than others through the consumption of plant foods and their bioactive components. Furthermore, interindividual variations in epigenetic profiles, endocrine system, and microbiome communities, along with the identification of "susceptibility profiles" in response to plant bioactive consumption can lead to customized dietary advice and intake specifically for different ethnic and population subgroups.

Therefore, we have the genome, which is the component of genes and their mutations that are available to be expressed; the transcriptome, consisting of protein-encoding and non–protein-encoding regions of RNA; and the proteome, composed of primary polypeptides, their posttranslational modifications, and protein complexes. This is followed by the epigenome, comprising DNA methylations and methylations and acetylations of histones and miRNA expression, and the metabolome, encompassing small endogenous molecules that create energy and are a major component of synthesis of compounded bioactive intermediates, including nutrients such as lipids, complex carbohydrates, proteins, and nucleic acids.

Furthermore, the nutribiome, resulting from food-derived xenobiotics, and the xenobiome, consisting of compounds derived from sources outside of the body, can be protected and preserved through personalized nutritional intake [96].

7 NOVEL THERAPEUTICS USING POLYPHENOLS

Several studies using epidemiological studies, animal studies, and human clinical trials show that polyphenols have therapeutic effects on a range of pathologies, such as cardiovascular disease including thrombosis [97], atherosclerosis [98], and inflammation [99]; they also exhibit anticancer [100] and neuroprotective properties [101]. These therapeutic mechanisms are achieved through their significant antioxidant properties [102], inhibiting intracellular kinase activity, disrupting the cell-to-cell signaling by binding to cell surface receptors [103], and disrupting the integrity of cell plasma membranes [104].

Several epidemiological and clinical studies demonstrate the therapeutic potential and benefits of polyphenols. However, the exact importance of these compounds is yet to be elucidated. Beneficial effects of dietary supplementation of polyphenols can be achieved at supraphysiological concentrations, which have been found to show toxicity [105]. Understanding the mechanisms, such as inhibitory activity/potency of these compounds of the individual functional group, allows more potent and selective analogues to be developed. Extensive investigations on structure-activity studies have provided a construction of more structurally selective analogues; an example is the polyphenolic metabolite quercetin-3-*O*-amino acid esters, which were more useful for Src tyrosine kinase than EGF receptor kinase [106]. To increase the efficacy of the compounds, one primary strategy adopted uses the analogues of the parent compound in combination with dietary supplementation.

Other therapeutic approaches involve administration of unmodified polyphenols, such as resveratrol, as a viable treatment for chronic diseases. For chronic complications such as lung injury, researchers have demonstrated that the phyto-estrogen or phytoallexin analogues and resveratrol (*trans*-3,5,4′-trihydroxystilbene) were found effective for treatment of staphylococcal enterotoxin B (SEB)-induced lung injury. The antiinflammatory properties of resveratrol can be attributed to a potentially significant therapeutic agent against some degenerative conditions (e.g., coronary artery disease, brain injury). A study conducted in preclinical administration of a grape extract containing 8 mg total resveratrol to a group of coronary artery disease patients has demonstrated an increase in antiinflammatory factors [107]. This study has not reported any adverse side effects, despite high-level administration of resveratrol. This indicates the use of polyphenols as beneficial therapeutics due to their rapid clearance rates. The biological mechanisms are influenced mainly by the structural features of resveratrol. This allows necessary information to be gathered regarding the synthesis of analogues and selection of these analogues as molecular targets and for the individual cellular process. Further, the development of biomaterial devices and drug combinations of parent compounds and analogues may enhance the bioavailability of polyphenols, thus having higher efficacy.

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Conflicts of Interests

No potential conflicts of interest were disclosed.

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CHAPTER

18

The Polyphenolic Compound Resveratrol Attenuates Pain: Neurophysiological Mechanisms

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Abbreviations

CAM	complementary and alternative medicine
PGE2	prostaglandine E2
Cox	cyclooxygenase
NSAIDs	nonsteroidal antiinflammatory drugs
CNS	central nervous system
C1-C2	upper cervical dorsal horn
SpVc	trigeminal spinal nucleus caudalis
VPM	ventral posterior medial thalamic nucleus
WDR	wide-dynamic rage
NS	nociceptive specific
EPSP	excitatory postsynaptic potentials
PBN	parabrachial nucleus
SI	1st somatosensory cortex
SII	2nd somatosensory cortex
ACC	anterior cingulate cortex
INS	insular cortex
TRP	transient receptor potential
TRPA1	transient receptor potential ankyrin 1
DRG	dorsal root ganglion
MO	mustard oil
TTX-S	TTX-sensitive
TTX-R	TTX-resistant
CCI	chronic constriction nerve injury
NMDA	*N*-methyl-D-aspartate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
SNL	spinal nerve ligation
NOS	nitric oxide synthetase
PAG	periaqueductal gray
NRM	nucleus raphe magnus
5HT	5-hydroxytryptamine
TMJ	temporomandibular joint
PKA	protein kinase A
PKC	protein kinase C
PNS	peripheral nervous system
SGCs	satellite glial cells
JOR	jaw opening reflex

1 INTRODUCTION

Complementary and alternative medicine (CAM) refers to the array of therapies that extend beyond conventional Western medical treatments. Recent reports have described patients choosing CAM, including herbal medicines and acupuncture, for the treatment of chronic clinical pain [1–3], particularly when other medical treatments are ineffective [2,3]. In addition, diet and dietary supplementation can potentially affect conditions associated with pain [4,5].

Polyphenols comprise a large variety of molecules including some with a unique phenol ring (phenolic acids, phenolic alcohols) or typically more than one. According to both the number of phenol rings and the structural elements binding these rings to one another, polyphenols can be divided into the following groups: flavonoids, phenolic acids, phenolic alcohols, stilbenes, and lignans [6,7]. Flavonoids, a natural polyphenol present in various fruits and vegetables, are an important therapeutic and chemopreventive agent used in the treatment of various illnesses, while resveretrol, the best-known compound of the stilbene group of polyphenols, has been associated with altering the progression of cancer and other diseases. Indeed, resveratrol possesses a variety of biological activities, including cardiovascular protection, neuroprotection, anticancer, and antiinflammatory [8,9] via roles in several pathways including inflammation, oxidative stress, apotosis, mitochondrial dysfunction, and angiogenesis. Additionally, resveratrol exerts cardioprotection by inhibiting platelet aggregation [10–12]. With no known toxic side effects, resveratrol is a potential CAM agent for the treatment of pain.

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Recent studies revealed that resveratrol modulates neuronal excitability in both the peripheral and central nervous systems via voltage-dependent ion channels [13–15], as well as synaptic transmission via ligand-gated channels. Resveratrol could thus potentially modulate peripheral to central sensory transmission, including pain perception. Tissue damage and inflammation can induce the production of proinflammatory agents, such as prostaglandin E_2 (PGE_2), which sensitizes peripheral nociceptor receptors and acts on the central nervous system including somatosensory neurons in the spinal dorsal horn [16–18]. Resveratrol reportedly decreases the production of PGE_2 by inhibition of cyclooxygenase (Cox)-2 cascades and inhibits inflammation-induced hyperalgesia via suppression of Cox-1 and Cox-2 activities [19–22]. It is well known that the acidic antipyretic analgesic nonsteroidal antiinflammatory drugs (NSAIDs) are analgesic drugs [23]. Current options for the pharmacological treatment of pain include NSAIDs, as potent inhibitors of Cox-2 [23], and opioids; however, both are associated with several possible side effects including an increased risk of stomach ulcers and heart attacks. Thus resveratrol could provide an alternative therapeutic agent for treating and preventing pathological pain, such as inflammatory pain, without side effects.

In this review, we aimed to summarize current knowledge for the mechanism by which resveratrol modulates nociceptive neuronal activity. In addition, we discuss the potential contribution of resveratrol to the relief of nociceptive and pathological pain, as well as its development as a functional food and CAM agent.

2 CLASSIFICATION OF PAIN

Pain is generally divided into two groups: physiological pain (nociceptive pain) and pathological pain. Physiological pain is adaptive, transient, and has a protective role as a warning signal of potential tissue damage in response to a noxious stimulus [24]. Conversely, pathological clinical pain is usually maladaptive, persistent, and serves no useful physiological purpose [24,25]. Peripheral tissue injury and inflammation can alter the properties of somatic sensory pathways, resulting in behavioral hypersensitivity and increased responses to pain caused by both noxious stimuli (hyperalgesia) and normally innocuous stimuli (allodynia) [17,26]. Peripheral inflammation or injury also induces the release of neurotransmitters from central terminals [27–29] and increases the excitability of neurons in the dorsal horn of the spinal cord, including the spinal trigeminal nucleus caudalis. Consequently, sensitization of the central nervous system (CNS) could contribute to inflammation- or injury-induced hyperalgesia [30–32], suggesting that changes in the excitability of primary afferents (peripheral sensitization) and/or altered information processing in the spinal cord or higher centers (central sensitization) are related to enhanced pain sensation [17,33].

3 PAIN PATHWAY IN THE TRIGEMINAL SYSTEM

This review is focused on the potential contribution of resveratrol to the relief of nociceptive and pathological pain. Based on our recent data demonstrating resveratrol's effect on the excitability of trigeminal nociceptive neurons in a trigeminal pain rat model, we will first introduce the trigeminal pain pathway and the general properties of nociceptive neurons.

Fig. 18.1 depicts the trigeminal nociceptive sensory pathway, including the lateral and medical systems. The lateral pain system conveys sensory information related to discriminative aspects of pain such as localization and intensity, whereas the medial pain system conveys emotional aspects of pain. The trigeminal nervous system possesses unique structures and functions for the processing of orofacial nociception as well as nonnoxious sensations. The oral mucosal membrane, tongue, tooth pulp, periodontal tissue, and temporomandibular joint (TMJ) are innervated by small-diameter myelinated

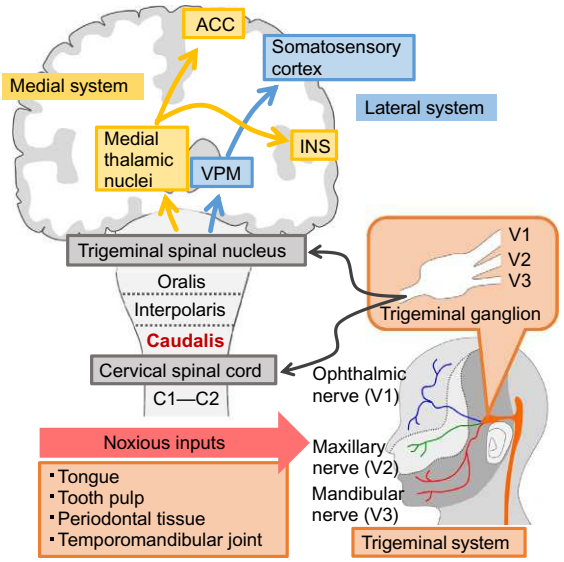

FIG. 18.1 Ascending pathway for trigeminal noxious information: The lateral pain system conveys sensory information for discriminating aspects of pain, while the medial pain system conveys emotional information with respect to pain. Orofacial noxious inputs are conveyed to SpVc and C1–C2 neurons, and then further relayed to somatosensory neurons (SI, SII) and the limbic cortices (the ACC and INS) via thalamic nuclei, the ventral posteromedial thalamic nucleus (VPM), and medial thalamic nuclei, respectively. *SI*, primary somatosensory cortex; *SII*, secondary somatosensory cortex; *ACC*, anterior cingulate cortex; *INS*, insular cortex.

Aδ-fibers and unmyelinated C-fibers that process orofacial nociception [34]. Noxious sensory information in the area innervated by the trigeminal nerves is relayed from trigeminal afferents to second-order neurons in the spinal trigeminal nucleus (SpVc) of the brainstem and the upper cervical (C1–C2) spinal cord [34,35]. The spinal trigeminal nucleus, an important relay station in the transmission of orofacial sensory information, is functionally subdivided into three nuclei: oralis, interpolaris, and caudalis [34]. In addition to the C1–C2 dorsal horn, the (SpVc) caudalis is a relay station for trigeminal nociceptive inputs induced by inflammation and tissue injury [34,35]. Projection neurons in the SpVc and C1–C2 then have axonal input into ventral posterior medial thalamic nucleus (VPM) and medial thalamic nuclei and parabrachial nucleus (PBN) [36–39]. Nociceptive neurons receiving noxious inputs from the orofacial region are somatotopically organized within the VPM, but not in the medial thalamic nuclei and PBN. Intraoral noxious information is conveyed to the somatosensory cortex via the medial portion of the VPM and to the face and head via the lateral portion of the VPM [39]. Nociceptive neurons in the VPM receiving inputs from the orofacial region send axons to the primary (SI) and secondary (SII) somatosensory cortical neurons, whereas inputs directed to the medial thalamic nuclei and PBN project to limbic cortices including the anterior cingulate cortex (ACC) and insula cortex (INS). INS neurons receive autonomic inputs (sympathetic and parasympathetic) and are involved in modulating autonomic responses, but little is known regarding the response properties of INS nociceptive neurons [40]. Although it is evident that these cortical areas are involved in pain, mechanistic details underlying the involvement of these higher brain regions in pain are still largely unknown.

The properties of somatic sensory pathways can be altered by chronic pathological conditions, such as tissue inflammation, leading to hyperalgesia and allodynia [17]. Information processing in the spinal trigeminal nucleus or higher centers is altered by changes in the excitability of primary afferent neurons, known as peripheral sensitization [33]. There are two types of nociceptive neurons in the SpVc and C1–C2, classified based on their sensitivity for mechanical stimulation applied to orofacial areas as follows: nociceptive-specific (NS) neurons (located in the superficial laminae I–II) and wide-dynamic-range (WDR) neurons (located in the deep laminae IV–V) [34]. NS neurons respond only to noxious stimulation (e.g., high-threshold mechanical stimulation) of receptive fields, suggesting that NS neurons send localization-encoding stimuli to higher centers [34]. WDR neurons respond to both noxious (via Aδ- and C-fibers) and non-noxious (via Aβ-fiber) stimulation and have large receptive fields [36,41]. Graded nociceptive stimulation applied to the most sensitive area of the receptive field induced increased firing frequency of SpVc WDR neurons in proportion to the stimulus intensity and many also responded to noxious heat stimulation, suggesting that WDR neurons send intensity-encoding stimulus to higher centers. Since the SpVc and C1–C2 WDR neurons can also have convergent inputs from tooth-pulp, facial skin, jaw, masseter muscle, and phrenic afferents [42], it is likely that WDR neurons might contribute to the phenomenon of referred pain, which is pain perceived at a location other than the site of painful stimulus.

4 MODULATORY MECHANISM OF RESVERATROL ON THE NOCICEPTIVE PAIN

4.1 Peripheral Mechanism

As shown in Fig. 18.2, nociceptive sensory signaling pathways comprise the following sequential steps: signal transduction from peripheral terminals that transduce external stimuli; generation of action potentials; propagation of action potentials along axons; and finally, transmission to central terminals, which form the presynaptic elements of the first synapses in the CNS sensory pathways [35,43]. When noxious mechanical stimulation is applied to the skin, mechanosensitive ion channels (transient receptor potential channel family) open and this depolarization further opens voltage-dependent Na^+ and K^+ channels, generating action potentials. The action potentials are then discharged through primary afferent fibers (Aδ- and C-fibers) to the central terminal in nociceptive neurons, located in the trigeminal SpVc, leading to presynaptic voltage-dependent calcium channel depolarization and the subsequent release of neurotransmitters (e.g., glutamate) into the synaptic cleft and binding to postsynaptic (glutamate) receptors, which activate excitatory post-synaptic potentials (EPSP). If the EPSP amplitudes exceed the action potential threshold, a barrage of action potentials is conducted to higher centers in the pain pathway and pain is perceived (Fig. 18.2).

With regard to the effect of resveratrol in the nervous system, previous reports implicate a role in modulating the excitability of peripheral nervous system (PNS) neurons via the activation of voltage-dependent and transient receptor potential (TRP) channels [13,14,44]. In vitro, mechanical stimuli induce currents via mechanosensitive channels such as TRP ankyrin 1 (TRPA1) to trigger mechanotransduction in trigeminal neurons innervating the inner walls of the anterior eye chamber [45]. In addition, TRPA1 can modulate mechanotransduction in primary sensory neurons [46], and resveratrol has been shown to be a potent inhibitor of TRPA1 in vitro and in vivo [47], suggesting that resveratrol attenuates the generator potential and inhibits action potential firing

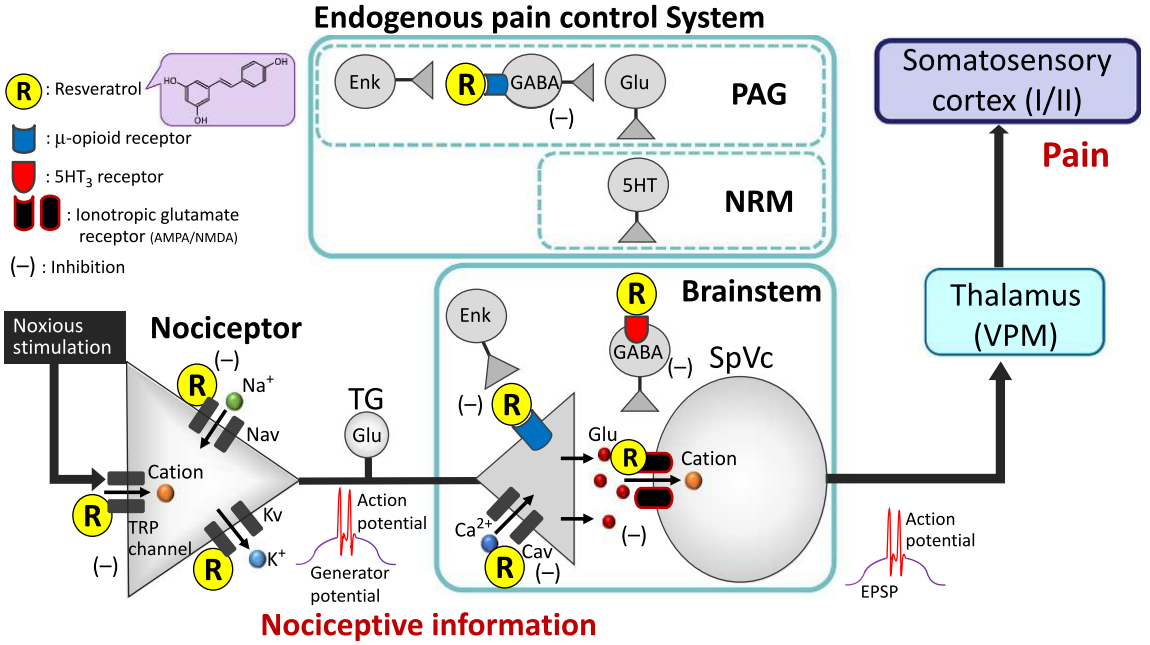

FIG. 18.2 Modulatory mechanism of nociceptive pain targeted by resveratrol: Schematic drawing of the possible mechanism underlying the mitigating effects of resveratrol on nociceptive pain. It is possible that resveratrol suppresses the excitability of peripheral terminals of the trigeminal nerve by modulating both the mechanical transduction and generation of action potentials and the glutaminergic excitatory synaptic transmission of SpVc neurons by inhibiting postsynaptic glutamate receptors and presynaptic Ca^{2+} channels, as follows. Firstly, resveratrol could inhibit nociceptive transmission via the endogenous opioid system, whereby resveratrol activates μ-opioid receptors in the presynaptic terminals of nociceptive trigeminal primary afferent neurons. These actions would inhibit the excitability of neuronal transmission in the SpVc by depressing presynaptic glutamate release. Secondly, secondary activation of μ-opioid receptors by resveratrol increases neuronal activity in the PAG via GABAergic disinhibition, which can then activate serotonergic neurons in the nucleus raphe magnus (endogenous opioid system). These actions inhibit synaptic transmission of SpV. Thirdly, resveratrol suppresses excitatory synaptic transmission of the SpVc via activation of 5-HT_3 receptor-mediated GABAergic inhibition. *TG*, trigeminal ganglion; *SpV*, trigeminal spinal nucleus; *PAG*, periaqueductal gray; *NRM*, nucleus raphe magnus; *Glu*, glutamate; *Enk*, enkepahalin; *GABA*, gamma-aminobutyric acid; *5HT*, 5-hydroxytryptamine; *R*, resveratrol; *Nav*, voltage-gated sodium channel; *Kv*, voltage-gated potassium channel; *Cav*, voltage-gated calcium channel; *WDR*, wide-dynamic-range neurons.

via the mechanical transduction process. Moreover, resveratrol modulates sodium and potassium currents in dorsal root ganglion (DRG) neurons associated with the generation of action potentials [13,44]. Indeed, a recent in vivo electrophysiological study demonstrated that local subcutaneous injection of resveratrol into the receptive field of SpVc WDR neurons could attenuate nonnoxious and noxious mechanical stimulation-induced excitability in an effect that was both dose dependent and reversible [48]. In addition, the mean magnitude of inhibition by resveratrol of SpVc neuron discharge frequency was comparable to that of the local anesthetic lidocaine (1%). Based on these results, it can be speculated that resveratrol inhibits the excitability of peripheral terminals of the trigeminal nerve by modulating both the noxious mechanical stimulation-induced generator potential and the initiation of action potential processes (Fig. 18.2).

4.2 Central Mechanisms

Resveratrol was recently shown to suppress the action potential firing of cerebral cortex neurons via the opening of large conductance Ca^{2+}-activated K^+ channels and inhibition of voltage-dependent Na^+ channels [49]. This aligns with a previous study demonstrating that in hippocampal slices resveratrol significantly suppresses glutamate-induced currents in postsynaptic CA1 pyramidal neurons without having any presynaptic effects [50]. These authors also reported that *N*-methyl-D-aspartate (NMDA) receptors were more sensitive to resveratrol than α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors [50]. Furthermore, resveratrol decreases action potential duration and L-type Ca^{2+} currents in ventricular myocytes [14]. Taken together, these findings suggest that resveratrol suppresses the glutamatergic excitatory synaptic transmission of SpVc by inhibiting post-synaptic glutamate receptors and presynaptic Ca^{2+} channels (Fig. 18.2).

We recently investigated whether acute intravenous administration of resveratrol to rats could attenuate the excitability of nociceptive WDR SpVc neuronal activity in vivo in response to mechanical stimulation [51]. In that study, the mean SpVc WDR neuronal firing rate in response to both nonnoxious and noxious mechanical stimuli was dose-dependently inhibited by resveratrol in a reversible manner, and the relative magnitude of inhibition of SpVc WDR neuronal discharge frequency

by resveratrol was significantly greater for noxious than nonnoxious stimuli. Takehana et al. [52] also reported that the mean firing frequency evoked by iontophoretic application of glutamate was mimicked by the application of noxious pinch mechanical stimulation, and that this evoked firing that was significantly inhibited by intravenous administration of resveratrol. The relative magnitude of this inhibition by resveratrol was similar to that for NMDA iontophoretic application. These results suggest that resveratrol suppresses glutamatergic neurotransmission of SpVc neurons responding to nociceptive mechanical stimulation via the NMDA receptor in vivo, highlighting its possible value as a CAM agent for the treatment of trigeminal nociceptive pain.

Importantly, descending pain modulation systems, including the endogenous pain control system, can also modulate the ascending noxious information related to pain, and systemically administered resveratrol has dose-dependent antinociceptive effects related to the endogenous pain system [53]. This effect occurs via an opioidergic mechanism, because pretreatment of rats with naloxone, an opioid antagonist, completely blocked the analgesic effect of resveratrol [53]. We also demonstrated that systemic administration of resveratrol could attenuate the rat nociceptive jaw-opening reflex (JOR) via the endogenous opioid system [54]. It is known that opiates acting via the μ-opioid receptor partially inhibit evoked inhibitory GABAergic pre- and postsynaptic potentials in the periaqueductal gray (PAG) [55], while blocking μ-opioid receptors increases neuronal activity in the PAG via GABAergic disinhibition, which can then activate serotonergic (5-hydroxytrypytamine [5-HT]) neurons in the nucleus raphe magnus (the PAG-nucleus raphe magnus-trigeminal pathway) [56]. In addition, resveratrol facilitates 5-HT_3 receptor-mediated ion currents [57]. Previous studies have reported that nociceptive stimulation-evoked SpVc/C1 neuronal activity is suppressed by conditioning peripheral nerve stimulation via 5-HT_3 receptor-mediated GABAergic inhibition [58]. Taken together, these observations suggest that resveratrol suppresses excitatory synaptic transmission of the SpVc via activation of 5-HT_3 receptor-mediated GABAergic inhibition and/or endogenous opioidergic mechanisms, and further studies are needed to clarify these possibilities (Fig. 18.2).

5 MODULATORY MECHANISM UNDERLYING THE EFFECT OF RESVERATROL ON PATHOLOGICAL PAIN

5.1 Inflammatory Pain

Peripheral tissue injury and inflammation of the innervating trigeminal nerve can alter the properties of trigeminal somatic sensory pathways, causing behavioral hypersensitivity and increased responses to pain caused by noxious stimuli (e.g., hyperalgesia) [17]. As shown in Fig. 18.3, following peripheral inflammation and/or nerve injury, inflammatory mediators such as PGE_2 bind to G protein-coupled proteinoid EP receptors and induce activation of protein kinase A and C (PKA and PKC, respectively) in nociceptive peripheral terminals, leading to phosphorylation of mechanosensitive Na^+ and K^+ channels and receptors. As a result, the activation threshold for transducer channels such as the TRP channel family is reduced and the membrane excitability of the peripheral terminals increases, resulting in a high frequency of action potentials being conducted to presynaptic central terminals of the SpVc. The end result is large amounts of glutamate being released into the synaptic cleft and binding to up-regulated postsynaptic glutamate receptors, augmenting EPSPs, and causing a barrage of action potentials to be conducted to higher pain pathway centers and create a state of heightened sensitivity termed peripheral sensitization.

5.1.1 *Acute Inflammation*

Resveratrol inhibits voltage-dependent Na^+ channels and acts as both a TRPA1 antagonist and Cox-2 inhibitor [22,45,46]. We recently investigated whether pretreatment with resveratrol attenuates acute inflammation-induced sensitization of nociceptive processing in rat SpVc and C1 dorsal horn neurons, via *c-fos* immunoreactivity [59]. Mustard oil (MO), a TRPA1 channel agonist, was injected into the whisker pads of rats to induce inflammation. Pretreatment with resveratrol significantly decreased the mean thickness of inflammation-induced edema in whisker pads compared to those of untreated, inflamed rats. Ipsilateral of both the superficial and deep laminae of SpVc and C1 dorsal horn, there were significantly more *c-fos*-immunoreactive SpVc/C1 neurons in inflamed rats compared to naïve rats, and resveratrol pretreatment significantly decreased that number relative to untreated, inflamed rats [59]. These results suggest that systemic administration of resveratrol attenuates acute inflammation-induced augmented nociceptive processing of trigeminal NS and WDR SpVc and C1 neurons. These findings support resveratrol as a potential therapeutic agent for use in CAM to attenuate or even prevent acute trigeminal inflammatory pain.

5.1.2 *Chronic Inflammation*

Based on resveratrol's inhibition of Cox-1 and Cox-2 activity [22], we recently tested the hypothesis that chronic administration of resveratrol would attenuate inflammation-induced hyperexcitability of trigeminal nociceptive neuronal activity associated with hyperalgesia in behavioral and electrophysiological experiments [60]. In that study, the threshold of escape from mechanical stimulation applied to the orofacial area in inflamed

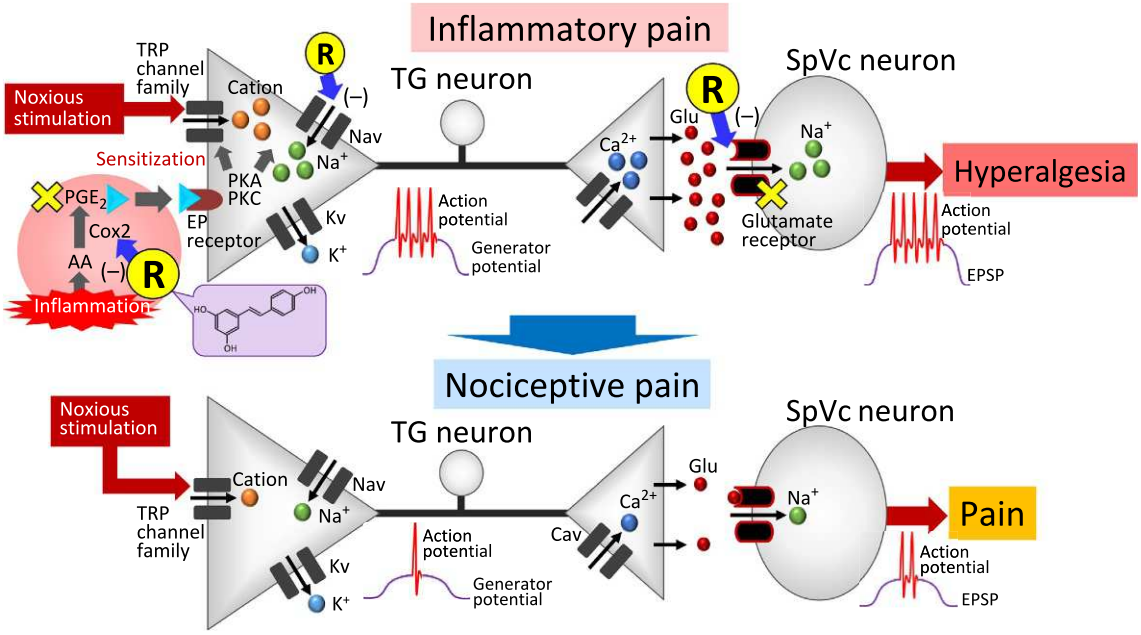

FIG. 18.3 Modulatory mechanism of pathological pain relief by resveratrol: Schematic drawing of the possible mechanism underlying the effects of resveratrol on inflammatory pain. *Inflammatory pain*: Following peripheral inflammation, inflammatory mediators, such as PGE_2, bind to G protein-coupled prostanoid EP receptors and induce activation of PKA and PKC, respectively, in nociceptive peripheral terminals, leading to phosphorylation of mechanosensitive Na^+ and K^+ channels and receptors. As a result, the activation threshold for transducer channels such as the TRP channel family is reduced and membrane excitability of the peripheral terminals increases (peripheral senatization), resulting in a high frequency of action potentials being conducted to presynaptic central terminals of the SpVc. This results in a substantial release of glutamate into the synaptic cleft, which binds to up-regulated postsynaptic glutamate receptors, augmenting EPSPs, causing a barrage of action potentials to be conducted to higher centers of pain pathways, and creating a state of heightened sensitivity termed central sensitization. *Nociceptive pain*: It is possible that chronic administration of resveratrol attenuates inflammation-induced mechanical inflammatory hyperalgesia, with this effect primarily involving suppression of SpVc WDR neuronal hyperexcitability via inhibition of both peripheral Cox-2 cascade signaling pathways, thus restoring the SpV neuronal hyperactivity activity to control levels. *TG*, trigeminal ganglion; *SpV*, trigeminal spinal nucleus; *PAG*, periaqueductal gray; *NRM*, nucleus raphe magnus; *Glu*, glutamate; *Nav*, voltage-gated sodium channel; *Kv*, voltage-gated potassium channel; *Cav*, voltage-gated calcium channel; *WDR*, wide-dynamic-range neurons. *AA*, arachidonic acid.

rats was significantly lower than in naïve rats, and the lowered mechanical threshold in inflamed rats was returned to control levels following chronic administration of resveratrol. In addition, after resveratrol administration, the mean discharge frequency of SpVc WDR neurons in inflamed rats was significantly decreased in response to both nonnoxious and noxious mechanical stimuli, and significant decreases were observed in both the inflammation-induced increased spontaneous discharge of SpVc WDR neurons and the frequency and occurrence of noxious pinch-evoked after discharge [60]. Finally, resveratrol administration restored the expanded receptive field of inflamed rats to control levels [60]. These results together suggest that chronic administration of resveratrol attenuates inflammation-induced mechanical inflammatory hyperalgesia and that this effect is due primarily to the suppression of the hyperexcitability of SpVc WDR neurons via inhibition of both peripheral and central Cox-2 cascade signaling pathways. These findings further support the potential therapeutic use of resveratrol in limiting trigeminal inflammatory hyperalgesia.

PGE_2 was reported to facilitate the activation of TRP vanilloid 1 (TRPV1) and tetrodotoxin (TTX)-resistant (TTX-R) Na^+ channels [13,17,61], and it has been reported that resveratrol inhibits both TTX-sensitive (TTX-S) and TTX-R Na^+ currents in acutely dissociated DRG neurons. It also seems that TTX-R Na^+ channels (e.g., Nav1.8 and Nav1.9) are selectively expressed in small- and medium-sized DRG neurons [62]. Compelling evidence indicates that these small DRG neurons are somata that give rise to thinly and unmyelinated C- and Aδ-fibers, which primarily conduct nociceptive stimuli. Modulation of these Na^+ channels involves activation of adenylate cyclase and increases in cAMP, possibly leading to PKA-dependent phosphorylation of the channels. By this mechanism, PGE_2 produced during an inflammatory response may significantly increase the excitability of nociceptive fibers (peripheral sensitization). Based on reports that the increased excitability of small-diameter trigeminal ganglion neurons seen after PGE_2 application involves increased TTX-R Na^+ currents [63], it can be assumed that resveratrol inhibits the excitability of small-diameter trigeminal ganglion neurons by

suppressing TTX-R Na^+ currents induced by increased production of PGE_2. As illustrated in Fig. 18.3, it thus seems reasonable to speculate that at least part of the peripheral antinociceptive action of resveratrol arises involves the prevention of peripheral sensitization, as is the case for antipyretic analgesics.

Conversely, PGE_2 can also act in the CNS, namely in the spinal dorsal horn and SpVc neurons, to produce hyperalgesia [18]. Inflammation-induced increases in Cox-2 mRNA and protein have been demonstrated in the spinal cord [20,64], where Cox-1 and Cox-2 are expressed constitutively. Recent evidence indicates that a major stimulus for the induction of Cox-2 is the proinflammatory cytokine interleukin-1β, which is found in the PNS and the CNS, and is produced in response to inflammation [20,65]. Two possible molecular mechanisms have been proposed to account for PGE_2-induced hyperalgesia via actions in the CNS: firstly, PGE_2 reduces inhibitory glycinergic neurotransmission via a postsynaptic mechanism [37], and secondly, direct depolarization of deep dorsal horn neurons by higher concentrations of PGE_2 [19]. Therefore, it is most likely that systemic administration of resveratrol has central antinociceptive effects by suppressing PGE_2-induced reductions in inhibitory glycinergic neurotransmission and enhanced depolarization of SpVc neurons through a postsynaptic mechanism. Finally, it is known that peripheral inflammation induces central sensitization, such as up-regulation of NMDA receptor signaling [25,33,35]. In addition, based on the suggested role for resveratrol in glutamatergic neurotransmission of the SpVc neurons responding to nociceptive mechanical stimulation via the NMDA receptor in vivo [52], resveratrol might serve as a useful CAM agent for the treatment of trigeminal inflammatory pain. Further studies are needed to confirm and further advance this hypothesis.

5.2 Neuropathic Pain

Until recently, chronic constriction nerve injury (CCI) in animal models was used to mimic neuropathic pain in humans [66–69], with CCI of the sciatic nerve serving as one of the most reliable models of neuropathic pain [68,69]. Following peripheral nerve injury, primary afferent neurons exhibit high frequency background activity or irregular burst discharges [66]. On the other hand, in the axotomy model of primary afferent neurons, hyperexcitability induced by peripheral nerve injury was attributed to extensive changes in the ionic currents of DRG neurons [69,70]. Moreover, changes in excitability of primary afferent neurons were also observed at the adjacent intact primary afferent neurons after nerve injury (axotomy spared neuron models) [71]. We have previously studied trigeminal neuropathic pain models, such as CCI, axotomy-spared, and axotomy regenerated neuron models [72–74]; unfortunately, resveratrol has not yet been studied in these contexts.

We recently introduced some data for the effect of resveratrol on neuropathic pain. In a rat spinal nerve ligation (SNL) model, Péres-Seyeriano et al. [75] found that intrathecal resveratrol reduced allodynia and reversed the reduction of constitutive Ca^{2+}-dependent nitric oxide synthetase (NOS) activity in the ipsilateral dorsal spinal cord following SNL. Furthermore, resveratrol significantly reversed reduction of NOS expression in the ipsilateral dorsal horn of the spinal cord, suggesting that resveratrol reduces tactile allodynia in neuropathic pain rat models by restoring the altered activity and expression of NOS [75]. On the other hand, intrathecal administration of resveratrol in a CCI pain model attenuated CCI-induced mechanical allodynia and thermal hyperalgesia [76], while chronic oral resveratrol treatment was shown to reduce thermal hyperalgesia. Although the precise neurophysiological mechanisms underlying these effects are unknown, spinal and supraspinal serotonergic systems were suggested to be differentially responsible for the antihyperalgesic and antidepressant characteristics of resveratrol [77]. Further studies are needed to elucidate a precise mechanism by which resveratrol attenuates neuropathic pain.

6 FUNCTIONAL SIGNIFICANCE FOR MODULATION OF PAIN RELIEF

6.1 Therapeutic Targets for Inflammatory Pain

A widely accepted trigeminal chronic pain model is the complete Freund's adjuvant (CFA) inflamed rat model [37,78]. Changes in neuronal properties following tissue injury and inflammation of the area innervating the orofacial area lead to pathological pain, such as hyperalgesia and allodynia [37,43]. In a previous study, we reported that TMJ inflammation-induced hyperexcitability of SpVc WDR neurons innervating the facial skin contributes to ectopic mechanical allodynia of this area [43,45]. Moreover, resveratrol restored the increased mean spontaneous discharge frequency of SpVc WDR neurons in inflamed rats to control levels [60]. Burnstein et al. [79] also reported that ongoing activity in the SpVc is responsible for ongoing headache (spontaneous pain), and the origin of such activity in the CNS is of considerable clinical interest, because of its suggested role in determining the level of posttraumatic injury and chronic pain [79]. A more recent study demonstrated that ongoing activity of WDR neurons in the SpVc is driven from the periphery, because microinjection of lidocaine into trigeminal ganglia significantly decreases ongoing activity [80]. Because chronic administration of resveratrol

attenuates the spontaneous activity in inflamed rats, these observations together suggest that resveratrol attenuates the spontaneous discharge activity (due to peripheral and/or trigeminal ganglion sensitization and probably contributing to spontaneous pain) of SpVc WDR neurons innervating the facial skin [81].

A dietary grapeseed polyphenol extract was previously shown to inhibit TMJ inflammation-induced pain [82] and we reported that in the absence of inflammatory or neuropathic pain, acute intravenous administration of resveratrol suppresses trigeminal sensory transmission, including nociception [52], and so resveratrol may contribute to the suite of CAMs as a therapeutic agent, without side effects, for the treatment of trigeminal nociceptive pain [83]. In a previous study under in vivo conditions, systemic administration of resveratrol attenuated inflammation-induced hyperexcitability of trigeminal SpVc neurons associated with hyperalgesia in rats [60]. Recently, there has been increased interest in the use of CAM for the treatment of persistent chronic pain [2]. Patients frequently turn to CAM therapies, such as herbal medicines and acupuncture, for pain control when other medical treatment is ineffective [1,2]. In recent studies, the potential effects of diet and dietary supplementation on conditions associated with pain have been the focus of considerable research [3,5]. Therefore we postulate a role for resveratrol in CAM to attenuate inflammatory hyperalgesia as pharmacological blockade of molecular targets for NSAIDs.

6.2 Therapeutic Targets for Nociceptive Pain

Surgical incisions are a potential source of chronic pain [84,85], and it is possible that resveratrol could effectively reduce this aspect of postoperative pain [86,87]. In recent work, resveratrol induced apotosis in numerous human cancer cells [88,89]. Specifically, Bai et al. [89] proposed resveratrol as a novel agent for the treatment of bladder cancer based on the inhibition of migration and invasion. Clinically, morphine has long been used to mitigate cancerous pain; however, morphine and its derivatives can cause major side effects including respiratory depression, sedation, and the development of addiction. In the present study, pretreatment of rats with the opioid antagonist naloxone attenuated the nociceptive JOR via the endogenous opioid system, supporting the hypothesis that the combination of resveratrol, which has no known toxic side effects, with an opioid could be a powerful therapeutic strategy for alleviating palliative trigeminal nociception, including clinical pain (e.g., postoperative and/or cancerous pain).

The results of the different studies into resveratrol contribute to the development of analgesic drugs with fewer side effects for the treatment of pathological pain, including orofacial pain. In particular, the findings from in vivo studies support resveratrol as a potential viable therapeutic alternative for alleviating nociceptive pain and preventing trigeminal inflammatory hyperalgesia.

7 FUTURE DIRECTION

Pain is generally recognized as involving many complicated aspects: sensory-discriminative, motivational, and affective aspects. The sensory discriminative aspect of pain is analogous to nonnoxious sensation and thought to be involved in the discrimination of pain features such as its location, intensity, and quality (lateral system in pain pathway). On the other hand, the motivational and affective aspects of pain are considered as related to emotional and autonomic responses due to long-lasting, intense noxious stimuli (medial system in pain pathway). Most studies of the effects of resveratrol on nociceptive and pathological pain have been focused on the lateral pain system (sensory-discriminative aspect of pain); however, future research should focus on how resveratrol might affect the medial pain system (emotional aspect of pain).

Regarding orofacial pain, most orthodontic patients experience pain and discomfort in their teeth as well as referred pain across other areas, including the cheek and masseter muscles [90]. Despite its detrimental impact on patients, little is known about the underlying mechanism of this referred pain in orthodontic patients, which makes prevention procedurally more difficult [91]. Tooth movement pain has been attributed to associated pressures, ischemia, inflammation, and edema [92], all of which can lead to the release of inflammatory mediators such as PGE2 [93]. PGE2 is synthesized by Cox-2 in response to inflammatory stimuli [94] and marked increases in Cox-2 expression have been reported not only locally at the site of inflammation, but also in neurons, glia, and endothelial cells in the spinal cord and brain during inflammation [20]. Recently Gao and Duan [91] demonstrated increased Cox-2 in the spinal trigeminal caudalis (SpVc) of experimental tooth movement (ETM) rat models, although not in the periodontal tissue, suggesting that central Cox-2 inhibition holds therapeutic promise in the treatment of orofacial pain induced by orthodontic tooth movement. In this context, we hypothesize that resveratrol administration could attenuate mechanical hyperalgesia via suppression SpVc WDR neuronal excitability and inhibition of the Cox-2 signaling cascade pathway.

Finally, there is strong evidence that activation of glia, such as astroglia and microglia within the dorsal horn of the spinal cord including the SpVc, and the subsequent production of cytokines play an important role in the creation and maintenance of pathological pain [95]. In addition, these pathological effects may not be limited to glial cells within the CNS, because sensory ganglia in the PNS

also contain satellite glial cells (SGCs). Anatomically, the SGCs form a distinct sheath around nearly every individual sensory neuron in the DRG, while trigeminal ganglia are associated with the transport and metabolism of various molecules, such as glutamate and glucose, and the maintenance of ionic homeostasis, including that of extracellular potassium [96]. Furthermore, increased neuron-glia interactions might play an important role in the induction and maintenance of peripheral sensitization of trigeminal nociceptors [35,81]. We therefore proposed that resveratrol could modulate potential therapeutic targets in glia-neuronal interactions for the prevention of trigeminal neuropathic and inflammatory pain.

8 CONCLUDING REMARKS

In conclusion, recent studies provide evidence supporting the dietary constituent resveratrol as a potential therapeutic CAM for the alleviation of nociceptive pain and pathological pain and the prevention of trigeminal inflammatory hyperalgesia. Experimentally, resveratrol modulates neuronal excitability in the PNS and CNS, including the sensory system, via various voltage-dependent ion/ligand-gated channels, neurotransmitter receptors, and inflammatory signaling cascades. Current data regarding the mechanisms underlying the modulatory effects of resveratrol on nociceptive neuronal activity associated with pain relief can be summarized as follows: (i) local resveratrol injection into the peripheral receptive field suppresses nociceptive spinal trigeminal neuron excitability, possibly by inhibiting generator and action potentials in the nociceptive nerve terminals of trigeminal ganglion neurons; (ii) trigeminal sensory transmission, including nociception, is suppressed by acute intravenous resveratrol; (iii) acute and chronic administration of resveratrol attenuates inflammation-induced mechanical hyperalgesia, primarily due to the suppression of hyperexcitability of SpVc WDR neurons via inhibition of peripheral and central Cox cascade signaling pathways; and, (iv) resveratrol suppresses excitatory synaptic transmission of the SpVc via activation of 5-HT_3 receptor-mediated GABAergic inhibition and/or endogenous opioidergic mechanisms. Recent studies thus suggest a potential contribution of resveratrol to the clinical relief of nociceptive and pathological pain, as well as its development as a functional food and CAM agent. Further study is needed to elucidate this possibility.

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CHAPTER

19

Possible Benefits and Risks of Polyphenols Supplementation During Pregnancy

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Acronyms and Abbreviations

cGMP	cyclic guanosine monophosphate
DNA	desoxyribonucleic acid
DOHaD	developmental origins of adult health and disease
EDC	endocrine-disrupting compound
eNOS or NOS3	endothelial constitutive synthase
IUGR	intrauterine growth restriction
LBW	low-birth-weight offspring
LGA	large-for-gestational-age offspring
NO	nitric oxide
PDE-5	phosphodiesterase type-5
PGI2	prostacyclin I2
PlGF	placental growth factor
SC	sildenafil citrate
SGA	small-for-gestational-age offspring
TXA2	thromboxane A2
VEGF	vascular endothelial growth factor

OUTLINE

The present review summarizes the current knowledge on realistic benefits and potential hazards of polyphenols supplementation during pregnancy.

1 INTRODUCTION

The fate of all living organisms is determined by the environment surrounding them. The main environmental limiting factors are weather conditions, water and food accessibility, and oxygen availability in the case of high-altitude regions. The ability to manage and to adapt to these features determines the viability and potential of individuals and populations. Such ability has been mastered by genetic evolution and adaptive behavior patterns through generations.

In the case of human beings, the efforts to understand and to master the environmental effects, generation after generation, have been focused on the period of life from birth to senescence and death (the postnatal life) and little attention has been paid to the period from conception to birth (the prenatal life). In fact, until the end of the 18th century, the most widespread beliefs were based on *preformationism* theories defending that, from Creation onwards, organisms develop from miniature versions of themselves (*animalcules* or, in the case of humans, *homunculi*) contained either inside the spermatozoid or the ovum (*spermism* and *ovism* concepts, respectively). The opposed theory, *epigenesis* or *neoformationism*, defended the existence of embryonic development through a process involving successive stages of formation of cell layers and gradual appearance of organs. Although epigenesis was first postulated by Aristotle in the 4th century BC, the theory only gained credit from the development of the microscope onwards, at the end of the 18th century, and only prevailed over preformationism near the end of the 19th century.

In both scenarios, the uterus was thought merely a space where the offspring grows in a latent state isolated from external influence, except for a few scientists like Sir Thomas Browne, who wrote in 1642:

> Some Divines count Adam 30 years old at his Creation, because they suppose him created in the perfect age and stature of man; and surely we are all out of the computation of our age, and every man is some months elder than he bethinks him; for we live, move, have a being, and are subject to the actions of the elements, and the malice of diseases, in that other World, the truest

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> Microcosm, the Womb of our Mother … In that obscure World and Womb of our Mother, our time is short, computed by the Moon, yet longer than the days of many creatures that behold the Sun; our selves being not yet without life, sense, and reason; though for the manifestation of its actions, it awaits the opportunity of objects, and seems to live there but in its root and soul of vegetation. Entering afterwards upon the scene of the World, we arise up and become another creature … (Religio Medici: The Religion of a Physician).

Unsurprisingly, the book was placed upon the Papal *Index Librorum Prohibitorum* (i.e., List of Prohibited Books).

The beginning of the 20th century was marked by an increasing interest in pregnancy events and, halfway through the middle of the past century, the study of fetal development and physiology evolved rapidly to become a major discipline within the biomedical sciences. The influence of the fetal environment, that is, of the intrauterine environment, was early acknowledged as critical for prenatal development. Therefore extensive research has been focused on its knowledge and amelioration. The present chapter aims to offer a noncomprehensive review of the current knowledge on the role of the intrauterine environment on pregnancy development and postnatal traits and the possible usefulness of polyphenols to improve pregnancy outputs.

2 THE ROLE OF INTRAUTERINE ENVIRONMENT IN PREGNANCY DEVELOPMENT

The main factor affecting the intrauterine environment is the availability of nutrients and oxygen that the fetus requires for development. Availability of oxygen and nutrients may be limited by maternal factors, like hypoxia (e.g., in the case of pregnant women living at or visiting high altitudes), undernutrition (e.g., in the case of socioeconomic disadvantage, food scarcity, emergency situations, and war) or environmental stressors (e.g., hyperthermia). Oxygen, nutrients, hormonal, and growth factors are transferred to the fetus through the placenta; hence, abnormal placental development and function cause, therefore, a fetal shortage of nutrients and oxygen (Fig. 19.1). In any of these cases, inadequate maternal conditions and/or inadequate placental function may result in insufficient supply of oxygen and nutrients to the fetus, causing intrauterine growth restriction (IUGR) and leading to the birth of small-for-gestational-age (SGA; also known as low-birth-weight, LBW) offspring [1]. Conversely, maternal overnutrition (e.g., in the case of maternal overweight and obesity with or without metabolic disorders) is usually related to excess of nutrient supply to the fetus and causes large-for-gestational-age (LGA) offspring [2]. However, in some cases, maternal obesity and metabolic disorders may also cause IUGR [3–5], mainly due to vascular alterations affecting the placental development and function, inducing fetal hypoxia [6].

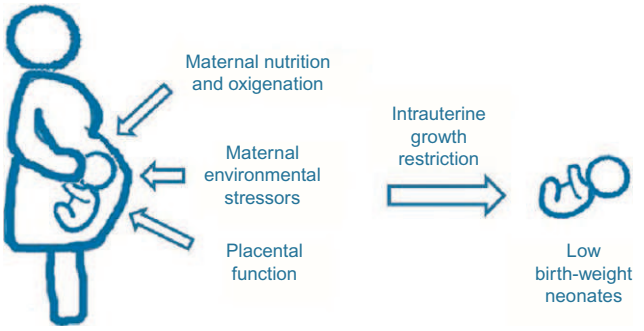

FIG. 19.1 Highlights of maternal and placental factors affecting offspring development and neonatal phenotype.

In humans, the incidence of LBW neonates ranges between 7% and 15% depending on sociodemographic issues [7]. Classically, IUGR has been associated with maternal malnutrition but, currently, 60% of IUGR offspring are identified as mainly caused by placental insufficiency [8,9]. Hence, at present, placental insufficiency is a concerning health issue [10,11], since it has been found to be linked to social, maternal, and gestational factors as diverse as postponement of childbearing, inadequate lifestyle, stress, or sedentary habits.

3 THE ROLE OF THE INTRAUTERINE ENVIRONMENT IN POSTNATAL TRAITS

The occurrence of IUGR, which is associated with an increased risk of perinatal morbidity and mortality, accounts for 800,000 neonatal deaths worldwide [12]. The problem is aggravated by the fact that surviving offspring are predisposed to lifelong chronic noncommunicable disorders due to changes in their metabolic phenotype [13–15].

The link among the intrauterine environment, fetal growth, and postnatal metabolic phenotype was evidenced by data obtained in individuals that were born in The Netherlands during the World War II, specifically during the period known as the Dutch Hunger Winter, from October 1944 to May 1945. These individuals, prenatally exposed to famine, showed a higher incidence of obesity and metabolic diseases during adulthood [16–21]. Concomitantly, this evidence was supported by the *Barker hypothesis*, stated by Prof. Barker and colleagues in the 1980s to 1990s. The Barker hypothesis is

based on a large set of epidemiological studies of human births affected by IUGR due to maternal famine, which were undoubtedly related to diabetes, vascular disease, and hypertension in adulthood [22,23].

The subsequent research introduced the concept of Prenatal or Fetal Programming, which, in brief, may be defined as long-term changes in the structure and/or function of developing organs in conceptuses exposed to an inadequate intrauterine environment, like deficiency of nutrients or oxygen [24,25]. Currently, the set of studies giving way to the concept of the developmental origins of adult health and disease (DOHaD) confirms that individuals prenatally undernourished, either by maternal famine or by deficiencies in placental function (which may be found in pregnant women with metabolic alterations), have a high predisposition to the development of metabolic syndrome, diabetes, and cardiovascular disease at adulthood [26–28].

4 PREVENTIVE STRATEGIES AND THERAPIES FOR IUGR

There are currently no effective preventive approaches or treatments for IUGR (Fig. 19.2), in spite of the many advances in obstetrics during the last years [29]. Most of the approaches have been based on nonpharmacological treatments focused on the mother (bed-rest, oxygen administration, nutrient therapy), with scarce success [30,31]. Pharmacological therapies have been based on the improvement of placental development and function, aiming to stimulate the processes of vasodilatation and angiogenesis required for adequate placental development.

Improvements in the processes of placental angiogenesis and vasodilatation may be obtained through acting on the route of nitric oxide (NO) and the vascular endothelial growth factor (VEGF). In healthy pregnancies, vascular dilatation and neoangiogenesis around the moment of implantation and during the later placental development are driven by proangiogenic factors, mainly by the placental growth factor (PlGF) and by VEGF. VEGF is a potent endothelial cell-specific mitogen that promotes angiogenesis, vascular hyperpermeability, and vasodilatation by autocrine mechanisms involving nitric oxide (NO) and prostacyclin (PGI2) production [32].

The first pharmacological attempts, during the 1990s, were based on the use of low doses of aspirin [33–35]. Acetylsalicylic acid inhibits cyclooxygenase activity in platelets and therefore increases the ratio between PGI2 and thromboxane A2 (TXA2). PGI2 acts as a vasodilator and platelet aggregation inhibitor while TXA2 acts as a vasoconstrictor and platelet aggregation stimulator, so increases in such ratio results in the improvement of systemic, and therefore utero-placental, circulation. The use of low doses of aspirin from the early pregnancy stage has been found to be useful to prevent IUGR in the case of early-onset preeclampsia [36,37], but the findings do not support routinely prophylactic or therapeutic use in other cases of IUGR.

Vasodilatation and angiogenesis are primarily driven by NO and its endothelial constitutive synthase (eNOS or NOS3), which can be found both at the trophoblast, the cells that adhere to and penetrate the uterine

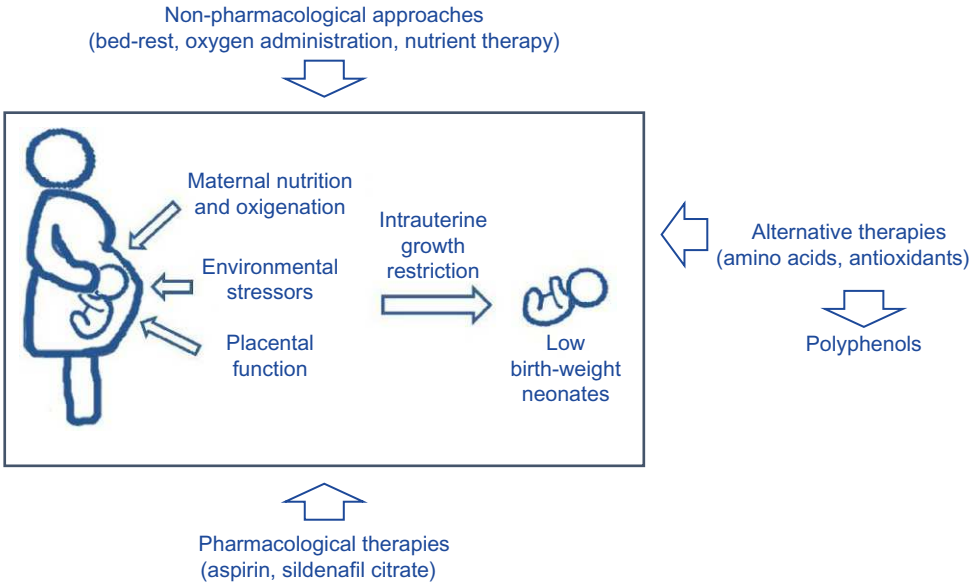

FIG. 19.2 Highlights of the different approaches for improving pregnancy traits

endometrium at implantation, and at the extravillous trophoblast, inducing vasodilatation and angiogenesis in maternal cells during the implantation process [38–40]. During the postimplantation period and subsequent placental development, NO and NOS3 are hypothesized to be involved in tissue remodeling, immunosuppression, and vasoregulation [41]. In consequence, a decreased NO bioavailability is recognized to be involved in the pathogenesis of IUGR [42]. Sildenafil citrate (SC, commonly known by its commercial name of Viagra) is a selective phosphodiesterase type-5 (PDE-5) inhibitor that prevents the hydrolysis of cyclic guanosine monophosphate (cGMP), the biological signaling for NO production [42], and therefore increases NO bioavailability. Thereby, SC therapy may optimize placental function and alleviate IUGR. Experimental trials with SC in different animal models have shown to improve IUGR phenotype [43–46]. In humans, SC has been tested with promising results, improving maternal and fetal blood flow velocimetry and fetal well-being [47–51]. However, our studies in a rabbit model [46] have shown that SC therapy produces blood overflow in the fetal brain, which may cause cerebral hypertension; hence, elucidating whether these changes can have consequences in the adult life needs further research.

5 ALTERNATIVE TOOLS FOR PREVENTION AND TREATMENT OF IUGR: AMINO ACIDS AND ANTIOXIDANTS

The route of NO and VEGF may be favored not only by pharmacological treatments but also through nutritional supplementation with NO precursors, mainly in the form of amino acids. The most known is arginine, the primary substrate of NOS, but there are several other amino acids that are precursors of NO (ornithine, leucine, glutamine, and proline). These amino acids also regulate the synthesis of polyamines and proteins; thus besides favoring the placental development and the nutrient transfer through the placenta, they also support conceptus development. The results obtained under experimental conditions in animal models (sheep and swine) suggest that amino acid supplementation may be a promising strategy to reduce the incidence of IUGR offspring [52–54], although some of the findings have been conflicting, which makes necessary further specific studies elucidating this issue. The same has been observed in human studies, which fact stresses the need for studies determining the actual requirements of amino acids and protein requirements during pregnancy [55], the real metabolic bioavailability of dietary amino acids following digestion and absorption [56], and the actual availability for the fetus after placental transport [57]. In fact, there is growing evidence indicating that IUGR is associated with a reduction in placental amino acid transport capacity and in metabolic pathways that prejudice the effectiveness of nutritional therapies with amino acids, such that the previous points need to be the focus of future studies.

IUGR involves not only inadequate nutrient supply. Concomitantly, imbalances in the levels of angiogenic regulators, which cause insufficient placental and fetal blood flow, compromise the supply of oxygen. In turn, inadequate supply of oxygen causes hypoxia and increases oxidative stress at the feto-placental unit [58], which aggravates the effects of IUGR. Moreover, the increased oxidative stress is exacerbated in the case of IUGR, since these fetuses have a weakened antioxidant defense system [59–61] but, moreover, because of the adaptive processes to placental undernutrition. Fetuses with IUGR are able to ensure better use of the scarce nutrients that they have by modifying their metabolic regulation, by displaying insulin resistance. Insulin resistance allows the IUGR fetuses to take advantage of the scarce supply of glucose that they are receiving. However, high levels of insulin increase the synthesis of NO inhibitors [62], prejudicing utero-placental blood flow [63] and reducing the supply of oxygen and nutrients to the fetuses even more, ending up in a vicious circle. Another consequence of fetal hypoxia is a related state of low-grade systemic inflammation affecting immune cell proliferation and serum cytokines, as well as increased susceptibility to infection [6,64,65].

Hence, from a seminal study of Chappell and coworkers in 1999 [66], several studies in animal models and humans have been focused on the prevention the deleterious effects of oxidative status at the fetoplacental unit by the use of antioxidant agents. A majority of the studies have been based on supplementation with the antioxidant vitamins C and E [67–71], which show a tendency to be lower in case of IUGR [72]. However, the clinical outputs are not fully conclusive [73] and therefore more studies are needed prior to supporting the use of these treatments in IUGR pregnancies. Alternative studies have been based on the use of melatonin since it acts as a direct free-radical scavenger, an indirect antioxidant, and a cytoprotective agent in pregnancy [74,75], but clinical evidence is likewise still scarce.

There is a different approach to ameliorate the antioxidant/oxidative status of the fetuses during pregnancy that would be based on a diverse source of antioxidant agents, polyphenols. Polyphenols are potent antioxidants with a double action, scavenging reactive oxygen species and inhibiting their formation, and may be useful for compromised pregnancies since they increase the plasma antioxidant capacity [76,77], and therefore they can improve the placental oxidative stress stage [78].

Polyphenols are the most abundant dietary antioxidants and currently are well-considered and freely consumed by the general population, because they are present at high concentrations in a great variety of natural

foods and beverages (e.g., herbal teas, pieces or derivatives of fruits, vegetables and seeds) and because of the general perception of their effectiveness as "natural" products for prevention and treatment of different diseases. In fact, most of the polyphenols have not only antioxidant activity but also metabolism-regulatory, antiinflammatory, and immunomodulatory properties. In consequence, there is increasing clinical and epidemiological evidence on their relevance against pathologies such as cancer, cardiovascular, metabolic, and neurodegenerative diseases [79].

Polyphenols are also perceived as beneficial for pregnancy and, as an example, 78% of the pregnant women in the United States take supplements during pregnancy [80]. However, possible usefulness of polyphenols for reproductive health and pregnancy is only beginning to be explored, and there is a scarcity of objective data on both realistic benefits and potential hazards of polyphenol supplementation during pregnancy [76,81,82].

6 IMPLICATIONS AND EVIDENCE FOR BENEFICIAL EFFECTS OF POLYPHENOLS SUPPLEMENTATION DURING PREGNANCY

The most important polyphenols are flavonoids (flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols), phenolic acids (e.g., gallic, p-coumaric, caffeic, and ferulic acids) and stilbenes (e.g., resveratrol). Possibly, flavonoids (specifically quercetin and genistein) and stilbenes (resveratrol) are the most extensively studied with regards to their effects on pregnancy features (Table 19.1).

TABLE 19.1 Summary of Main Polyphenols and Current Evidence on Possible Beneficial Effects and Risks Derived From Their Administration During Pregnancy

			Supplementation during pregnancy	
		Foods	**Possible benefits**	**Possible risks**
Flavonoids	Flavonols, e.g., quercetin	Broccoli, Onions, Apples, Tea (black tea) and Red wine	Improves the oxidative state and dyslipidemia in pregnant diabetic rodents [83,84]	May affect embryo implantation by increasing estradiol/progesterone ratio
			Restoration of fetal development and prevention of postnatal hyperglycemia, insulin resistance, obesity, hypertension and early osteoporosis [84–87]	Accelerated reproductive development in female offspring [88,89]
			Improves antioxidant activity and decreased oxidative stress-induced DNA damage in the offspring [87,90,91]	Affects the fetal erythropoiesis and exerts lifelong alterations in gene expression and DNA methylation of hematopoietic cells [92]
			Improves the systemic and utero-placental circulation [93]	Increased expression of inflammation associated cytokines of the offspring in adulthood [94]
			Reverses adverse outcomes of preeclampsia [95]	Inhibits vitamin C transport across trophoblast in a dose-dependent and noncompetitive manner [96]
			Increased folic acid uptake [97,98]	Constriction of fetal *ductus arteriosus* [99–101]
	Flavones	Parsley and Celery		
	Isoflavones, e.g., genistein	Soybeans and Soy-containing food	Decreases oxidative stress and exerts cardiovascular protection, lowering incidence of hypertension [102]	Accelerated reproductive development in female offspring [88,89]
				Disrupting sexual differentiation in the offspring [89]
				Abnormal DNA-methylation and other epigenetic modifications in the dams and in the offspring [103]
				Altered expression of genes important for the development and function of reproductive tissues in the offspring [103]

Continued

TABLE 19.1 Summary of Main Polyphenols and Current Evidence on Possible Beneficial Effects and Risks Derived From Their Administration During Pregnancy—cont'd

			Supplementation during pregnancy	
		Foods	**Possible benefits**	**Possible risks**
				Altered expression of genes important for metabolism regulation in the offspring [104]
				Altered expression of genes important for stimulation of appearance estrogen-dependent tumors in the offspring [105]
				Induction of epigenetic changes by histone modifications or changes in miRNA expression [106]
				Affects the fetal erythropoiesis and exerts lifelong alterations in gene expression and DNA methylation of hematopoietic cells [92]
				DNA breaks and rearrangements in the mixed-lineage leukemia gene in the offspring [107,108]
				Inhibits vitamin C transport across trophoblast in a dose-dependent and noncompetitive manner [96]
	Flavanones, e.g., hesperidin or naringin	Citrus fruits and juices		
	Anthocyanidins	Blueberry, Raspberry, Black rice and Black soybean		
	Flavanols	Apples, Pears, Tea, Cocoa, Grapes and Blueberries		
Phenolic acids	Gallic	Gallnuts, Sumac, Witch hazel, Tea leaves, Oak bark and other plants		
	P-coumaric	Peanuts, Navy beans, Tomatoes, Carrots, Basil and Garlic		
	Caffeic	Thyme, Sage, Spearmint, Ceylon cinnamon and Star anise		
	Ferulic acids	Corn and Bamboo shoots		
Stilbenes	Resveratrol	Red grapes and Blueberries	Ameliorates embryonic oxidative stress and apoptosis associated with diabetic embryopathy. Prevents developmental delays in embryos of diabetic dams [109,110]	
			Improving antioxidant status and vascularization of the kidney of the IUGR offspring [111]	
Phenylethanoids	Hydroxytyrosol	Olive fruits, Virgin olive oil	Decreases the appearance of low-birth-weight neonates [112]	

Flavonoids are usually consumed in the human diet due to their ample presence in fruits and vegetables (e.g., blueberries and other berries, black and green teas, red grapes and wine, bananas, pomegranate, parsley, onions, all citrus fruits and dark chocolate). The flavonol *quercetin* (3,3′,4′,5,7-pentahydroxyflavone), possibly the most consumed flavonoid (found in broccoli, onions, apples, tea, and red wine) is a potent antioxidant. There are studies addressing its positive effects on the in vitro viability of oxidized endometrial cells [113], and preclinical studies in pregnant diabetic rodents have evidenced improvement of oxidative stress and dyslipidemia in the mother [83,84]. The effects on the offspring from pregnancies affected by oxidative stress and metabolic disturbances mainly consist of the restoration of the fetal development and the prevention of postnatal hyperglycemia, insulin resistance, obesity, hypertension and early osteoporosis [84–87]. The enhancement of the antioxidant defense system in utero seems to persist during the postnatal stages and, as a result, offspring exposed to quercetin have also increased antioxidant activity and decreased oxidative stress-induced DNA damage [87,90,91]; a similar effect has been found after exposure to genistein (4′,5,7-trihydroxyisoflavone). The isoflavone genistein is another crucial flavonoid the intake of which is traditionally high in Asia and low in Western countries, since it is mainly found in soybeans and soy-containing food. Currently, the intake of genistein is becoming higher worldwide due to the increasing consumption of soy beverage as a replacement for cow's milk and the high concentration in such soymilk (30 and 175 mg/L isoflavones). There are some studies in rodents addressing that feeding soy isoflavone-rich diet during pregnancy decreases oxidative stress and exerts cardiovascular protection, lowering incidence of hypertension [102].

Quercetin is also a potent inhibitor of inflammatory responses by inhibiting cyclooxygenase expression [93], which increases the ratio PGI2/TXA2 and may therefore improve systemic and utero-placental circulation in a similar way as previously described for acetylsalicylic acid. In this way, studies in rodent models showed that quercetin reverses adverse outcomes of preeclampsia, such as proteinuria and high neonatal death rate, although without significant improvements in the birthweight of the pups [95]. Finally, quercetin also evidences a positive action on pregnancy by increasing folic acid uptake, which is a critically important precursor of nucleic acids [97] and amino acids [98].

The stilbene resveratrol (*trans*-3,5,4′-trihydroxystilbene), which is found in red grapes and blueberries, has similar effects to quercetin on oxidative stress and metabolism. In adults, the intake of resveratrol increases antioxidant activity, lowers blood sugar, and improves lipid profile [114] without any observable side effects [115]. Hence, resveratrol would be especially useful for obesity, metabolic syndrome, insulin resistance, and type 2 diabetes [116,117]. The use of resveratrol supplementation may be considered a useful tool to improve maternal metabolic profile in pregnancies affected by obesity and associated disorders. In fact, there is experimental evidence in rodents that the compound ameliorates embryonic oxidative stress and apoptosis associated with diabetic embryopathy and prevents developmental delays in embryos of diabetic dams [109,110]. The use of grape skin extracts in low-protein maternal diets used as a model for IUGR specifically prevents glomerular loss and renal immaturity, which is commonly found in IUGR offspring, by improving antioxidant status and vascularization of the kidney [111]. These effects of resveratrol would be exerted not only through improving maternal metabolic status, which would indirectly affect the offspring, but also through direct effects on the fetus, as it is known to pass the placenta [118].

Other polyphenols showing positive effects on pregnancy outputs, with lower consumption at a worldwide level but frequently consumed in some certain areas (e.g., Mediterranean countries in which diet is strongly based on the use of olive oil), are hydroxytyrosol (4-(2-Hydroxyethyl)-1,2-benzenediol) and its precursor oleuropein. Hydroxytyrosol is a polyphenol present in olive fruits (and, hence, in virgin olive oil) with prominent antioxidant, metabolism-regulatory, antiinflammatory, and immunomodulatory properties. There is increasing clinical and epidemiological evidence on its relevance against different pathologies, i.e., cancer, cardiovascular, metabolic, and neurodegenerative diseases [119]. With regards to its effects on pregnancy, our group has recently credited the usefulness of its maternal supplementation during pregnancy to counteract IUGR, and therefore the appearance of LBW neonates [112].

7 POSSIBLE RISKS OF POLYPHENOLS SUPPLEMENTATION DURING PREGNANCY

The use of polyphenols during pregnancy is perceived as beneficial and therefore it is currently increasing worldwide. However, in spite of such popularity, there is a scarcity of experimental data on potential hazards of polyphenol supplementation during pregnancy. There are, in fact, some evidence supporting the induction of deleterious effects when supplying polyphenols during pregnancy.

First, polyphenols may even affect pregnancy success. There is evidence that high doses of quercetin may affect embryo implantation and hence pregnancy success, by increasing estradiol/progesterone ratio [120]. Such changes in estradiol levels during pregnancy may also be related to the accelerated reproductive development

described in the case of female offspring prenatally exposed to quercetin [88]. These effects are most classically described for genistein, a well-known phytoestrogen; in fact, the most potent phytoestrogens are polyphenols: genistein, daidzein, and coumestrol [121,122]. Phytoestrogens are compounds produced by plants which, because of their structural similarity to estradiol, may cause estrogenic and/or antiestrogenic effects by stimulating or blocking estradiol receptors. Exposure to exogenous estrogens may affect reproductive function of both males and females but, moreover, its exposure during development and early life can have marked effects on the reproductive system of the offspring [89], by disrupting sexual differentiation and reproductive function in adulthood. These considerations need to be taken into account having in mind the current popularity of soy-derived products and mainly soymilk (in which concentration of genistein is particularly high) as a replacement for cow's milk, especially in pregnant women and afterwards in their infants [105].

In fact, genistein is currently considered an endocrine-disrupting compound (EDC). Its exposure is associated with abnormal DNA-methylation and other epigenetic modifications, as well as altered expression of genes important for the development and function of reproductive tissues [103], for metabolism regulation (being also considered an obesogenic compound, [104]) and for stimulation of appearance of estrogen-dependent tumors in the offspring, like mammary tumors [105]. There is other evidence supporting that the use of polyphenols during pregnancy may be related to the induction of epigenetic changes, mainly through DNA methylation [119], but also by histone modifications or variations in miRNA expression [106]. Most of them are adaptive and beneficial, but deleterious effects cannot be discarded, such as increased incidence of different types of cancer [123]. Specifically, exposure to isoflavones (i.e., genistein and quercetin) has also been found to affect fetal erythropoiesis and exerts lifelong alterations in gene expression and DNA methylation of hematopoietic cells [92]. Exposure to isoflavones also induces DNA breaks and rearrangements in the mixed-lineage leukemia gene, which are frequently observed in childhood leukemia [107,108]. Concomitantly, prenatal exposure to quercetin has been related to increased expression of inflammation-associated cytokines at adult age [94]. These considerations are of most importance since it is currently well-known that polyphenols drive activation and/or silencing of transcription factors and consequently influence gene expression, with miRNA being a main target of their action [87].

Other deleterious effects of polyphenols are related to interferences in the transport of other substances. Quercetin exerts a positive action on pregnancy by increasing folic acid uptake by the fetus, but there was early evidence that quercetin and also genistein inhibit vitamin C transport across trophoblasts in a dose-dependent and noncompetitive manner [96]. Subsequent studies indicate that different classes of polyphenols, various combinations of polyphenols, and different doses and different periods of exposure may affect the transport of several bioactive compounds (organic cations, glucose, thiamin, and even folic acid) at the placenta [124].

Finally, other negative effects from exposure to polyphenols, mainly during the third trimester of pregnancy, are also related to their antiinflammatory actions which, in a similar way to some antiinflammatory drugs, may trigger changes in the fetal vascular system, specifically constriction of *ductus arteriosus* [99–101]. The *ductus arteriosus* drives the fetal blood flow from the right ventricle to the descending aorta. Its constriction/obliteration may cause complications such as fetal and neonatal heart failure, hydrops, and neonatal pulmonary hypertension, which may cause death.

8 CONCLUDING REMARKS AND FUTURE RESEARCH

There is a strong trend for an abundant use of polyphenols during pregnancy, based on their beneficial effects on some disorders occurring during adulthood and related to systemic or local increases in oxidative stress and low-grade inflammation. The use during pregnancy is a priori supported by clear evidence of beneficial effects on development and metabolic traits of the fetus. However, there is also evidence pointing to the induction of deleterious effects due to xenoestrogenic and epigenetic properties. Hence, profound research on the properties, benefits, and safety of the use of the different polyphenols is strongly needed, which may rely on interventional studies in adequate preclinical models for a rapid translation to human medicine. Such preclinical studies need to be performed in animal models and based on two main pillars: a profound knowledge of the physiology and the translational value of the different species and a strong respect for animal welfare and health.

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CHAPTER

20

Flavonoids as Modulators of Neutrophils' Oxidative Burst: Structure-Activity Relationship

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1 INTRODUCTION

The immune response is divided into two components, determined by the speed and specificity of the reaction: the adaptive and the innate responses. Adaptive immunity is the hallmark of the immune system of higher animals. This response consists of antigen-specific reactions through T and B lymphocytes. The adaptive response is precise but takes several days or weeks to develop. However, this type of response has memory. Thus, a subsequent exposure to the pathogen leads to a more vigorous and rapid response, although still not immediate [1,2]. Speed is a defining characteristic of the innate immune system. Within minutes of pathogen or foreign body exposure, the innate immune system starts generating a protective inflammatory response. Activation of the innate immune system results in an inflammatory response, which is essential for a rapid control of infections before they can spread. In addition, it is now clear that cells of the innate immune system contribute to the initiation and subsequent focus of the ensuing adaptive immune response [3]. Phagocytes are especially critical to the acute inflammatory response, as a result of their ability to efficiently engulf and destroy a variety of pathogens. These cells are also known as professional phagocytes and comprise neutrophils, monocytes, macrophages, and eosinophils. Among this group, neutrophils, also known as polymorphonuclear leukocytes (PMNs), are the most numerous and are usually the first cells to arrive at the inflammation site, being possibly the most important cell type of the innate response during acute infection or exposure to foreign bodies [4–6]. Neutrophils generate reactive oxygen (ROS) and nitrogen (RNS) species during phagocytosis and in response to soluble agonists. This functional response, termed oxidative burst, largely contributes to host defense, but it can also result in collateral damage to host tissues, their modulation being of extreme importance for the maintenance of the organisms homeostasis [6,7].

This chapter provides a general overview of the role of human neutrophils during the inflammatory process. It also analyzes the available data about the different types of flavonoids and their potential effectiveness against neutrophils' oxidative burst, establishing, whenever possible, an accurate structure-activity relationship.

2 NEUTROPHILS MORPHOLOGY

Neutrophils have been viewed as a swift, short-lived effector cell type of the immune system, which serve solely to perform phagocytosis, recruit other effector cells, and then commit cell suicide via apoptosis. Nevertheless, this is far from a complete picture, since neutrophils are a complex and mysterious cell type whose functions are not yet totally clarified [8]. On one hand, neutrophils provide the first line defense to our organism. The importance of this role is underscored by the high risk of infection in individuals with leukopenia. On the other hand, inappropriate or prolonged activation of neutrophils leads to tissue injury associated with a number of autoimmune and inflammatory diseases, including rheumatoid arthritis, Wegener's granulomatosis, systemic

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lupus erythematosus, multisystem organ failure associated with sepsis, and ischemia-reperfusion injury [9].

Neutrophils represent 40%–70% of white blood cells, being the most abundant circulating leukocytes. These cells are produced daily from hematopoietic stem cells, in a process called granulopoiesis, in large quantities (5×10^{10} to 10×10^{10} neutrophils/day) and reside mainly in the peripheral vasculature [8]. Neutrophil homeostasis is maintained by a fine balance between granulopoiesis, bone marrow storage and release, intravascular margination, clearance, and destruction [10].

Neutrophils have a brief (6–8 h) circulating half-life. However, the advantage to the host of this rapid turnover is uncertain [10]. A longer lifespan may allow neutrophils to carry out more complex activities in a tissue, such as contributing to the resolution of inflammation or shaping adaptive immune responses, nonetheless their persistence in tissues may lead to bystander cell injury [11].

There are several structural characteristics that set the neutrophils apart from most other cells. First, the most easily discerned feature, their multilobulated and oddly shaped nucleus, which has given them the name of polymorphonuclear cells (which also include the closely related basophil and eosinophil). It has been speculated that the several lobes of the neutrophil nucleus facilitate their extravasation from the capillaries and/or participate in the release of granule proteins and chromatin that together form extracellular fibers, known as neutrophil extracellular traps (NETs), used as a way to trap and kill pathogens. In theory, this would make possible the release of one lobe after another, while still maintaining the integrity of the cell [12].

The presence of granules in neutrophil cytoplasm is probably the most distinctive feature of neutrophils. Neutrophil granules are formed sequentially during myeloid cell differentiation. The granules were originally defined by the presence or absence of myeloperoxidase (MPO) and consequently named "peroxidase-positive granules" or "peroxidase-negative granules," but over the years, the advances in subcellular fractionation and biochemical analysis allowed the understanding that neutrophils contain several distinct granule populations. So far, it has been possible to distinguish four categories of granules: primary (or azurophilic) granules characterized by the presence of high content of MPO and membrane-bound CD63; secondary granules (also known as specific granules) whose contents include lactoferrin and membrane-bound CD66b; tertiary granules, which lack CD66b but contain gelatinase; and secretory vesicles, which contain albumin and express alkaline phosphatase and CD35 on their membranes [13].

In healthy individuals, circulating neutrophils are poorly responsive to proinflammatory stimuli. A complex and interactive array of extracellular stimuli and intracellular signal transduction pathways regulate the transition of resting neutrophils to cells primed for enhanced responses and, finally, to fully activated cells. Neutrophil activation results in a series of events that together culminate in the elimination of the pathogen, namely phagocytosis, NETs release, and the production of reactive species [9,14]. The phagocytosis process is intimately related with reactive species production as it triggers the initiation of neutrophils' oxidative burst.

3 NEUTROPHIL PHAGOCYTOSIS

Upon contact with a pathogen, the neutrophil membrane can internalize both opsonized and nonopsonized particles, by a process known as phagocytosis. This is a process mediated by a complex interplay of membrane lipids, intracellular signaling cascades, and cytoskeletal rearrangements. The main opsonin receptors of neutrophils, Fc receptors and a subgroup of β2 integrins, bind to immunoglobulin or complement-coated particles, respectively. While complement-opsonized particles are internalized by gently "sinking" into the cell, Fcγ receptor ligation initiates the vigorous extension of pseudopods that surround and ultimately entrap the particle into the phagosome [13,14]. Upon formation, the phagosome initiates a process of "maturation," characterized by a series of events developed in a dynamic process that involves an alteration in its composition, which allows the elimination of pathogens and disposal of debris [13,14]. Subsequently, primary and secondary granules fuse with the phagosome and discharge their antimicrobial contents [14]. The elevated cytosolic free Ca^{2+}, mainly from the endoplasmic reticulum, which in turn activates the influx of extracellular Ca^{2+}, can trigger the fusion of granules in neutrophils and is also required for the granule secretion [13]. The role of protein kinase C (PKC) isoforms in the secretion of granules process is not yet well defined. Although several isoforms of PKC, as PKC-α, PKC-β, PKC-δ, translocate to the plasma membrane upon phagocytosis, it appears that while PKC-δ is essential for the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase assembly, the role of the other kinases in adhesion, degranulation, and phagocytosis is not yet conclusive [15].

4 PRODUCTION OF REACTIVE OXYGEN SPECIES

After phagocytosis, neutrophils produce an array of ROS and RNS by a process known as oxidative burst, where the activation of NADPH oxidase plays a central role [15] (Fig. 20.1).

So far, there are seven described homologs of NADPH oxidase (NOX1–NOX5, and Duox1 and 2). The catalytic core of phagocyte oxidase (phox) (NOX2) is an enzymatic complex that includes five subunits: $gp91^{phox}$, $p22^{phox}$,

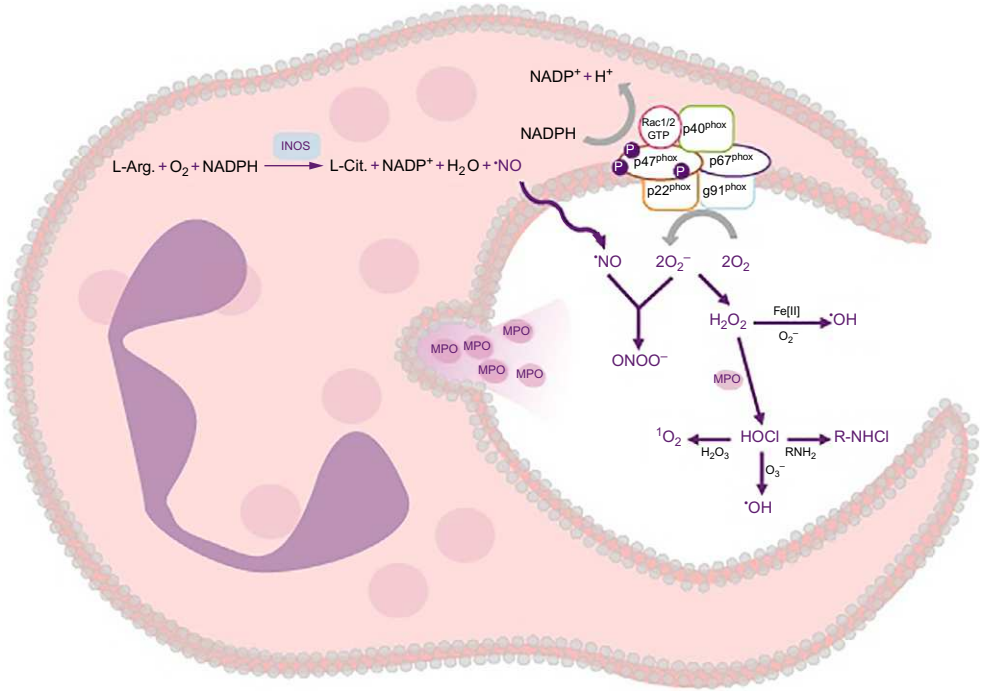


FIG. 20.1 Production of prooxidant reactive species in activated neutrophils. *Reprinted from Ribeiro D, Freitas M, Lima JL, Fernandes E. Proinflammatory pathways: the modulation by flavonoids. Med Res Rev 2015;35(5):877–936, copyright (2015), with permission from John Wiley and Sons.*

p40^{phox}, p47^{phox}, and p67^{phox}. In the inactive form, the catalytic core of NADPH oxidase, the heterodimeric subunit flavocytochrome b558 (cytb558), constituted by the subunits gp91^{phox} and p22^{phox}, is located at cellular membranes including the membranes of phagosomes, secretory vesicles, specific granules, and the plasma membrane. The gp91^{phox} is the electron transferase of NADPH oxidase. The p22^{phox} acts as a docking site for the regulatory trimeric complex, p40^{phox}, p47^{phox}, and p67^{phox}, located in the cytosol of latent cells, via its interaction with p47^{phox}. However, the p47^{phox} may also exist separately from the trimer [16]. Priming and activation of the NADPH oxidase by phagocytosis or stimulation with some bacterial products results in the complete assembly of oxidase components with cytb558 and the cytosolic subunits [15]. A number of components of the NADPH oxidase complex can be phosphorylated during activation, being able to directly modulate NADPH oxidase activity and their assembly. Nevertheless, the phosphorylation of p47^{phox} initiates all the process that results in the assembly and activation of the enzyme. The clinical importance of NADPH oxidase and its products is supported by the susceptibility of patients with chronic granulomatous disease (CGD), who have defective oxidase function, to recurrent bacterial and fungal infections [13].

The assembled oxidase facilitates the transfer of one electron from cytosolic NADPH to molecular oxygen captured from the medium. This reaction results in the production of superoxide anion radical ($O_2^{\bullet-}$) (~10 nmol/min per million of neutrophils), during oxidative burst (Eq. 20.1).

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet-} \qquad (20.1)$$

$O_2^{\bullet-}$ has a half-life of 10 ms, and it spontaneously originates hydrogen peroxide (H_2O_2). This reaction can be accelerated 10^4-fold by the action of the enzyme superoxide dismutase (SOD). There is no evidence that SOD from leukocytes is released into the phagosome. However, it can be introduced as a component of the ingested organism. In turn, in the presence of the transition metal iron, and in reaction with $O_2^{\bullet-}$ and H_2O_2, an extremely reactive radical is formed, the hydroxyl radical ($HO^{\bullet}$) (Haber Weiss reaction). In the first step of this reaction (Eq. 20.2), $O_2^{\bullet-}$ reacts with Fe^{3+}, originating Fe^{2+} and O_2, though this reaction is thermodynamically unfavorable under physiological conditions. The second step of this reaction (Eq. 20.3), also known as Fenton's reaction, occurs under biological conditions, where Fe^{2+} reacts with H_2O_2 to form both $HO^{\bullet}$ and HO^- [17].

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad (20.2)$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{2+} + HO^{\bullet} + HO^- \qquad (20.3)$$

Myeloperoxidase (MPO), which was originally named verdoperoxidase due to its intense green color, is a highly cationic, heme-containing, glycosylated enzyme [18]. It is the most abundant proinflammatory enzyme stored in the azurophilic granules of fully differentiated neutrophils, accounting for approximately 5% of their cell weight. It makes up to about 25% of the granule protein, and it achieves concentrations of about 100 mg/mL (1 mM) in the granule [19].

In the presence of H_2O_2 and a low molecular-weight intermediate (halide: chloride, bromide, or thiocyanate; tyrosine; or nitrite) MPO catalyzes the formation of powerful reactive intermediates including hypochlorous (HOCl) (Eq. 20.4), hypobromous (HOBr), and hypothiocyanous (HOSCN) acids, tyrosyl radical, and reactive nitrogen intermediates, with significant biological effects. Chloride is believed to be the physiological halide and therefore a preferred substrate for MPO, essentially due to its abundance in physiological fluids, HOCl being highly produced under these circumstances [18]. HOCl can react with other compounds as amines before reaching the microorganism, originating chloramines, which are less reactive than HOCl, but are long-lived oxidants that can contribute to cell damage [18].

$$H_2O_2 + Cl^- \rightarrow H_2O + HOCl \leftrightarrow OCl^- + H^+ \qquad (20.4)$$

Importantly, neutrophils can rapidly release MPO by degranulation, by cell death pathways including apoptosis and necrosis, or via the extrusion of NETs. This MPO can be deposited in the vasculature during inflammatory responses, being responsible for vascular cell injury, due to its capacity to generate damaging oxidants [18]. Despite that, the relevance of this enzyme is shown in patients without MPO who are more susceptible to fungal infections, particularly those caused by *Candida albicans* [18].

Singlet oxygen ($^1O_2$), is an electronically excited state of oxygen that spontaneously decays to the normal triplet state with emission of a photon of red light. It has been assumed that neutrophils produce $^1O_2$ also through MPO activity, which catalyzes the reaction of H_2O_2 with $HOCl/OCl^-$ (or Cl_2) (Eqs. 20.4 and 20.5), which exhibits chemiluminescence [20]:

$$H_2O_2 + OCl^- \rightarrow {}^1O_2 + H_2O + Cl^- \qquad (20.5)$$

It has also been argued that the production of $^1O_2$ by neutrophils is essential for NETs formation [21].

5 PRODUCTION OF REACTIVE NITROGEN SPECIES

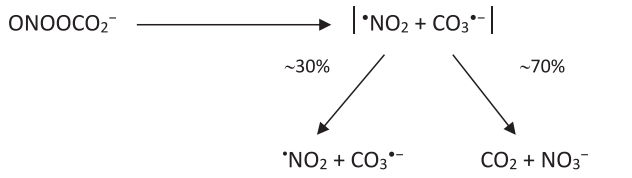
The production of nitric oxide ($^{\bullet}NO$) depends on the activity of the enzyme oxide nitric synthase (NOS). There are two known isoforms that generally fall into two categories: (i) constitutive NOS (which categorically includes endothelial NOS [eNOS] and neuronal NOS [nNOS]), dependent on Ca^{2+} levels and calmodulin binding, and (ii) inducible NOS (NOS2 or iNOS), the activity of which is independent of Ca^{2+} levels. The quantity of $^{\bullet}NO$ produced by constitutive NOS is low (nanomolar level) and transient. $^{\bullet}NO$ at this level has a relatively long half-life and is mainly involved in homeostatic processes such as neurotransmission, peristalsis, and blood pressure regulation [22]. Despite the 50% identity in the amino acid sequence of these three isoforms, iNOS is expressed by leukocytes, namely macrophages, in response to inflammatory stimulus as cytokines and/or microbial toxins. The production of $^{\bullet}NO$ occurs for long periods of time and its activity is dependent on calmodulin binding, which occurs at basal levels of Ca^{2+} and in the presence of L-arginine [22]. It is currently described that, during the inflammatory process, neutrophils and macrophages are responsible for the production of $^{\bullet}NO$. However, the concentration of nitrite/nitrate produced by neutrophils is substantially lower than that produced by macrophages and consequently it is yet unclear whether neutrophils produce $^{\bullet}NO$ in enough quantity to exert a pathological effect [23]. $^{\bullet}NO$ is an uncharged lipophilic molecule containing a single unpaired electron, making it reactive with other molecules. The reactions of $^{\bullet}NO$ can be resumed to three main processes that control the fate of $^{\bullet}NO$ in biological systems: [1] diffusion and intracellular consumption; [2] autooxidation to form nitrous anhydride (N_2O_3); and [3] reaction with $O_2^{\bullet-}$ to form ($ONOO^-$) (Eq. 20.6), a potent cytotoxic oxidant that has many biological targets: inactivating ion channels, damaging DNA, and nitrating tyrosine residues that can potentially inactivate enzymes and disrupt signal transduction [24]. $ONOO^-$ is known to exist in the relatively stable *cis*-conformation or to gain a proton to form peroxynitrous acid (ONOOH), $pK_a = 6.8$ (Eq. 20.7) [25].

$$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^- \qquad (20.6)$$

$$ONOO^- \underset{pKa=6.8}{\overset{H^+}{\leftrightarrow}} ONOOH \qquad (20.7)$$

The fate of $ONOO^-$ is not consensual due to its complex chemistry and the impact of carbon dioxide (CO_2). For instance, at pH 7.4, 80% of $ONOO^-$ will be in the anionic form; conversely, at pH 6.2 (e.g., inside phagocytic vacuole), up to 80% will be in the protonated form [26]. It was demonstrated that CO_2, ubiquitously present in biological systems, reacts catalytically with $ONOO^-$ resulting in the formation of nitrosoperoxycarbonate anion ($ONOOCO_2^-$). The homolysis of the O—O bond produces carbonate radical anion ($CO_3^{\bullet-}$) and (nitrogen dioxide radical) ($^{\bullet}NO_2$) as a caged radical pair. In this case, the relative kinetics of cage return and escape dictates that 67% of the $ONOOCO_2^-$ will decompose to NO_3^-, while 33% each of $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ become free radicals, able to react with suitable substrates, or in the absence of such, recombining to give NO_3^- and CO_2 (Eq. 20.8) [22]. Although this process is relatively well accepted in macrophages, in neutrophils these mechanisms remain to be fully defined [27]. Moreover, some studies have failed to show any $^{\bullet}NO$ production, and this could be related to the lower levels of $^{\bullet}NO$ produced by neutrophils or by the rapid reaction of $^{\bullet}NO$ to give $ONOO^-$ [28]. Interestingly, the reaction of $^{\bullet}NO$ with $O_2^{\bullet-}$ occurs even in the presence of SOD, indicating that it is extremely fast to

outcompete the enzyme-catalyzed dismutation and, as such, it is conceivable [26].

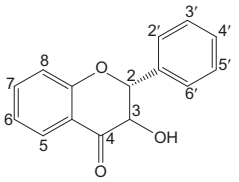
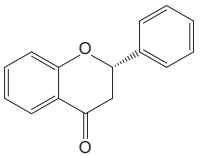
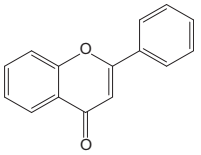
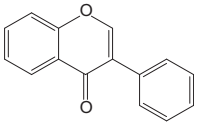
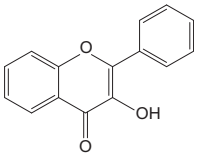
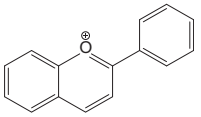


(20.8)

6 FLAVONOIDS

Polyphenols are secondary metabolites of plants involved in pigmentation, reproduction, and protection against pathogens. In 1930 a new substance was isolated from oranges. At that time, it was believed to be a member of a new class of vitamins and was designated as vitamin P. Later on, it became clear that this substance was a flavonoid (rutin) and until now more than 4000 varieties of flavonoids have been identified [29]. Flavonoids are the most abundant and most studied class of polyphenols and represent a class of molecules that are characterized based upon a 15-carbon skeleton consisting of two benzene rings (A and B) linked via a heterocyclic pyrane ring (C) [29]. They are synthesized through the phenylpropanoid pathway [30]. Based on the differences in the structure of the C ring, flavonoids can be classified into seven groups: flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols (catechins), and anthocyanidins [31]. The structural variation of flavonoids may come from various patterns of substitution through enzymatic reactions including hydroxylation, methoxylation, sulfonation, acylation, prenylation, or glycosylation. They can be found in almost all foods of vegetable origin and are present in high amounts in apples, onions, red wine, grapes, citrus fruits, tea, berries, and olive oil [32]. In Table 20.1 are represented the structures of the

TABLE 20.1 Subclasses and Prominent Flavonoids and Typical Food Sources [33]

Flavonoid subclass	Structure	Examples	Food sources
Flavanols		Catechin, gallocatechin, epicatechin	Teas, red grapes, and red wines
Flavanones		Naringenin, hesperetin, eriodictyol	Citrus foods
Flavones		Apigenin, luteolin	Green leafy spices
Isoflavones		Daidzein, genistein, glycitein, biochanin A	Soybeans, soy foods, and legumes
Flavonols		Kaempferol, myricetin, quercetin, isorhamnetin	Nearly ubiquitous in foods
Anthocyanidins		Cyanidin, delphinidin, pelargonidin	Red, purple, and blue berries

flavonoids' various subclasses and their prominent and typical food sources. It is reported that human intake of all flavonoids varies from a few milligrams to several hundreds of milligrams per day. Significant scientific evidence shows that flavonoids have many beneficial health effects for human beings. Among the biological properties of flavonoids, the antiinflammatory activity is attracting growing interest, the modulation of neutrophils' oxidative burst being an important part of that function [34] that will be discussed in the following section.

7 EFFECT OF FLAVONOIDS ON NEUTROPHILS' OXIDATIVE BURST

Herein we describe the effect of pure flavonoids, isolated and purified from natural sources or chemically synthesized, on neutrophils' oxidative burst. An extensive bibliographic search resulted in the finding of a large panel of flavonoids whose chemical structures and effects are described in Figs. 20.2–20.4.

From the analysis of the studies embedded in this research field, it is obvious that there are a myriad of experimental variations that condition the discussion and comparison of the results of the different studies. In this sense, Table 20.2 summarizes the type of stimulus most commonly used (phorbol 12-myristate 13-acetate [PMA], *N*-formylmethionine-leucyl-phenylalanine [fMLP], opsonized zymosan), which differ in their mechanisms of action. Table 20.3 summarizes the detection probes used in this type of studies, which can be selective or unselective, or even detect the intracellular and/or extracellular production of reactive species.

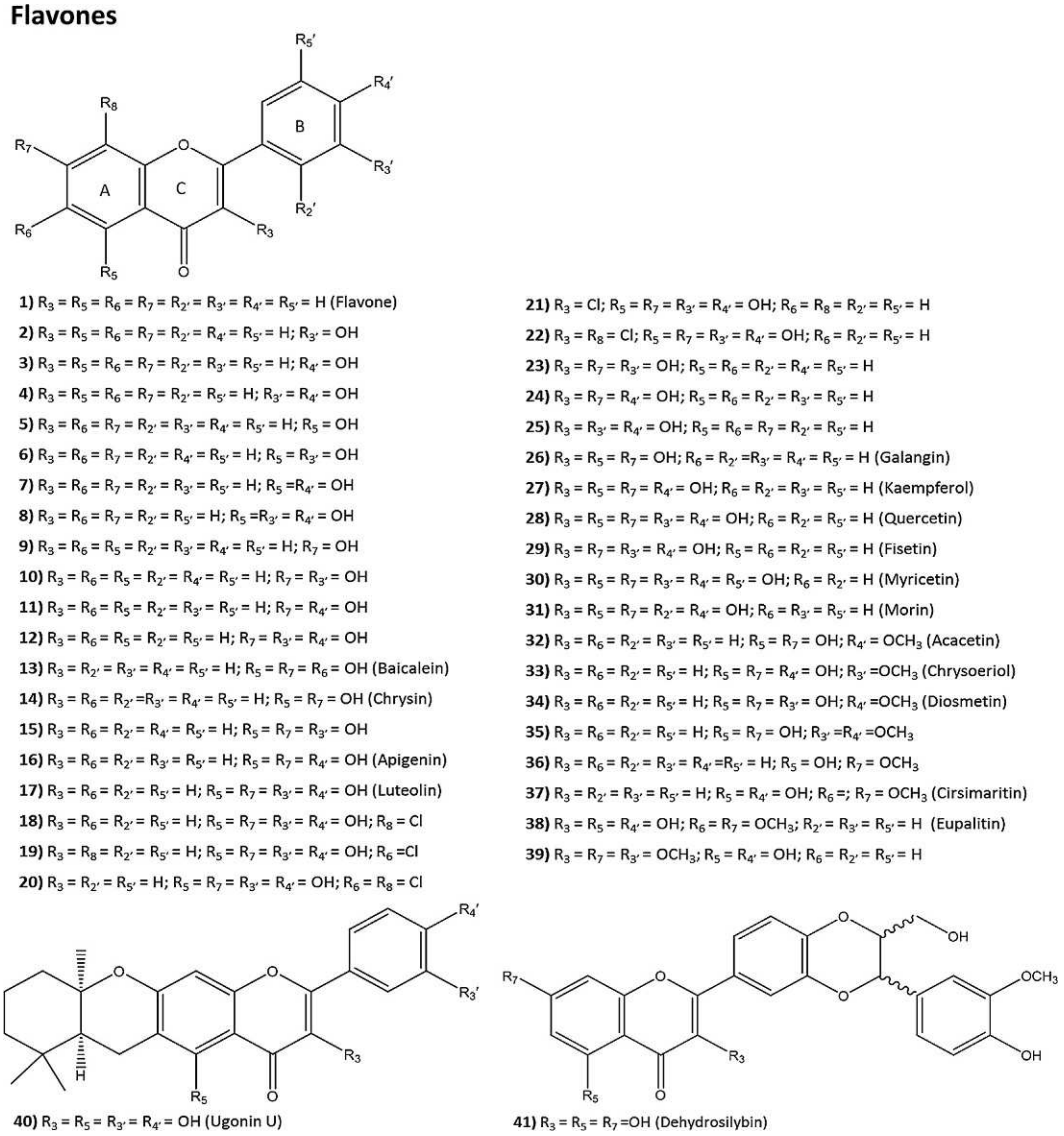
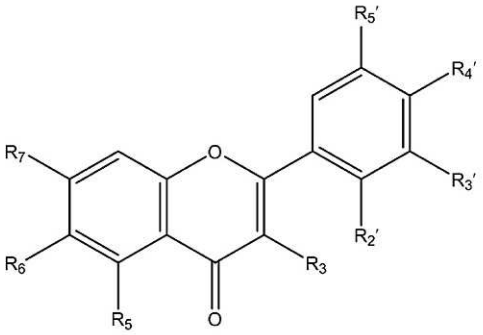


FIG. 20.2 Chemical structures of the flavones tested in the analyzed studies.

Glycosylated flavones



42) R_3 = *O*-rutinose; $R_5 = R_7 = R_{3'} = R_{4'}$ = OH; $R_6 = R_{2'} = R_{5'}$ = H (Rutin)

43) $R_3 = R_{2'} = R_{3'} = R_{4'} = R_{5'}$ = H; $R_5 = R_6$ = OH; R_7 = *O*-glucuronide (Baicalin)

44) R_3 = *O*-glucose-6''-acetyl; $R_5 = R_7 = R_{4'}$ = OH; $R_6 = R_{2'} = R_{5'}$ = H; $R_{3'} = OCH_3$ [Isorhamnetin-3-*O*-(6''*O*-acetyl)glucoside]

45) R_3 = *O*-rhamnoside; $R_5 = R_7$ = OH; $R_6 = R_{2'}$ = H; $R_{3'} = R_{5'}$ = H; $R_{4'}$ = OH (Afzelin)

46) R_3 = *O*-rhamnoside-methylgallate; $R_5 = R_7$ = OH; $R_6 = R_{2'} = R_{3'} = R_{5'}$ = H; $R_{4'}$ = OH (Pterogynoside)

47) R_3 = *O*-rhamnoside-methylgallate; R_5 = OH; $R_6 = R_{2'} = R_{3'} = R_{5'}$ = H; R_7 = *O*-rhamnoside; $R_{4'}$ = OH (Kaempferitrin)

48) R_3 = β-D-galactopyranoside; R_5 = OH; $R_6 = R_7 = OCH_3$; $R_{2'} = R_{3'} = R_{5'}$ = H; $R_{4'}$ = OH (Eupalitin-3-*O*-β-D-galactopyranoside)

49) R_3 = *O*-glucose-6''-galloyl; $R_5 = R_7 = R_{3'} = R_{4'}$ = OH; $R_6 = R_{2'} = R_{5'}$ = H [Quercetin-3-*O*-(6''*O*-galloyl)glucoside]

50) R_3 = O-glucuronide; $R_5 = R_7 = R_{3'} = R_{4'} = R_{5'}$ = OH; $R_6 = R_{2'}$ = H (Myricetin 3-*O*-β-D-glucuronide)

51) R_3 = *O*-glucuronide; $R_5 = R_7 = R_{3'} = R_{5'}$ = OH; $R_6 = R_{2'}$ = H; $R_{4'} = OCH_3$ (Mearsetin 3-*O*-β-D-glucuronide)

52) R_3 = *O*-glucuronide; $R_5 = R_7 = R_{3'} = R_{4'}$ = OH; $R_6 = R_{2'} = R_{5'}$ = H (Quercetin 3-*O*-β-D-glucuronide)

53) R_3 = *O*-glucuronide; $R_5 = R_7 = R_{4'}$ = OH; $R_6 = R_{2'} = R_{5'}$ = H; $R_{3'} = OCH_3$ (Isorhamnetin 3-*O*-β-D-glucuronide)

54) R_3 = *O*-glucuronide; $R_5 = R_7$ = OH; $R_6 = R_{2'} = R_{3'} = R_{5'}$ = H; $R_{4'} = OCH_3$ (Kaempferide 3-*O*-β-D-glucuronide)

55) R_3 = *O*-glucuronide; $R_5 = R_7 = R_{4'}$ = OH; $R_6 = R_{2'} = R_{3'} = R_{5'}$ = H (Kaempferol 3-*O*-β-D-glucuronide)

56) R_3 = *O*-2''-*O*-acetyl-glucuronide; $R_5 = R_7 = R_{4'}$ = OH; $R_6 = R_{2'} = R_{3'} = R_{5'}$ = H

57) R_3 = *O*-2''-*O*-acetyl-glucuronide; $R_5 = R_7 = R_{4'}$ = OH; $R_6 = R_{2'} = R_{5'}$ = H; $R_{3'} = OCH_3$

58) R_3 = *O*-2''-*O*-acetyl-glucuronide; $R_5 = R_7 = R_{3'} = R_{4'}$ = OH; $R_6 = R_{2'} = R_{5'}$ = H (Quercetin 3-*O*-β-(2''-*O*-acetyl- β-D-glucuronide)

59) R_3 = *O*-3''-*O*-acetyl-glucuronide; $R_5 = R_7 = R_{3'} = R_{4'}$ = OH; $R_6 = R_{2'} = R_{5'}$ = H (Quercetin 3-*O*-β-(3''-*O*-acetyl- β-D-glucuronide)

60) R_3 = *O*-3''-*O*-acetyl-glucuronide; $R_5 = R_7 = R_{4'}$ = OH; $R_6 = R_{2'} = R_{3'} = R_{5'}$ = H

FIG. 20.3 Chemical structures of the glycosylated flavones tested in the analyzed studies.

From the analyzed studies, luminol is the most commonly used detection probe (Table 20.4). Luminol detects several reactive species, in the extra- and intracellular medium (Table 20.3). This detection probe is useful due to its sensitivity and provides a good estimation of the general production of reactive species. However, if authors need to focus on one specific reactive specie, selective probes have to be assayed. Following luminol, the detection probes most frequently used by the authors are lucigenin and cytochrome c (Table 20.4). These probes selectively detect extracellular $O_2^{\bullet -}$, which is the first ROS produced by neutrophils in the cascade of the reactive species production. In fact, this type of probe shows the effect of flavonoids in the early events of the signaling cascade of reactive species production. However, it is important to understand the effect of these compounds in the later stages of reactive species production or even in other relevant enzymes, such as MPO. There are just a few authors that explore more specific and selective probes, such as APF and, for that reason, the effect of flavonoids in HOCl production, has not yet been accurately explored.

Table 20.4 summarizes the source, tested concentrations, stimulus, and the detection probe/method, as well as the effect(s) of all of the reported flavonoids. According to the table, it is clear that almost all flavonoids were able to modulate the neutrophils' oxidative burst. However, despite their undeniable activity, the establishment of an accurate structure-activity relationship is difficult due to several experimental variables, as already mentioned. Moreover, from the interpretation of the results it is also difficult to disclose the mechanism by which flavonoids modulate neutrophils' oxidative burst, since these compounds can act by:

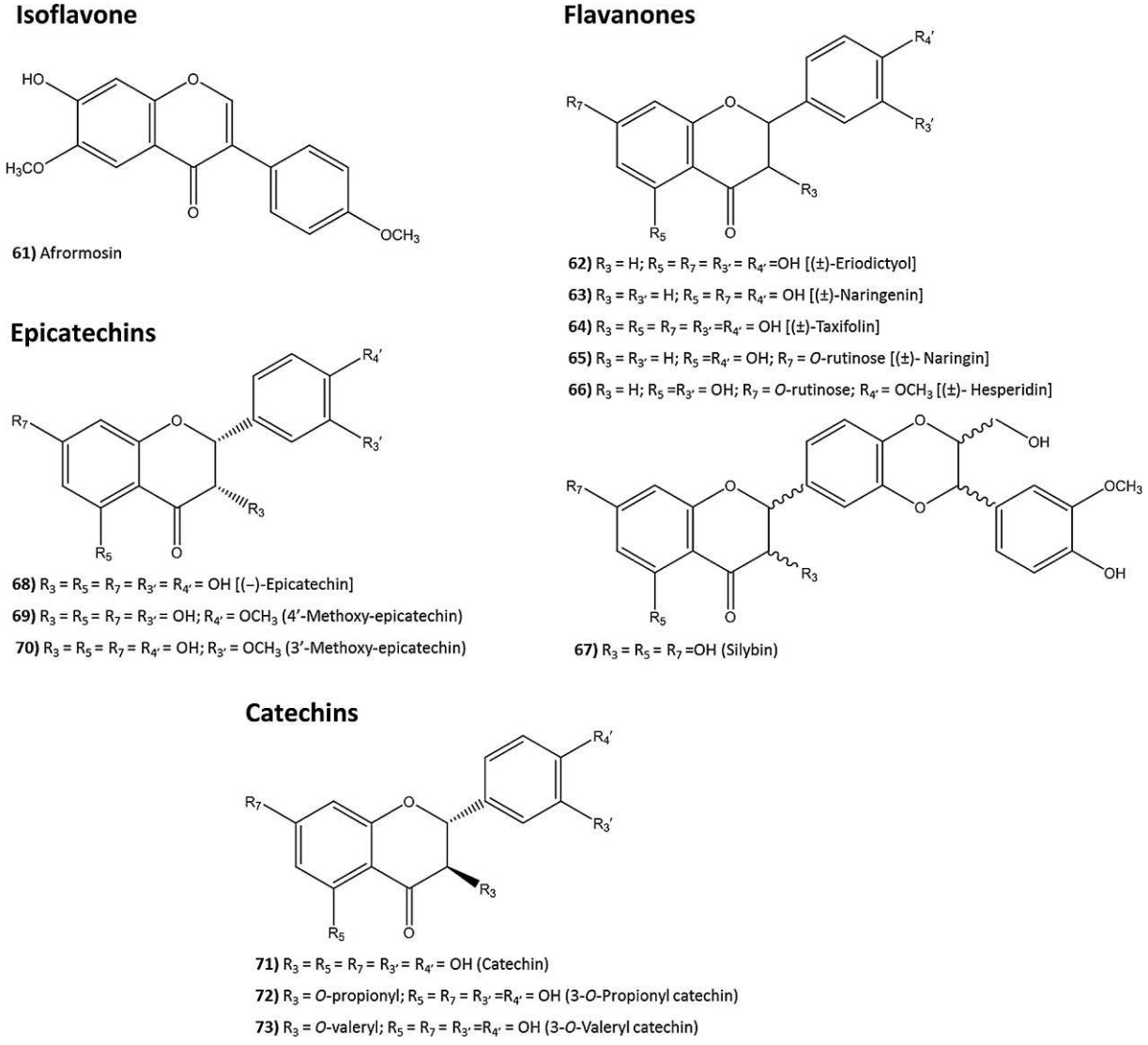


FIG. 20.4 Chemical structures of the isoflavone, flavanones, epicatechins, and catechins tested in the analyzed studies.

TABLE 20.2 Stimulus Used to Induce the Production of Reactive Species to Test the Modulatory Effect of Flavonoids on Neutrophils' Oxidative Burst

Stimulus	Tested concentrations	Mechanism of action	References
PMA	0–50 μM	Activates NADPH oxidase through redistribution of PKC and phosphorylation of several proteins, including the cytosolic NADPH oxidase component $p47^{phox}$. Two pools of NADPH oxidase, in the plasma membrane and in the specific granule membrane, can be regulated dependently or independently of phosphoinositide 3-kinase	[35,36]
fMLP	0–10 μM	Activates PLC and hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 1,4,5-triphosphate, the former being an activator of PKC	[37]
LPS	0–100 μg/mL	Up-regulates NADPH oxidase assembly by inducing flavocytochrome b558 translocation from the specific granules to the plasma membrane	[38]
Opsonized zymosan	0–2.5 mg/mL	It is an insoluble cell-wall preparation from the fungi *Saccharomyces cerevisiae*. Induces neutrophil NADPH oxidase activation by phosphorylation of $p47^{phox}$ on selective sites (Ser345, 328 and 315) or Rac2 activation	[39]

fMLP, N-formylmethionine-leucyl-phenylalanine; *LPS*, lipopolysaccharide; *NADPH*, nicotinamide adenine dinucleotide phosphate; *PKC*, protein kinase C; *PLC*, phospholipase C; *PMA*, phorbol 12-myristate 13-acetate.

TABLE 20.3 Detection Probes Used to Study the Modulatory Effect of Flavonoids Against Neutrophils' Oxidative Burst [40,41]

Probe	Tested concentrations	Cellular localization	Reactive species detected
FLUORESCENT			
DCFH-DA	5–300 μM	Intracellular	H_2O_2, $HO^{\bullet}$, $^{\bullet}NO$, $ONOO^-$
HE	10 μM	Intracellular	$O_2^{\bullet-}$
APF	1–5 μM	Intracellular	$HO^{\bullet}$, $ONOO^-$, HOCl
Amplex red	25 μM	Extracellular	H_2O_2
CHEMILUMINESCENT			
Lucigenin	25–200 μM	Extracellular	$O_2^{\bullet-}$
Luminol	60–1000 μM	Extracellular and intracellular	$O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$, HOCl, $^{\bullet}NO$, $ONOO^-$
Pholasin	0.7 nM	Extracellular	$O_2^{\bullet-}$, $^1O_2$, $HO^{\bullet}$, HOCl, $ONOO^-$
COLORIMETRIC			
Cytochrome c	40–200 μM	Extracelullar	$O_2^{\bullet-}$
Taurine	5–15 mM	Extracellular	HOCl
TMB	2.8 mM		Taurine chloramine and HOCl

Amplex red, *N*-acetyl-3,7-dihydroxyphenoxazine; *APF*, 2-[6-(4′-amino)-phenoxy-3H-xanthen-3-on-9-yl] benzoic acid; *DCFH*, DA-2′,7′-dichlorodihydrofluorescein diacetate; *HE*, hydroethidine; *TMB*, 3,3′,5,5′-tetramethylbenzidine.

TABLE 20.4 Summary of the Analyzed Studies of the Flavonoid Effects on the Modulation of Neutrophils' Oxidative Burst, Including Flavonoid Sources (Natural, Synthesis), Tested Concentrations, Type of Stimulus, Detection Probe Used, and the Produced Effects

Flavonoid(s)	Source	Tested concentrations	Stimulant	Probe	Effect	References
FLAVONES						
1, 5, 9, 11, 32	Synthesis/ commercial	0–100 μM	PMA	Luminol	NE	[42–44]
2–4, 6–8, 10, 12, 14–18, 20–26, 33–35, 28					↓ ROS production	
3				Lucigenin	NE	[43]
1–2, 4–12, 14–17, 32–35, 28					↓ $O_2^{\bullet-}$ production	
1–3, 5–7, 9, 11, 14, 15, 32–34				Amplex red	NE	[42–44]
4, 8, 10, 12, 16, 17,18, 20–26, 35, 28					↓ H_2O_2 production	
2, 3, 5–7, 9–11				APF	IM	[42–45]
14, 32,					NE	
1, 4, 8, 12, 15–26, 33–35, 22, 28					↓ HOCl production[a]	

Continued

TABLE 20.4 Summary of the Analyzed Studies of the Flavonoid Effects on the Modulation of Neutrophils' Oxidative Burst, Including Flavonoid Sources (Natural, Synthesis), Tested Concentrations, Type of Stimulus, Detection Probe Used, and the Produced Effects—cont'd

Flavonoid(s)	Source	Tested concentrations	Stimulant	Probe	Effect	References
13	Commercial	0–100 μM	fMLP/PMA	Lucigenin/ DCFH-DA	↓ ROS production, ↓ MPO activity and no effect on NADPH oxidase assembly	[46]
26–28, 30	Commercial	0–40 μM	Immune complex of ovalbumin and rabbit polyclonal anti-ovalbumin IgG	Luminol	↓ ROS production	[47]
				Lucigenin	↓ $O_2^{\bullet-}$ production, did not affect the NADPH oxidase activity and strongly inhibited MPO activity	
27, 28, 30	Commercial	0–0.1 μM	fMLP	Pholasin	↓ ROS production	[48]
27–31	Synthesis/commercial	0–10 μM	fMLP	Luminol	↓ ROS production (**29** > **28** > **31** > **30** > **27**)	[49]
28	Commercial	0–0.45 mM	PMA	Tau/TMB	↓ TauCl production	[50]
		0–100 μM	Opsonized zymosan	Ferricytochrome c	↓ $O_2^{\bullet-}$ production	[51]
				Taurine chlorination	↓ HOCl production	
			PMA	DCFH-DA	↓ ROS production (slight ↓ H_2O_2 production)	
		0–8 μM	fMLP, LPS	DCFH-DA	↓ ROS production	[52]
		0–100 μM	PMA	Lucigenin	↓ $O_2^{\bullet-}$ production (**41** > **28** > **64** > **67**)	[53]
		0–10 μM	fMLP	Luminol	↓ ROS production	[54]
36	Isolated from *Muntingia calabura* L.	0–1 μM	fMLP	Cytochrome c/ cytochalasin B	↓ $O_2^{\bullet-}$ production	[55]
			PMA		NE	
37	Isolated from *Eriodictyon californicum*	0–30 μM	fMLP	Ferricytochrome c	↓ $O_2^{\bullet-}$ production	[56]
39	Isolated from *Melicope semecarpifolia*	0–50 μM	fMLP	Ferricytochrome c/cytochalasin B	↓ $O_2^{\bullet-}$ production	[57]
			PMA		NE	
40	Isolated from *Helminthostachyszeylanica* (L) Hook	0–10 μM	–	Ferricytochrome c	↑ $O_2^{\bullet-}$ release	[58]
			fMLP	Hydroethidine/ Ferricytochrome c	↑ $O_2^{\bullet-}$ production and release	
41	Synthesis	0–100 μM	PMA	Lucigenin	↓ $O_2^{\bullet-}$ production (**41** > **28** > **64** > **67**)	[53]

TABLE 20.4 Summary of the Analyzed Studies of the Flavonoid Effects on the Modulation of Neutrophils' Oxidative Burst, Including Flavonoid Sources (Natural, Synthesis), Tested Concentrations, Type of Stimulus, Detection Probe Used, and the Produced Effects—cont'd

Flavonoid(s)	Source	Tested concentrations	Stimulant	Probe	Effect	References
GLYCOSYLATED FLAVONES						
42	Commercial	0–0.1 μM	fMLP	Pholasin	↓ ROS production	[48]
		0–150 μM	PMA	Luminol	↓ ROS production	[59]
43	Commercial	0–100 μM	fMLP/PMA	Lucigenin/ DCFH-DA	↓ ROS production, ↓ MPO activity and no effect in NADPH oxidase assembly	[46]
44	Isolated from *Solidago canadensis var. scabra*	0–100 μM	Opsonized zymosan	Ferricytochrome c	↓ $O_2^{\bullet -}$ production	[51]
				Taurine chlorination	NE	
			PMA	DCFH-DA	↓ ROS production (slight ↓ H2O2 production)	
49	Isolated from *Drosera aliciae* Hamet		Opsonized zymosan	Ferricytochrome c	↓ $O_2^{\bullet -}$ production	
				Taurine chlorination	↓ HOCl production	
			PMA	DCFH-DA	↓ ROS production (slight ↓ H_2O_2 production)	
46–47	Isolated from *Pterogyne nitens*	0–0.45 mM	PMA	Tau/TMB	↓ TauCl production	[50]
48	Isolated from *Boerhaavia diffusa*	0–500 μg/mL	PMA	DCFH-DA	NE	[60]
50–60	Isolated from *Polygonumaviculare* L.	0–10 μM	fMLP	Luminol	↓ ROS production	[54]
ISOFLAVONES						
61	Isolated from *Amburana cearensis* A.C. Smith	0–335.2 μM	PMA	Lumimol	↓ ROS production	[61]
				Lucigenin	↓ $O_2^{\bullet -}$ production	
FLAVANONES						
62–64	Synthesis/commercial	0–100 μM	PMA	Luminol	↓ ROS production	[43]
				Lucigenin	↓ $O_2^{\bullet -}$ production	
63				Amplex red	NE	
62, 64					↓ H_2O_2 production	
63				APF	IM	
62, 64					↓ HOCl production	

Continued

TABLE 20.4 Summary of the Analyzed Studies of the Flavonoid Effects on the Modulation of Neutrophils' Oxidative Burst, Including Flavonoid Sources (Natural, Synthesis), Tested Concentrations, Type of Stimulus, Detection Probe Used, and the Produced Effects—cont'd

Flavonoid(s)	Source	Tested concentrations	Stimulant	Probe	Effect	References
63, 65, 66				Luminol	↓ ROS production	[62]
			Opsonized zymosan	Ferricytochrome c	Only heperidin (**54**) significantly ↓ $O_2^{\bullet-}$ production	
64, 67			PMA	Lucigenin	↓ $O_2^{\bullet-}$ production (**41** > **28** > **64** > **67**)	[53]
EPICATHECHINS						
68	Isolated from cocoa extract	0–8 μM	fMLP, LPS	DCFH-DA	↓ ROS production (**55** > **28**)	[52]
69, 70	Synthesis				NE	
68	Commercial	0–150 μM	PMA	Luminol	↓ ROS production	[59]
CATECHINS						
71–73	Synthesis	0–100 μM	PMA	Luminol	↓ ROS production (**60** > **59** > **58**)	[63]
71	Isolated from cocoa extract	0–8 μM	fMLP, LPS	DCFH-DA	↓ ROS production (**58** > **28**)	[52]
	Synthesis/commercial	0–150 μM	PMA	Luminol	NE	[59]

NE—no effect, or less than 40% no significant activity, found under the tested conditions.
IM—Interference with the methodology.
DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate; *fMLP*, *N*-formylmethionyl-leucyl-phenylalanine; *MPO*, myeloperoxidase; *NADPH*, nicotinamide adenine dinucleotide phosphate; *PMA*, phorbol 12-myristate 13-acetate; *Tau*, taurine; *TauCl*, taurine chloramine; *TMB*, 3,3′,5,5′-tetramethylbenzidine.
[a] *In the work of Freitas et al. [42], flavone (**1**) depicted no activity.*

a) inhibition of key enzymes (NADPH oxidase, MPO, etc.);
b) scavenging of reactive species;
c) chelation of metals;
d) causing cell toxicity.

Flavonoids can also act only in one target (a, b, c, or d) or even in all the targets at the same time.

Analyzing Table 20.4, it is possible to observe that none of the authors studied the effect of flavonoids against neutrophil production of RNS. Actually, the concentration of nitrite/nitrate produced by neutrophils is low and consequently it is yet unclear whether neutrophils produce $^{\bullet}$NO in enough quantities to exert a pathological effect [23] or even to be detected. This assumption is corroborated by the fact that among all the authors cited in this chapter none of them studied the production of RNS.

Despite these difficulties, it is possible to generally infer the most favorable substitutions that render flavonoids good modulators of the neutrophils' oxidative burst. Our assumptions were based on reports that studied a panel of compounds, which allow us to infer a structure-activity relationship, and without cross comparisons (e.g., the same flavonoid studied by different authors).

8 CATECHOL GROUP IN THE B-RING

Several authors report that the *ortho* arrangement of the two hydroxyl groups (free catechol grouping) in the B-ring of the flavonoid core is essential for the modulatory effect of flavonoids against neutrophils' oxidative burst. Ribeiro et al. [43] studied a panel of 24 flavonoids against human neutrophils' oxidative burst, under the same experimental conditions, using different detection probes, luminol, lucigenin, amplex red, and APF. Focusing on luminol, it is possible to understand some important structure-activity relationships. Luteolin and chrysin

differ in the presence of the catechol group in the B-ring. Comparing the IC_{50} of luteolin (9.3 ± 1.2 μM) with chrysin (53.0 ± 4.6 μM), it is possible to infer that luteolin, with a catechol group in B-ring, has an almost six times lower IC_{50} than the obtained for chrysin, with no substitution in the B ring. The relevance of the presence of the catechol group was also demonstrated by the same authors, comparing flavonoid **1** vs. **4**, flavonoid **5** vs. **8**, flavonoid **9** vs. **12**, for all the detection probes used. Wang et al. corroborated these results reporting the following order of potencies: fisetin (**29**)>quercetin (**28**)>morin (**31**)>myrecitin (**30**)>kaempferol (**27**), in fMLP-stimulated neutrophils. These results showed that the presence of catechol on the B-ring seems to be an important feature, since both fisetin (**29**) followed by quercetin (**28**) were the most active compounds [49]. Also using glycosylated flavonoids, it is possible to support this finding. Zielinska et al. [51] studied the effect of quercetin (**28**) and its derivatives quercetin-3-*O*-(6″-*O*-galloyl)glucoside (**49**) and isorhamnetin-3-*O*-(6″-*O*-acetyl)glucoside (**44**). The less efficient compound was flavonoid **44**, which is deprived of the free catechol function due to the substitution of the C3′ hydroxyl group by a methoxyl group. Additionally, the similar activity of quercetin-3-*O*-(6″-*O*-galloyl)glucoside (**49**) and quercetin reinforces the idea that the the *ortho* arrangement of the catechol group in the B-ring is essential for the activity.

It is known that diffusion of compounds into the cell is a function of their lipophilicity, i.e., their ability to incorporate in the lipids of the cell membrane. According to Ribeiro et al. [43], replacement of a hydroxyl by a methoxyl group increases the lipophilicity, thus enhancing the inhibitory activity of chrysoeriol (**33**) (IC_{50} = 2.8 ± 0.5 μM) and diosmetin (**34**) (IC_{50} = 3.6 ± 0.4 μM) when compared with luteolin (**17**) (IC_{50} = 9.3 ± 1.2 μM). Flavone (**35**), which has two methoxyl groups in the B-ring, presented a low activity (IC_{50} = 57.6 ± 0.2 μM), which may indicate that the lipophilicity is not, by itself, determinant for the activity observed. In fact, the more hydrophobic flavonoids can partitionate in the hydrophobic core of the membranes and the more hydrophilic flavonoids interact by hydrogen bonding with the polar head groups at the lipid-water interface of membranes. This type of interaction may provide a level of protection for the bilayer from external and internal aggressors (i.e., oxidants) contributing to preserve the structure and function of biological membranes, namely by inhibiting the lipid oxidation chain reaction [43].

9 3-OH IN THE C-RING

The presence of a 3-OH in the C ring contributes to the modulatory effect of flavonoids against neutrophils' oxidative burst. Ribeiro et al. and Freitas et al. studied a panel of flavonoids, and among them, luteolin (**17**) and quercetin (**28**), using luminol, amplex red and APF as detection probes. Both flavonoids are structurally similar, except in the presence of 3-OH in the C-ring on quercetin (**28**). These studies proved that we should be careful with comparisons among studies with different experimental conditions. Ribeiro et al. reported that, using luminol and amplex red, luteolin (**17**) is more effective in decreasing the production of reactive species by neutrophils. In turn, Freitas et al. [42] demonstrated that quercetin is more potent than luteolin. These discrepancies could be related to the number of neutrophils used in the assay. Freitas et al. [42] used the double of the cells used by Ribeiro et al. and these could explain the differences found in the results. Corroborating this, in the assay of APF, both authors used the same density of cells, and obtained the same results, quercetin being more potent than luteolin, showing the relevance of the presence of 3-OH in the C ring. Taurine is one of the most abundant free amino acid in inflammatory cells, including neutrophils. Taurine chloramine (TauCl) is produced by the MPO system in activated neutrophils, during the inflammatory process. The activity of several flavonoids on ROS generation by neutrophils was assessed through TauCl production by neutrophils activated with PMA [50]. Quercetin (**28**) (IC_{50} = 7 μM) was the most potent inhibitor of TauCl formation, followed by pterogynoside (**46**) (IC_{50} = 17 μM) and kaempferitrin (**47**) (IC_{50} = 17 μM), whereas afzelin (**45**) caused less inhibition (IC_{50} = 45 μM). These results corroborate the idea that the 3-OH of the C-ring is favorable for the effect [50].

In fact, in 1988 Pincemail and coworkers [64] already stated that quercetin (**28**) inhibited MPO activity in a competitive type of inhibition. Rutin (**42**), the rutinoside of quercetin (**28**), was less active than its aglycone. Moreover, it is interesting to note that the substitution of a few OH groups by more hydrophilic groups dramatically lowers the inhibitory effect as compared to quercetin, which is a lipophilic compound. These results suggest that the hydrophobicity is an essential requirement for the inhibitory effect of quercetin (**28**). According to a docking study, quercetin (**28**) binds to the hydrophobic region at the entrance of the distal heme pocket of MPO, with the B-ring of quercetin being oriented to the heme center. These results also indicated that the B-ring hydroxyl group(s) may be important to enter in the pocket, reaching the active site of MPO [65].

Santos et al. compared galagin (**26**), kaempferol (**27**), quercetin (**28**) and myrcetin (**30**), and concluded that quercetin (**28**) was the most active compound, when lucigenin was used as the detection probe. These results show that quercetin (**28**) can also act at the beginning of the cascade of reactive species production, through

inhibition of NADPH oxidase or by scavenging $O_2^{\bullet -}$. However, the four flavonols did not decrease the oxygen consumption by stimulated neutrophils, so they do not act on the assembly or catalytic activity of the NADPH oxidase complex in neutrophils, suggesting that the scavenging effect against reactive species is the most probable mechanism [47].

Vuotto et al. studied three flavonoids: catechin (**71**), 3-*O*-propionyl catechin (**72**) and 3-*O*-valeryl catechin (**73**). The order of potencies for the inhibition of neutrophils' oxidative burst, measured with luminol, was *O*-valeryl catechin (**73**)>3-*O*-propionyl catechin (**72**)>catechin (**71**). The low effect of catechin (**71**) may be due to its limited diffusion into the cell. Since propionic and valeric acid increase the lipophilicity of catechin, these substitutions favor intracellular availability of the compounds, and therefore may favor their effectiveness. Moreover, the authors showed, by the use of specific inhibitors, that the extracellular chemiluminescence was similar among the compounds and the intracellular was greater with the esterified catechins [63]. Freitas et al. [42] studied the effect of a group of chlorinated flavonoids [flavone (**1**), luteolin (**17**), **18**, **20**, **21**, and quercetin (**28**)] due to the known reactivity of flavonoids with HOCl during an inflammatory process [66] against neutrophils' oxidative burst. Flavone **21**, with a 3-Cl substituent, was the one that demonstrated the best inhibition of the luminol-induced chemiluminescence ($IC_{50}=2.0\pm0.4\,\mu M$), followed by quercetin (**28**) ($IC_{50}=2.8\pm0.4\,\mu M$). It was reported that the combination of 3- and 5-OH groups with the 4-oxo group allows electron delocalization from the 4-oxo group to both substituents, favoring the free radical scavenging by flavonoids. Taking into account that flavonoids with a 3-Cl (**21**) or a 3-OH (**28**) in the C-ring presented almost the same effect, the electron delocalization could be favored not only by the presence of an OH in position 3 but also by a Cl in the same position. Interestingly, when the probes amplex red and APF were used, flavone **21** was more effective than quercetin (**28**). These results showed that 3-Cl substituent of the C-ring could be as or even more relevant than the 3-OH for the modulatory effect of flavonoids against H_2O_2 and HOCl produced during neutrophils' oxidative burst.

10 C2–C3 DOUBLE BOND

The relevance of the presence of a C2–C3 double bond for the inhibitory activity of flavonoids was demonstrated by Ribeiro et al. [43]. Flavonoids apigenin (**16**), luteolin (**17**) and quercetin (**28**) and their structurally related flavanones, without the C2–C3 double bond, (±)-naringenin (**63**), (±)-eriodictyol (**62**), (±)-taxifolin (**64**), respectively, were studied. In general, the flavonoids with the C2–C3 double bound were more active than their flavanones, where the double bond lacks, for all the detection probes used, luminol, lucigenin, amplex red, and APF. These results were corroborated by Zholobenko et al. [53], who reported a higher effectiveness of quercetin (**28**) than (±)-taxifolin (**64**)] in the inhibition of lucigenin-induced chemiluminescence. The most pronounced effect of quercetin (**28**) in comparison with (±)-taxifolin (**64**)] could be related to the less planar structure of (±)-taxifolin] (**64**), due to the lack of the C2–C3 double bond, which results in a higher ability to undergo inactivation through the formation of strong hydrogen bonds with macromolecules [43,53].

11 CONCLUSION

The information presented in this chapter provides evidence that flavonoids are strong inhibitors of neutrophils' oxidative burst. It is difficult to establish an accurate structure-activity relationship due to the variables in experimental conditions found among the studies, and, most of the time, the reduced number of flavonoids studied under the same experimental conditions. Despite that, it was possible to infer that a catechol group in the B-ring, the presence of the 3-OH in the C-ring, and the double bond between C2 and C3, are pivotal characteristics that renders flavonoids good modulators of the neutrophils' oxidative burst.

Therefore, flavonoids may constitute an alternative anti-inflammatory therapy, due to the proven ability to suppress the production of reactive species, one of the most relevant mechanisms engaged at the onset and progression of the inflammatory process.

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CHAPTER

21

Manipulation of Mitochondrial Function by Polyphenols for New Treatment Strategies

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Acronyms and Abbreviations

ACF	aberrant crypt foci
ALS	amyotrophic lateral sclerosis
AMPK	adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
CAT	catalase
DRP1	dynamin-1-like protein 1
EC	epicatechin
ECG	epicatechin gallate
EGCG	epigallocatechin gallate
EMRE	essential MCU regulator
ER	endoplasmic reticulum
ETC	electron transport chain
GPx	glutathione peroxidase
HUVEC	human umbilical vein endothelial cells
IGF-1	insulin-growth factor-1
IMM	inner mitochondrial membrane
IMS	intermembrane space
IP3	inositol-1,4,5 trisphosphate
IP3R	inositol-1,4,5 trisphosphate receptor
MAM	mitochondria-associated ER membranes
MCU	mitochondrial Ca^{2+} uniporter
MFN	mitofusin
MICU1	mitochondrial Ca^{2+} uptake 1
MICU2	mitochondrial Ca^{2+} uptake 2
mPTP	mitochondrial permeability transition pore
mtDNA	mitochondria deoxyribonucleic acid
NAD	nicotinamide dinucleotide
NADH	reduced nicotinamide dinucleotide
NRF	nuclear respiratory factor
OMM	outer mitochondrial membrane
OPA1	optic atrophy 1
PGC-1α	peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PINK1	PTEN-induced putative kinase 1
PTEN	phosphatase and tensin homolog
RNA	ribosomal ribonucleic acids
ROS	reactive oxygen species
RSV	resveratrol
SERCA	sarco/ER Ca^{2+} ATPases
SIRT1	sirtuin 1
SMAC	second mitochondria-derived activators of caspase
SOD	superoxide dismutase
TCA	tricarboxylic acid cycle
TFAM	mitochondrial transcription factor A
VDAC	voltage-dependent anion channel

1 INTRODUCTION

A constantly increasing human lifespan brings the urgent need for new treatment strategies to fight cancer and the various cardiovascular and neurodegenerative diseases associated with aging. Many of these illnesses, such as Alzheimer's and Parkinson's disease, are still incurable, and the development of drug resistance, e.g., in cancer treatment, makes even the treatment of healable diseases very difficult [1]. To understand the difficulties in treatment of these diseases, it is indispensable to reveal the molecular mechanisms that form the basis for development of the diseases and their progression. Within the cell, mitochondria are in a powerful position to modulate physiological and pathophysiological processes. As cellular power plants and executors of programmed cell death, they offer numerous targets to control and modulate the function of healthy or damaged cells. Mitochondria house the most efficient production site for adenosine triphosphate (ATP), yielding about 15 times more ATP than glycolysis within the cell. Besides energy supply, they control and crucially contribute to intracellular signaling, modulate Ca^{2+} homeostasis, and initiate

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apoptosis via the mitochondrial apoptotic pathway. Despite their unique and central role within the cell, they have until recently rarely been used as drug targets. Polyphenols are a diverse group of compounds that were shown to directly or indirectly interfere with mitochondrial proteins and mitochondrial function. in vitro as well as in vivo experiments revealed a wide range of actions. For instance, the most prominent representative of polyphenols, resveratrol (RSV), was shown to display cytoprotective as well as cytotoxic effects in vitro and in vivo in a dose-dependent manner, making this compound a promising drug against neurodegenerative and cardiovascular diseases as well as cancer [2]. However, conclusive clinical trials are still rare and are challenged by the poor bioavailability of polyphenols as well as strong interindividual variance in the metabolism [3].

In this work we give an overview of the mitochondrial targets for different polyphenols, including RSV, quercetin, tea catechins and curcumin, and discuss how these compounds manipulate mitochondrial function in vivo and in vitro. Moreover, we will conclude how these mitochondrial modulations can contribute to the treatment of cardiovascular and neurodegenerative diseases as well as cancer and consider the limitations that must be overcome to establish these compounds successfully as drugs.

2 MITOCHONDRIA

2.1 Origin and Structure

Eukaryotic cells are characterized by a compartmentalization that allows them to fulfill multiple tasks, including protein synthesis, processing of nutrients, and energy production. Mitochondria have evolved from cellular invaders into irreplaceable cellular compartments. Their double membrane and their own 16-kb mitochondrial deoxyribonucleic acid (mtDNA), encoding 13 protein subunits of the mitochondrial electron transport chain and F_0F_1-ATP synthase as well as components of ribosomal ribonucleic acids (RNA) and transfer RNAs, offer evidence for their independent existence more than a billion years ago [4]. However, mitochondria underwent a genetic exchange with their host, ending up with 85% of all mitochondrial proteins encoded in the nucleus. Consequently, the independent existence of mitochondria became impossible, and mitochondria turned out to be the perfect match for the vast majority of eukaryotic cells, allowing their hosts to use nutrients more efficiently [5]. Besides energy metabolism, mitochondria have established a crucial role in apoptosis, cell differentiation, cellular signaling, and Ca^{2+} homeostasis [2]. The structure of mitochondria is well adapted to fulfill these diverse duties, and undergoes constant changes to meet the actual demand. The two membranes of the mitochondrial double membrane consist of an outer mitochondrial membrane (OMM), linking mitochondria to other organelles and containing many passive and nonselective transporters that loosely regulate the transport of molecules from the cytosol into the intermembrane space (IMS) [6], and an inner mitochondrial membrane (IMM) with a strongly restricted transport system [7]. The IMM infolds into the mitochondrial matrix as cristae structures. The IMM protein optic atrophy 1 (OPA1) stabilizes cristae structure and is required for fusion of IMMs, while mitofusins 1 (MFN1) and (MFN2) mediate the fusion of the OMMs. In contrast, dynamin-1-like protein 1 (DRP1) induces the process of mitochondrial fission via translocation from the cytosol to the OMM and interaction with proteins like human fission protein-1. A continuous interplay between fusion and fission allows mitochondria to react spontaneously to occurring events [8]. Moreover, the amount of mitochondria within a cell is regulated constantly by the balance of mitochondrial biogenesis and mitophagy, which degrades mitochondria to prevent accumulation of damaged organelles. Mitochondrial biogenesis is a multistep process that involves mtDNA transcription and translation as well as translation of transcripts from the nucleus. Furthermore, newly synthesized proteins and lipids are imported and assembled in the expanding mitochondrial network. These processes are crucially orchestrated by the peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α), which regulates the activity of several transcription factors linked to mitochondrial biogenesis and function, such as the nuclear respiratory factors (NRF1 and NRF2). PGC-1α itself is strongly regulated by metabolic sensors and modulators, including adenosine monophosphate-activated protein kinase (AMPK) and sirtuin 1 (SIRT1), which link mitochondrial content to the current energy demand of the cell. In contrast, clearance of damaged mitochondria was also found to be essential for the maintenance of mitochondrial function and its importance was demonstrated by mitophagy-induced extension of lifespan [9].

2.2 Function

Mitochondria have a wide range of tasks to fulfill within the cell. They supply cellular energy, control cellular signaling and differentiation, sequester and buffer Ca^{2+} ions, and contribute to the process of apoptosis [2].

As cellular power plants, mitochondria are responsible for the cell's most efficient production of ATP via the mitochondrial respiration chain [10]. Oxidative decarboxylation of pyruvate or beta oxidation yield acetyl

coenzyme A, which fuels the tricarboxylic acid (TCA) cycle in the mitochondrial matrix to produce the nicotineamide adenine dinucleotide (NAD) and flavin adenine dinucleotide. These reducing equivalents feed electrons into the electron transport chain (ETC) at the IMM, where the electron transport is coupled to proton pumping from the mitochondrial matrix to the IMS. The electrochemical gradient thereby produced is used for the proton transport from the IMS into the matrix through F_0F_1-ATP synthase, causing a release of energy and phosphorylation of adenosine diphosphate to ATP [11]. If electrons are incompletely transferred to complex I or complex III of the ETC, reactive oxygen species (ROS) are generated [12].

These by-products of mitochondrial respiration work as critical signaling molecules and regulate various biological processes, including autophagy, stem cell proliferation, and hormone signaling [13]. Moreover, low levels of mitochondrial ROS evoke life-prolonging effects through the development of an antioxidant defense shield [14]. In contrast, high ROS levels harm mtDNA and proteins [15] and trigger the initiation of cellular senescence [15]. Consequently, various enzymes ensure the neutralization of ROS, including superoxide dismutases (SODs), catalases (CAT), glutathione peroxidases (GPx) and peroxiredoxins [16].

Mitochondria's ability to sequester Ca^{2+} from the cytosol upon either Ca^{2+} influx through plasma membrane Ca^{2+} channels [17] or Ca^{2+} release from the endoplasmic reticulum (ER), the biggest internal Ca^{2+} store [18], is another crucial signaling function. The Ca^{2+} exchange between mitochondria and the ER happens in specialized regions with so-called mitochondria-associated ER membranes (MAMs), which ensure proximity between these two organelles [19]. The Ca^{2+} transfer from the cytosol into the lumen of the ER is accomplished by the sarco/ER Ca^{2+} ATPases (SERCAs) at the expense of ATP hydrolysis. Various ER Ca^{2+} release channels, including the inositol-1,4,5-trisphosphate receptors (IP3Rs), ryanodine receptors and presenilins, act as signaling counterparts [20]. Once cytosolic Ca^{2+} levels rise, mitochondria take up Ca^{2+} into the IMS via the nonselective voltage-dependent anion channel (VDAC) in the OMM [10]. In contrast, the mitochondrial Ca^{2+} uptake through the IMM is strictly controlled by a large complex of proteins. The mitochondrial Ca^{2+} uniporter (MCU) forms the pore in the IMM and forms heterooligomers with its negative regulator mitochondrial Ca^{2+} uniporter b [21]. The essential MCU regulator (EMRE) is linked to the Ca^{2+}-sensing EF-hand-containing proteins mitochondrial Ca^{2+} uptake 1 (MICU1) and mitochondrial Ca^{2+} uptake 2 (MICU2), which fine-tune the activity of MCU [22]. Ca^{2+} uptake by mitochondria is a powerful regulator of Ca^{2+}-dependent enzymes in the cytosol as well as in mitochondria, where Ca^{2+} boosts the activity of Ca^{2+}-dependent dehydrogenases of the TCA cycle and thereby mitochondrial metabolism [23]. To avoid mitochondrial Ca^{2+} overload-induced apoptosis, tight control of mitochondrial Ca^{2+} uptake and release through the mitochondrial Na^+/Ca^{2+} exchanger is essential [24].

As executors of programmed cell death, mitochondria react to death-inducing stimuli like oncogene activation or metabolic disorder. Disruption of OPA1 heterooligomers by proapoptotic B-cell lymphoma 2 (Bcl-2) family members causes widening of cristae junctions and thereby enhanced permeability [25]. In parallel, this induces the release of second mitochondria-derived activators of caspases (SMACs) and cytochrome c into the cytosol via a still largely uncharacterized mechanism [26]. While SMACs deactivate proteins that inhibit apoptosis and thereby indirectly induce the activation of caspases, cytochrome c forms the apoptosome complex with apoptotic protease activating factor 1 and ATP, which causes cleavage of pro-caspase 9 into its active form caspase-9. This initiator caspase initiates a chain reaction, culminating in the activation of executioner caspases, which degrade cellular compartments [27].

2.3 Mitochondria's Role in Disease and Aging

2.3.1 Aging and Cardiovascular Diseases

The hallmark of aging is a progressive decline in the ability of the cell or organism to counteract stress, damage, and disease, resulting in impaired physiological function, pathologies, and death [28]. Cardiovascular diseases such as stroke, coronary artery diseases, or hypertension, are still the leading causes of death in the Western world. Mitochondria were found to be key determinants in age-related pathological alterations of the heart and the vascular system by mitochondrial oxidative stress or altered signaling processes [29]. Interestingly, mice overexpressing catalase in mitochondria were protected from the phenotypes of cardiac aging such as impairment of myocardial performance, and lived significantly longer [29]. Several reports suggest biphasic alteration of mitochondrial activity during aging [15], including a reduced ratio of oxidized to reduced nicotinamide adenine dinucleotide in rhesus monkeys during middle age and a subsequent increase in old age [30]. Notably, an increase in mitochondrial activity might trigger the production of harmful ROS levels affecting mitochondrial and nuclear DNA as well as protein function, ultimately leading to cellular dysfunction and pathologies [31]. In support of this hypothesis, lifespan-prolonging interventions, such as caloric restriction, are frequently linked to a decrease in overall energy production [32]. Furthermore, reduction of mitochondrial

number in vivo has been reported to reduce the spectrum of senescence effectors and phenotypes in mice [33], highlighting the crucial role of mitochondria in cellular senescence and their potential to serve as a target for therapeutic interventions against age-related diseases. Moreover, changes in mitochondrial dynamics in fusion and fission as well as alterations in the mitochondrial membrane potential have been reported to induce cellular dysfunction during senescence [31]. Hence, changes in mitochondrial Ca^{2+} homeostasis as well as in the ER-mitochondria crosstalk have been reported to reduce the capacity of cells to adapt to stress and increase vulnerability to age-related diseases [34], again emphasizing the need for therapeutic strategies to improve mitochondrial function.

2.3.2 *Neurodegenerative Diseases*

The heterogeneous group of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS), is characterized by a progressive degeneration of the central nervous system [35]. Although representing just 2% of the body weight, the brain consumes 20% of the total body O_2, highlighting the critical dependency of brain function on O_2 supply [36] and the high rate of mitochondrial respiration within the brain. Therefore, mitochondrial dysfunction is an ever-present issue in neurodegenerative disorders. Notably, the by-product of mitochondrial respiration, ROS, was found to be a main factor for neuronal loss and damaged mtDNA, associated with mitochondrial dysfunction and neurodegeneration [35]. Moreover, mutations of mitochondrial proteins were found to be critical in neurodegenerative diseases. For instance, mutations occurring in Parkinson's disease disrupt the interplay between mitochondrial phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) and parkin, shown to regulate the clearance of dysfunctional mitochondria by mitophagy [37]. Furthermore, numerous mutations in cytosolic copper-zinc SOD were found in ALS and linked to an induction of respiratory deficiencies. In addition, a decrease in the signaling of PGC-1α signaling, usually promoting mitochondrial activity and biogenesis, was found in the skeletal muscle of ALS patients [35]. These examples demonstrate that mitochondrial dysfunction often forms the molecular basis for the development and/or progression of neurodegenerative diseases.

2.3.3 *Cancer*

Despite the huge variety of cancer types, cancer cells share molecular characteristics such as loss of cell cycle control and changed metabolism, resulting in uncontrolled proliferation, invasion, and metastasis [38]. In the 1920s, Otto Warburg proposed that a dysfunction of mitochondria rewires the cancer cell's metabolism toward increased glucose uptake and fermentation of glucose to lactate. Indeed, the high glucose uptake of cancer cells associated with the "Warburg effect" forms the basis for tumor imaging by fluorodeoxyglucose-positron emission tomography. Nevertheless, proper mitochondrial function was found to be absolutely central in various cancer types [39]. This might be due to a less active form of pyruvate kinase isoenzyme M2 in cancer cells, causing a reduced conversion of phosphoenolpyruvate to pyruvate. This bottleneck leads to an accumulation of intermediate products like nucleic acids, phospholipids, and serins, contributing to uncontrolled proliferation of cancer cells [40], but makes mitochondrial ATP production essential. Under these conditions, amino acids and fatty acids were suggested to fuel the TCA cycle and thereby boost respiration [40]. Indeed, cancer cells were found to increase their uptake of glutamine and/or even enhance glutamine synthesis [39]. Consistent with these findings, cancer cell survival was strongly dependent on proper mitochondrial Ca^{2+} supply from the ER [41], possibly by fueling Ca^{2+}-dependent dehydrogenases of the TCA cycle in the mitochondrial matrix [42]. Notably, it was recently also demonstrated that ER-mitochondria tethering was enhanced in cancer cells [43]. Consistent with this, tumor suppressors like p53 were found to manipulate the interaction between mitochondria and ER [44,45]. Moreover, experiments in hybrid cells demonstrated that normal cytoplasm, containing mitochondria from nontumorigenic cells, could suppress the malignant phenotype of tumor cells [46], which highlighted the fundamental role of mitochondria in the development and progression of cancer.

3 POLYPHENOLS

3.1 Occurrence in Nature

Currently more than 8000 compounds of the diverse group of polyphenols, characterized as compounds with at least one phenol function, are known. These include flavonoids like genistein, catechin, epicatechin (EC), quercetin, and kampferol as well as nonflavonoid polyphenols, such as RSV, curcumin, and rosmarinic acid [47]. Polyphenols are largely abundant in fruits, vegetables, and cereals. For instance, fruits like grapes and berries contain up to 200–300 mg polyphenols in 100 g fresh weight and one glass of red wine holds about 100 mg polyphenols. Notably, polyphenols are produced to protect plants against ultraviolet radiation and pathogens [48]. Since mitochondria are descendants of ancient bacteria, it seems consistent that these organelles are strongly affected by polyphenols. The polyphenols'

modes of action have been studied intensively in in vivo and in vitro approaches, revealing antioxidant, antiinflammatory, and antiaging effects, but also cytotoxic effects, which makes them promising compounds in the treatment of neurodegenerative diseases, cardiovascular diseases, and cancer [2]. Nevertheless, in clinical trials these effects are often weak, and interindividual variability is rather high. This might be due to a poor bioavailability of polyphenols and interindividual variances in the metabolism of polyphenols, for instance by different gut microbiota composition or metabolic status [3].

3.2 Characteristics and Bioavailability of Polyphenols

Resveratrol: As the most popular representative of the large group of polyphenols, the red wine ingredient RSV has received special attention in numerous in vitro and in vivo studies. Besides in *Vitis vinifera*, this polyphenol was found in peanut root, strawberry, blueberry, and mulberry. Numerous human clinical trials aimed to understand RSV's impact on various diseases and the human health span have also been conducted. Even rather high doses of RSV were proven to be safe, but RSV's bioavailability was found to be very low. The major metabolites, glucuronide and sulfate conjugation of *trans*-RSV, were found to increase RSV's aqueous solubility and thereby reduce the flux across membranes, increasing excretion by the kidney. However, these metabolites of RSV were also speculated to have similar effects as RSV and, therefore, possibly also contribute to RSV's impact [49].

Quercetin: Quercetin is a flavonoid compound present in various plants, including the trees *Sophora japonica L.* and *Crataegus pennatifida bunge.* Similar to RSV, quercetin has also been considered a potential drug against various diseases, but the clinical use of this compound is limited due to its poor water solubility and instability in physiological media, resulting in poor bioavailability. Therefore, attempts to overcome this issue are under investigation. For instance, nanomicelle delivery systems were developed to solubilize quercetin in aqueous media and reach the clinically relevant concentration and targets [50].

Tea catechins: Catechins found in green tea, *Camellia sinensis,* are EC, epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). Most catechins are metabolized in the gut to phase I and phase II metabolites, making their bioavailability low, which was also confirmed by a pharmacokinetic study in rats. Consequently, new pharmaceutical forms have been tested, including a nanoemulsion, which resulted in increased bioaccessibility of the compounds in comparison to aqueous solution [51].

Curcumin: Curcumin is a natural yellow phenolic compound, synthesized in many different plants, including turmeric, *Curcuma longa*. While it has been used as a spice and dietary supplement for centuries, its pharmacological activities are currently of major interest. Curcumin was found to be well-tolerated at a dose of 12 g/day, but due to its very low aqueous solubility of 0.6 μg/mL and its susceptibility to degradation under alkaline conditions, the compound's bioavailability is very low. Therefore, new pharmaceutical forms are needed to improve its poor pharmaceutical properties. For instance, nanocarriers may be used to increase curcumin's bioavailability and to target tumors, an approach that is currently under intensive investigation in several clinical trials [52].

4 MANIPULATION OF MITOCHONDRIAL FUNCTION BY POLYPHENOLS

4.1 Mitochondrial Respiration and ATP Production

The production of ATP via mitochondrial respiration is the central task of mitochondria. Consequently, changes in mitochondrial respiration activity have been associated with various dysfunctions and diseases [53]. Notably, the machinery of mitochondrial respiration offers numerous targets for interaction with compounds, and many chemicals, including oligomycin and antimycin A, are known to strongly manipulate the functionality and activity of mitochondrial respiration [54]. Polyphenols have also been demonstrated to have a direct and indirect impact on mitochondrial respiration, which extensively impacts cellular function and dysfunction (described in Section 5). Notably, many polyphenols induce opposing effects on mitochondrial respiration dependent on the concentration used. This might be due to compensatory mechanisms. For instance, inhibition of a protein by low levels of polyphenols might lead to the protein's up-regulation and consequently to a measurable increase in its activity [2].

Resveratrol: The effects of RSV on the mitochondrial respiration machinery were found to be strongly dependent on the dosage. While low levels of RSV (1–5 μM for 48 h) triggered the activity of complex I and caused an elevated ratio of oxidized to reduced nicotinamide dinucleotide (NAD^+/NADH) ratio in immortalized human hepatoblastoma cells HepG2, high concentrations of RSV (>50 μM) hampered complex I activity in the same cell type. Consistent with this finding, 100 μM of RSV was found to inhibit complex III. While manipulation of complex I and III were described as indirect, RSV was demonstrated to bind to the F_1 subunit of the F_0F_1-

ATP synthase directly. An RSV dosage in the pM–nM range caused activation while higher concentrations inhibited this enzyme, again highlighting the critical role of concentration. Consistent with these findings, 10 μM RSV was reported to cause an initial increase in mitochondrial ATP levels, while 100 μM of RSV resulted in a strong mitochondrial ATP drop after only several minutes [2].

Quercetin: Similar to RSV, it was proposed that quercetin binds to the F_1 subunit of the F_0F_1-ATP synthase. Quercetin inhibited F_0F_1-ATP synthase (55–65 μM) as well as complex I (10–100 μM) and complex III activity (IC_{50} = 5.49 nM) in rat brain mitochondria [55]. Consistent with this finding, quercetin (50 μM) was found to inhibit mitochondrial respiration and significantly decrease ATP levels in rat liver mitochondria [56].

Tea catechins: EGCG (IC_{50} = 17 μM) and ECG (IC_{50} = 45 μM) were also found to be potent inhibitors of rat brain F_0F_1-ATP synthase, while catechin, EC, and epigallocatechin had little effect on the activity of this enzyme. Notably, addition of a phenolic group to catechins converted inactive catechins to active catechins, suggesting that two or more phenolic structures are needed to inhibit F_0F_1-ATP [57].

Curcumin: Curcumin (IC_{50} = 45 μM) was found to inhibit F_0F_1-ATP synthase activity in rat brain mitochondria as well [57]. Moreover, curcumin treatment (25 μM, 2 h) increased the activity of cytochrome c oxidase in PC12 cells [58].

4.2 ROS Production/ROS Defense

Mitochondrial respiration is one of the main production sites for ROS, and manipulation of complex I and complex III activity has been proven to induce mitochondrial ROS production [12]. Consequently, the polyphenols' interaction with constituents of the mitochondrial respiration machinery and the partly opposing effects on the complexes' activities is also likely to affect ROS levels. Indeed, polyphenols were reported to display pro- as well as antioxidant features in vitro and in vivo. The opposing effects might be dependent on the applied concentration, the treated cell type, and the individual ability of a cell to develop ROS defense mechanisms [2]. The polyphenols' positive impact on cardiovascular and neurodegenerative diseases was often reported to be associated with the antioxidant features of polyphenols, as described in Section 5.

Resveratrol: Low concentrations of RSV were linked to a moderate boost in ROS formation and an increased development of ROS defense mechanisms, displaying cytoprotective effects. For instance, 5 μM RSV treatment for 2 h attenuated oxidative stress and neoplastic transformation triggered by the carcinogenic compound of cigarette smoke, benzo[a]pyrene, in Bhas 42 fibroblasts. Moreover, 20 μM RSV protected renal proximal tubule cells MRK52E from nicotine-triggered oxidative injury by enhancing the activity of mitochondrial manganese SOD (MnSOD) [2]. In contrast, treatment with 50 μM RSV for 24 h resulted in oxidative stress-related damage and reduced viability of hepatic stellate cells GRX. Notably, in the same experiment, SOD activity increased again and cytotoxicity was attenuated after 120 h of 50 μM RSV [59], highlighting the importance of the development of an antioxidant defense shield for RSV's antioxidant effect.

Quercetin: Quercetin was reported to increase ROS levels in various cancer cells in amounts sufficient to induce apoptosis [60]. For instance, treatment for 72 h with 80 μM quercetin significantly increased levels of superoxide and H_2O_2 levels in HepG2 and HA22T hepatoma cells, further enhancing the ROS-induced apoptosis of paclitaxel [61]. In contrast, quercetin protected against the ROS-induced toxicity of gentamicin by inducing ROS defense via the enhancement of SOD and CAT activities in whole blood of rats [62]. In this case the compound's low bioavailability may have allowed it to have only reached concentrations sufficient to induce ROS defense by low levels of ROS production. Moreover, quercetin was also suggested to scavenge ROS directly in concentrations of 5 to 50 μM because this compound is equipped with two pharmacophores with optimal configurations for free radical scavenging [63].

Tea catechins: Modest concentrations of nM to 10 μM of EGCG were found to produce low levels of intracellular ROS, which promote cellular protective mechanisms. In contrast, cytotoxic concentrations of EGCG in tumor cells were linked to high levels of H_2O_2. For instance, 50 μM EGCG generated about 1 μM of H_2O_2 in Jurkat cells, contributing to the induction of apoptosis in this cell type [64]. Moreover, green tea catechins (100 μM, 48 h), including EGCG and ECG, also initiated mitochondrial apoptotic pathways by boosting ROS formation [65].

Curcumin: Curcumin was also found to have prooxidant as well as antioxidant effects. For instance, treatment with 25 μM curcumin for 2 h did not raise ROS levels, but it did significantly increase SOD activity in the mitochondrial fraction of PC12 cells and altered glutathione metabolism [58]. Moreover, another study highlighted a strong cell-type-dependent effect. While 50 μM curcumin had no effect in normal rat hepatocytes, this same treatment induced apoptosis in a time-dependent manner in various cancer cells, including MCF-7, MDA-MB, and HepG2 cells, by overwhelming production of ROS [66].

4.3 Mitochondrial Biogenesis and Mitophagy

A well-controlled balance between mitochondrial biogenesis and mitophagy ensures proper mitochondrial content to sustain energy metabolism in response to various conditions. The disequilibrium of these two mechanisms is consequently often linked to the onset and progression of pathological conditions like neurodegenerative or cardiovascular diseases [9]. By induction of specific pathways, including AMPK, SIRT1, and PGC-1α, polyphenols were reported to induce mitochondrial biogenesis and attenuate the cellular dysfunction [67], for instance in neurodegenerative diseases (described in Section 5). In contrast, it was also shown that polyphenols trigger mitophagy at certain concentrations [68], again pointing to the strong cell-type- and concentration-dependency of these compounds.

Resveratrol: RSV treatment (10 μM) was found to significantly increase mitochondrial number and mtDNA content in human umbilical vein endothelial cells (HUVECs). This concentration of RSV was found to boost the activity of SIRT1 and mitochondrial biogenesis factors, including PGC-1α, mitochondrial transcription factor A (TFAM) and NRF1 in HUVECs as well as in human coronary arterial endothelial cells (CAECs). Moreover, a similar mechanism was described for colon carcinoma cell line SW620, in which 10 μM of RSV initiated up-regulation of PGC-1α, TFAM and NRF1 via sirtuin 3. Importantly, higher concentrations of RSV (50 μM) resulted in autophagy of HMrSV5 human peritoneal mesothelial cells. Also mitochondrial ultrastructure was affected by RSV in a concentration-dependent way: A highly branched mitochondrial network was established via increased MFN2 expression in C2C12 myoblasts, in P3 prostate cancer cells as well as in mouse embryonic fibroblasts after treatment with 10–20 μM RSV. Consistent with this, RSV treatment with a concentration of 1–10 μM for 24 h increased the expression of mitochondrial fusion proteins, including MFN1, MFN2, and OPA1, in a hepatic stellate cell model (GPx). In contrast, in the same cell type the expression of these fusion proteins was largely down-regulated by an RSV concentration of 50 μM [2].

Quercetin: Treatment of HepG2 cells with quercetin (15 μM) significantly elevated levels of PGC-1α, NRF-1, and TFAM mRNA. Moreover, mtDNA copy number also increased by quercetin treatment [69]. Consistent with this, treatment of mice with 25 mg/kg quercetin for 7 days induced up-regulation of PGC-1α, SIRT1, mtDNA, and cytochrome c, which was linked to an increase in maximal endurance capacity [70]. In contrast, 100 mg/kg treatment for 15 weeks enhanced mitophagy and thereby prevented chronic ethanol-induced hepatic mitochondrial damage in mice [68], indicating that quercetin impacts the opposing processes of mitochondrial biogenesis and mitophagy in a concentration/dose-dependent manner.

Tea catechins: Consistent with findings using quercetin, treatment of rats with 25 mg/kg of green tea polyphenols for 3 days was reported to stimulate mitochondrial biogenesis via increase in the expression of PGC-1α and TFAM, and also improved renal function after cyclosporine A treatment [71]. The correlation between catechins and mitophagy has not yet been investigated.

Curcumin: Curcumin treatment (1–20 μM) for 6 to 8 days was reported to up-regulate PGC-1α and to enhance AMPK activity in 3T3-L1 and primary white adipocytes, inducing a brown fat-like phenotype in these two cell types. Moreover, 30 μM of curcumin (24 h) caused the translocation of NRF2 to the nucleus and restored PGC-1α levels in a gentamicin-treated kidney cell line LLC-PK1. Curcumin (50 or 100 mg/kg per day, for 28 days) intraperitoneally injected also potentiated the effects of training upon up-regulation of complexes of the mitochondrial respiration chain induced via the AMPK/SIRT1/PGC-1α signaling pathway. Consistent with this, increased numbers of mitochondria were found in the cerebral cortex of rats treated with curcumin (50 or 100 mg/kg per day, intraperitoneally injected, for 5 days) by up-regulation of NRF-1 and TFAM. Conclusive evidence for mitophagy-induction by curcumin in higher doses is still lacking [72].

4.4 Mitochondrial Apoptotic Pathways

At least at higher concentrations, all polyphenols described in the following list were found to induce apoptosis. Interestingly, cancer cells seem to be far more susceptible to apoptosis-induction by polyphenols, making these compounds promising drugs for chemoprevention and possibly even cancer therapy (described in Section 5).

Resveratrol: In concentrations higher than 50 μM, RSV was demonstrated to kill various cancer cells, including human breast cancer cells MCF-7 and MDA-MB-213 as well as HepG2 cells, via a mitochondrial apoptotic pathway by mitochondrial Ca^{2+} overload, disruption of mitochondrial membrane potential and caspase activation. Notably, RSV at concentrations >50 μM was reported to be especially toxic for cancer cells, while noncancer cells were largely unaffected. For instance, viability of human prostate carcinoma cells LNCaP was strongly reduced by 24-h treatment with 50 μM of RSV, while RSV did not affect human prostate epithelial cells [2]. An increased tethering between ER and mitochondria was suggested as a possible reason for the increased susceptibility to RSV-induced mitochondrial Ca^{2+} overload of cancer cells such as the cervical cancer cell line HeLa and the hybridoma cell line Ea.hy926 in comparison to noncancer cells like HUVECs [43].

Quercetin: Quercetin was also found to have a minor impact on normal, nontransformed cells, but to trigger

apoptosis in various cancer cells via the mitochondrial pathway involving the loss of mitochondrial membrane potential, cytochrome c release, and activation of caspases, potentially by depletion of intracellular glutathione and increased intracellular ROS [60]. For instance, in HepG2 cells, quercetin ($IC_{50}=87\,\mu M$) incubation for 18 h induced apoptosis by translocation of proapoptotic Bcl-2-associated X protein (BAX) to the mitochondrial membrane and induction of apoptosis by caspase activation [73].

Tea catechins: Green tea catechins were reported to induce cell death in numerous cancer cells [74]. For instance, green tea catechins (100 μM, 48 h), including EGCG and ECG, were found to induce apoptosis by an increase in ROS formation and mitochondrial depolarization, which were correlating with the rate of apoptosis, in human prostate cancer DU145 cells [65]. Similar to RSV and quercetin, a cancer cell-specific effect was also revealed for EGCG. While EGCG treatment (10 μM and 20 μM) induced apoptotic cell death and growth arrest in a multiple myeloma cell line as well as in multiple myeloma cells from bone marrow aspirate samples within 5 to 7 days, peripheral blood mononuclear cells and normal fibroblasts were largely unaffected [74].

Curcumin: The same cancer specificity was demonstrated for curcumin. While 50 μM curcumin had no effect in normal rat hepatocytes, treatment with this compound induced apoptosis in a time-dependent manner in various cancer cells, including MCF-7, MDA-MB, and HepG2 cells, by overwhelming production of ROS [66]. In HepG2 cells, only 1 h incubation with concentrations higher than 10 μM curcumin were reported to induce elevation of mitochondrial membrane potential, after a 2-h incubation mtDNA as well as nuclear DNA were damaged, and after 6 h mitochondrial membrane potential was disrupted and cytochrome c released [75]. Another paper showed that 20 μM of curcumin also induced swelling of rat liver mitochondria and opening of the mitochondrial permeability transition pore via oxidizing thiol groups [76].

5 POLYPHENOLS AS TREATMENT STRATEGIES TO MANIPULATE MITOCHONDRIAL (DYS)FUNCTION IN DISEASES

5.1 Aging and Cardiovascular Diseases

The impact of polyphenols on cardiovascular diseases and lifespan has been the subject of numerous studies and much debate since the hypothesis of the so-called French paradox arose. Consumption of RSV-rich red wine was speculated to protect French people from the negative impact of rather high intake of dietary cholesterol and saturated fatty acids, causing a surprisingly low incidence of coronary heart disease in French people. Although several clinical trials struggled to conclusively confirm the hypothesis about RSV's crucial role in this so-called French paradox, research about the impact of RSV as well as of other polyphenols on health and lifespan was strongly boosted, and cell culture as well as animal experiments revealed promising results [2]. Recently, the TOSCA.IT study demonstrated that high polyphenol intake is associated with a more favorable cardiovascular risk factor profile in a population with type 2 diabetes [77], pointing out the high potential of polyphenolic compounds in the prevention or treatment of cardiovascular diseases.

Resveratrol: A clinical trial revealed that RSV treatment (150 mg/day) for 30 days induces a caloric restriction-like effect on energy metabolism, known to positively affect lifespan, of nonobese postmenopausal women, including enhanced activation or expression of AMPK, SIRT1, and PGC-1α and an increase in fatty acid oxidative capacity [78]. Furthermore, RSV supplementation indeed increased lifespan and insulin sensitivity of mice fed a high-fat diet by increasing AMPK and PGC-1α activity, leading to an increased number of mitochondria [79]. Moreover, RSV (50 mg/kg/day, 16 weeks) attenuated cardiac injury in diabetic rats through normalization of mitochondrial function, again partly via activation of SIRT1 and PGC-1α and increased ROS defense via MnSOD up-regulation [80]. In a rat model of hypertension leading to heart failure, RSV treatment (18 mg/kg/day, 8 weeks) was reported to preserve mitochondrial mass and biogenesis as well as mitochondrial fatty acid oxidation and PPARα expression, which extended survival and counteracted the development of cardiac dysfunction [81].

Besides improving cardiac function, RSV was also tested for its effect on vasculature. In a randomized, double-blind crossover study, RSV supplementation for 6 weeks (2–3 g/day) increased mitochondrial number as well as vascular function, but did not affect glucose metabolism in older glucose-intolerant adults [82].

Moreover, RSV also displayed protective effects against toxic substances that potentially boost the aging process. For instance, pretreatment of human umbilical vein endothelial cells (HUVECs) with RSV attenuated the cigarette smoke extract-induced oxidative stress by up-regulating SIRT1 and increasing nitric oxide-mediated signaling [83]. In mice, RSV protected cardiomyocytes from doxorubicin-induced apoptosis through

SIRT1-mediated deacetylation of p53, shown to bind and activate in its acetylated form the BAX promoter, enhancing apoptosis [84].

Quercetin: In a double-blind, placebo-controlled trial, quercetin-3-glucoside (160 mg/day, for 4 weeks) as well as quercetin supplementation (1 g/day, for 28 days) failed to impact risk factors for heart disease positively in healthy subjects [85,86]. Nevertheless, animal experiments revealed promising results on the cardioprotective effects of quercetin. Quercetin treatment (0.033 mg/kg per day, for 4 days) significantly increased cardiac function associated with improved mitochondrial function after ischemia-reperfusion injury [87]. Moreover, combined pretreatment with quercetin (10 mg/kg, for 14 days) and alpha-tocopherol (10 mg/kg, for 14 days) preserved mitochondrial integrity, including the activity of TCA cycle dehydrogenases and cytochrome c oxidase as well as mitochondrial Ca^{2+} and ATP levels, and also preserved the integrity of heart tissue in myocardial-infarcted rats [88]. Besides cardioprotection, quercetin was also reported to attenuate vascular calcification by inhibiting oxidative stress and mitochondria fission via decreasing Drp1 activity and expression [89].

Tea catechins: In contrast to quercetin, the flavonoid EC (100 mg/day, for 4 weeks) was found to contribute to the cardioprotective effects of cocoa and tea by improving insulin resistance [86]. In addition, a prospective cohort study demonstrated an inverse risk between oral intake of EC (approx. 15 mg/day) and coronary heart disease mortality in elderly men [90]. Green tea catechin also protected guinea pig hearts from postischemic myocardial dysfunction, probably by inhibition of mitochondrial permeability transition pore (mPTP) opening and mitochondrial Ca^{2+} overload-triggered apoptosis [91]. Consistent with this, intravenous pretreatment of rats with EC (10 mg/kg) dramatically reduced the size of the infarct induced by occlusion of the left anterior descending coronary artery followed by reperfusion and was associated with preservation of mitochondria integrity, including increased respiratory activity, lower mitochondrial Ca^{2+} levels, and higher levels of NADH and ATP [92]. The green tea polyphenol EGCG was also demonstrated to exhibit cardioprotective effects by preventing myocardial ischemia/reperfusion injury via release of mtDNA from damaged mitochondria in a rat ischemia/reperfusion animal model [93]. Another study suggested that the beneficial effects of EGCG are due to the inhibition of mtDNA release from damaged mitochondria, thereby inhibiting inflammatory damage [94].

Curcumin: The impact of curcumin treatment on cardiovascular risk factors was tested in several clinical trials, but no apparent effect was detected [95]. Nevertheless, animal experiments were successful in demonstrating cardioprotective effects of this compound. For instance, pretreatment of rats with a daily dose of 200 mg/kg curcumin for 7 days attenuated ischemic/reperfusion injury by decreasing oxidative stress and mitochondrial dysfunction [96]. Furthermore, one single treatment with curcumin (60 mg/kg) also counteracted catecholamine-induced cardiotoxicity by inhibition of mitochondrial swelling, prevention of mPTP opening, and normalizing mitochondrial biogenesis, respiration, and network dynamics [97].

5.2 Neurodegenerative Diseases

The polyphenols' antioxidant effects, especially present at low treatment concentrations (as described in Section 4), make these compounds potential drugs in the treatment of neurodegenerative diseases, often characterized by increased ROS levels and ROS-induced damage of mitochondrial and cellular function. Moreover, induction of mitochondrial biogenesis by polyphenols might also promote cellular function in neurodegenerative diseases [72]. Nevertheless, conclusive results from clinical trials are still missing, which might be due to the poor bioavailability of polyphenolic compounds. Moreover, it has to be questioned to what extent the transport of these compounds through the blood-brain barrier might even be possible [98].

Resveratrol: Interestingly, a clinical trial demonstrated that two different doses of trans-RSV (250 and 500 mg) resulted in dose-dependent increases in cerebral blood flow and an increase in deoxyhemoglobin, suggesting enhanced oxygen extraction [99]. Nevertheless, clinical trials directly demonstrating the attenuation of neurodegenerative disease-associated dysfunction, such as cognitive impairment in Alzheimer's disease, are still missing [100]. Interestingly, 5 μM of RSV for 6 h scavenged amyloid-β-triggered alterations in mitochondrial structure of mouse neuroblastoma cells Neuro-2A, but could not normalize elevated H_2O_2 production. In contrast, the electron scavenger MitoQ, which is a ubiquinone derivative, accumulating in mitochondria, reduced elevated H_2O_2 levels under this condition [101]. These results may possibly indicate that RSV's protective effect against ROS is based on an organism's potential to form an ROS defense shield, which is likely to be hampered in case of severe cellular degeneration [2]. However, RSV was shown to decrease intracellular ROS levels by enhanced ROS defense, such as elevation of MnSOD levels, in fibroblasts of patients with complex I deficiency. Moreover, RSV treatment (250 mg/kg/day) for 3 weeks also enhanced SOD activity and GSH levels of wild-type as well as of cuprizone-intoxicated mice, a mouse model mimicking features of multiple sclerosis due to demyelination.

Other mechanisms of RSV, positively counteracting cellular dysfunction, were activation of the mitochondrial

respiration as well as induction of mitochondrial biogenesis. For instance, RSV restored oxidative phosphorylation efficiency and mitochondrial biogenesis, affecting the proliferation of neural progenitor cells isolated from Ts65Dn mice, a widely used model of Down syndrome [102]. Furthermore, RSV treatment was described to increase complex I activity and mitochondrial ATP production through activation of AMPK and sirtuin 1 (SIRT1), which also raised the mRNA expression of PGC-1α's target genes in Park2 mutant fibroblasts of early-onset PD patients [67].

Quercetin: It was suggested that quercetin may act as a direct antioxidant via free radical scavenging. Indeed, intraperitoneal quercetin (25 mg/kg) administration every 40 h for 3 months ameliorated Alzheimer's disease pathology and protected cognitive function in aged triple transgenic Alzheimer's disease model mice [103]. Moreover, treatment with quercetin prevented mitochondrial dysfunction, recovered normal mitochondrial morphology and decreased ROS levels in hippocampal neurons treated with H_2O_2 and to a lesser extent also in cells treated with amyloid-β peptide [104].

In addition, quercetin—similar to RSV—was also demonstrated to enhance mitochondrial biogenesis: 10 μM of quercetin significantly increased mitochondrial biogenesis and mitochondrial bioenergetics capacity by stimulating PGC-1α promoter activity in N27 dopaminergic neuronal cells, possibly explaining the protection against neurodegeneration found in quercetin-treated MitoPark mice [105].

Tea catechins: Within the cell, 90%–95% of EGCG was found to accumulate in mitochondria. Since neurons were largely protected from oxidative stress-induced apoptosis by EGCG, while the expression and activity of antioxidant enzymes like MnSOD or glutathione peroxidase was unchanged, EGCG was proposed to work as a direct free radical scavenger [106]. Moreover, EC and quercetin were found to reduce synergistically ischemic neuronal cell death by preserving mitochondrial spare respiratory capacity after an ischemic insult [107].

In addition, EGCG was also found to improve oxidative phosphorylation efficiency and mitochondrial biogenesis, increasing proliferation of neural progenitor cells isolated from Ts65Dn mice, a widely used model of Down syndrome [102].

Curcumin: In clinical studies, curcumin was administered in different doses (1–4 g) to Alzheimer's disease patients. One study found an increase in vitamin E levels after curcumin supplementation and suggested that the antioxidant activity of curcumin might decrease the need for the antioxidant vitamin E. However, mental status was not found to be improved. In contrast, chronic curcumin treatment of old rats resulted in significant increases in SOD and GPx activities and decreased the lipid peroxide and lipofuscin content of brain, which was linked to the neuroprotection and antiaging properties of curcumin [108]. Moreover, curcumin (2 μM for 1 h) was also shown to protect PINK1 knockdown SH-SY5Y cells that mimic Parkinson's disease from mitochondrial dysfunction and cell death by normalizing mitochondrial membrane potential as well as mitochondrial respiration [109].

5.3 Cancer

The cancer cell-specific induction of the mitochondrial apoptotic pathways by polyphenols was impressively demonstrated by numerous in vitro studies, as described in Section 4. Consequently, in vivo studies were performed testing these effects. Clinical trials demonstrated that polyphenol supplementation was well tolerated even in rather high concentrations [49]. Nevertheless, it seems to be challenging to generate pharmaceutical formulations that ensure that polyphenols reach the respective cancer tissues in concentrations high enough to induce apoptosis. However, chemopreventive effects of polyphenols were already impressively demonstrated [110].

Resveratrol: RSV treatment was found to be successful in the treatment of cancer types, in which the low bioavailability of RSV could be circumvented. Analysis of surgically removed tumor tissues revealed that oral administration of 0.5 g or 1.0 g RSV for 8 days reduced tumor cell proliferation in patients with colorectal cancer. Moreover, RSV successfully inhibited tumorigenesis in a chemically induced skin carcinogenesis mouse model [2]. In addition, RSV might help to overcome drug resistance. For instance, RSV enhanced chemosensitivity of doxorubicin in human breast cancer cells by down-regulation of multidrug resistance protein and multidrug resistance associated protein [111]. Nevertheless, there is a need for new pharmaceutical forms to overcome RSV's low bioavailability and to reach concentrations in cancerous tissues high enough to induce apoptosis. However, studies also tested the cancer chemopreventive properties of RSV. For instance, daily intake of 2.5 g of RSV for 29 days decreased circulating insulin-like growth factor-1 (IGF-1) and IGF-1-binding protein-3, shown to have antiapoptotic properties and linked to risk of colorectal, prostate, breast, or lung cancer [112].

Quercetin: Consistent with the chemopreventive properties of RSV, a clinical trial revealed that quercetin supplementation (>24 μg/day) was found to reduce the risk for prostate cancer by 27% [110]. Moreover, an oral combination treatment of curcumin (480 mg) with quercetin (20 mg) for 6 months was reported to reduce the number and size of ileal and rectal adenomas in patients with familial adenomatous polyposis, an autosomal disorder characterized by the development of adenomas and colorectal cancer [113]. However, treatment with 6 doses of orally administered quercetin (1 mg/kg) reduced tumor

volume in mice with induced breast adenocarcinoma by activating the mitochondrial pathway of apoptosis and increased the lifespan five-fold without notable side effects, highlighting the potential of quercetin as a cancer therapy [114].

Tea catechins: A clinical trial tested the effects of green tea catechins in men with high-grade prostatic intraepithelial neoplasia and/or atypical small acinar proliferation. Daily intake of a mixture of green tea catechins, containing 400 mg EGCG per day, for 1 year did not reduce the number of prostate cancers, but caused a decrease in the serum prostate-specific antigen (PSA), possibly linked to a decrease in inflammation, which is potentially associated with the development and progression of prostate cancer [115]. Consistent with the potentially cancer-preventive role of tea catechins in prostate cancer, histology of patients with high-risk oral premalignant lesions tend to improve after treatment with green tea extract (500 mg–1000 mg/m^2) for 12 weeks [116], highlighting the importance of longer-term clinical testing.

Curcumin: Treatment with oral curcumin (4 g) for 30 days significantly reduced the occurrence of aberrant crypt foci (ACF), which are clusters of abnormal glands in the colon and rectum and the earliest neoplasia in the colon, possibly leading to colon cancer [117]. The effect of curcumin was tested in several in vivo studies on mouse models of pancreatic cancer. It was demonstrated that oral curcumin treatment (1 g/kg) potentiated the antitumor activity of gemcitabine in a mouse model of pancreatic cancer. Moreover, a curcumin-containing diet (0.6%) reduced the tumor size in mice injected with MIA PaCa-2 cells. Several clinical trials in patients of pancreatic cancer proved curcumin's safety, but due to low bioavailability, results were inconclusive and new formulations, e.g., nanoparticle-based curcumin, are being tested [118].

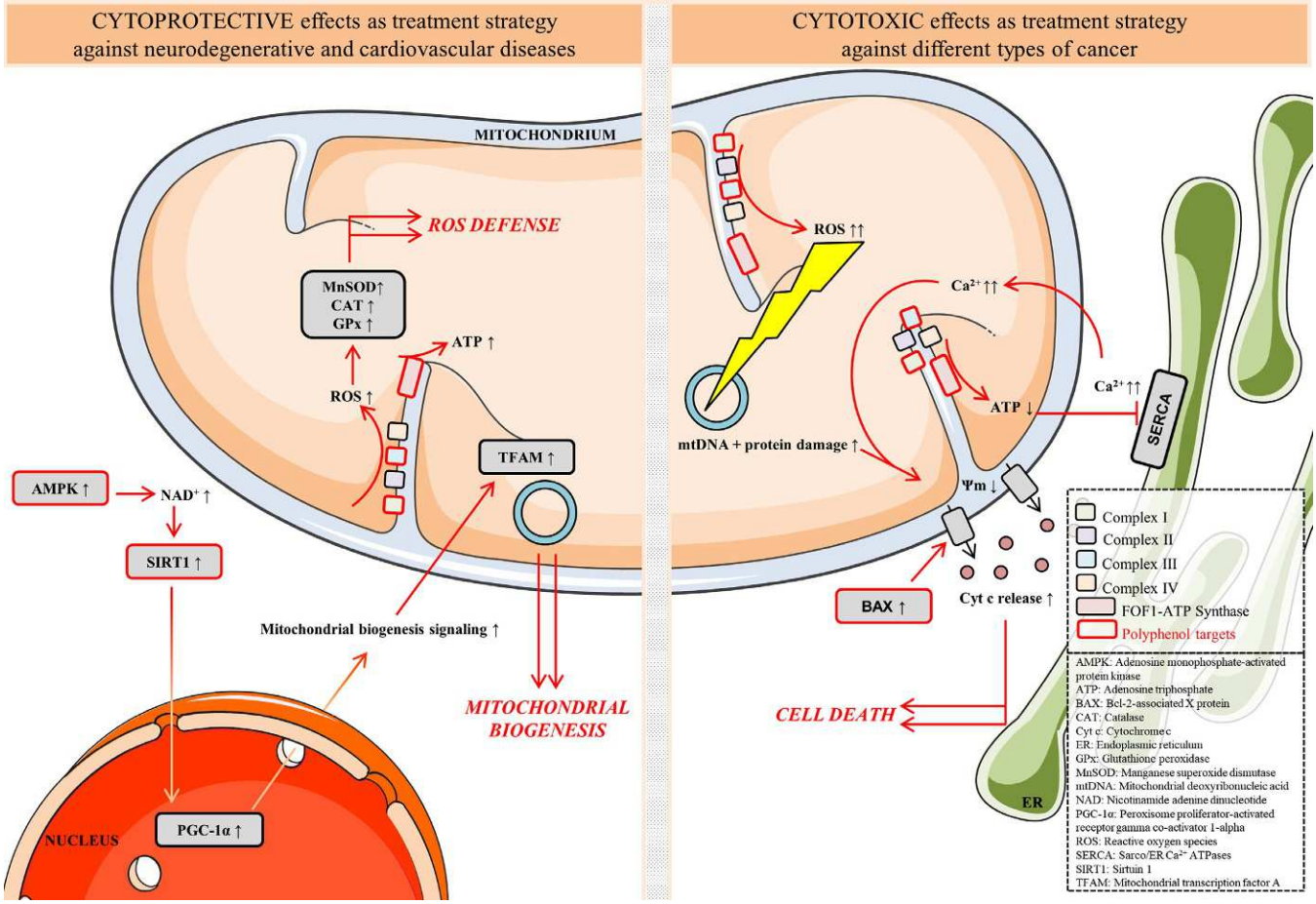
6 CONCLUSION

As cellular power plants and executors of programmed cell death, mitochondria are irreplaceable compartments of the eukaryotic cell and consequently crucially involved in cellular function and dysfunction. in vitro and in vivo studies about polyphenols' crucial impact on physiological and pathophysiological processes demonstrate the great potential of drugs directly or indirectly targeting and modulating mitochondria [2]. As described in Section 4, in vitro studies revealed that polyphenols like RSV, quercetin, tea catechins, and curcumin manipulate mitochondrial respiration, ROS production and ROS defense mechanisms, mitochondrial biogenesis and mitophagy, as well as mitochondria-induced apoptotic pathways in a concentration-, time- and cell-type-specific manner. Notably, different concentrations of the same compound were found to have opposing effects in the same cell type, which makes the dosage critical. Moreover, cancer cells were demonstrated to be especially susceptible to polyphenol-induced apoptosis, while noncancerous cells were largely unaffected by polyphenols [2].

As described in Section 5, in vivo studies in mice and rats further demonstrated polyphenols' diverse effects on mitochondrial proteins and mitochondrial function and revealed that these modulations impaired diseases, dysfunctions, and effects of intoxication. In contrast, clinical studies did not show conclusive results regarding the impact of mitochondrial manipulation by polyphenols on healthy or sick human beings, but could demonstrate the safety of these compounds even in rather high dosages and revealed pharmacokinetic characteristics. Accordingly, the bioavailability of polyphenolic compounds is rather low, which makes sophisticated pharmaceutical formulations necessary to profit from this group of compounds, as described in Section 3.2. Moreover, it seems to be crucial to unveil the right time point for treatment with polyphenols. Consistent with the theory of a biphasic alteration of mitochondria, associated with an increased mitochondrial activity linked to, for instance, enhanced mitochondrial respiration and ROS production in middle age and a decline of mitochondrial function in old age [15], further studies must investigate at which time point treatment with polyphenolic compounds is most effective to protect health and prevent aging-related diseases.

Despite the fact that we know about only a few direct mitochondrial targets of polyphenols, such as components of the mitochondrial respiration chain [57], polyphenols display multiple effects on mitochondrial function. Therefore, it seems of major importance to further investigate mechanisms of mitochondrial manipulation initiated by inhibition or activation of nonmitochondrial proteins. Moreover, it is likely that the polyphenols' positive impact on cellular function goes beyond direct mitochondrial effects.

As described in Section 2.3, the crucial role of mitochondria in various diseases and during aging is well known, but strategies to finely tune mitochondrial function in order to counteract mitochondrial and cellular dysfunction are still lacking. Through polyphenols' diverse mode of actions found in cell culture experiments and studies with rodents, we can define and study mechanisms of direct or indirect mitochondrial modulation to counteract or even prevent cardiovascular and degenerative diseases as well as cancer. In the face of an aging population, new treatment strategies seem to be indispensable to fight against incurable or drug resistant diseases (Scheme 21.1).



SCHEME 21.1 Cytoprotective and cytotoxic actions of polyphenols by manipulation of mitochondrial function. Cytoprotective actions, which are possible treatment strategies against neurodegenerative and cardiovascular diseases: Manipulation of mitochondrial respiration by polyphenols leads to low levels of ROS production, enhancing activity or expression of MnSOD, CAT, and GPx and thereby boosting antioxidant defense mechanisms. Besides, low levels of polyphenols cause increased ATP production via F_0F_1-ATP synthase activation. Moreover, polyphenol-induced activation of AMPK and SIRT1 causes increased mitochondrial biogenesis via PGC-1α and TFAM. Cytotoxic effects, which might be treatment strategies against different types of cancer: Polyphenols, especially in high concentrations, cause overwhelming ROS production by manipulation of mitochondrial respiration, which causes damage of mtDNA and proteins. Besides, a drop in ATP levels by inhibition of F_0F_1-ATP synthase causes SERCA inhibition and thereby mitochondrial Ca^{2+} overload, including cell death, possibly via localization of proapoptotic BAX to the mitochondrial membrane, disruption of mitochondrial membrane potential and cytochrome c release and cell death. "Created using Servier Medical Art."

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Glossary

Adenosine triphosphate
Molecular energy carrier.
Antioxidant effect
Inhibition of molecule oxidation.
Apoptosis
Programmed cell death.
Bioaccessibility
The fraction of an administered drug that becomes available for absorption.
Bioavailability
The fraction of an administered drug that reaches the systemic circulation.
Cellular differentiation
A process where a cell undergoes changes from one cell type to another.
Cellular proliferation
An increase in the number of cells.
Chemoprevention
Reduction of health risks by use of drugs.
Chemosensitivity
Increased susceptibility for treatment by use of drugs.
Clinical trial
Experiment done on humans in clinical research.
Cytoprotection
Protection by compounds against harmful agents or processes.
Cytotoxicity
Toxicity induced by compounds.
Demyelination
Damage of myelin.
Deoxyribonucleic acid
Chain of nucleotides carrying genetic information.
Dosage
Quantity of administered drug.
Drug resistance
Effectiveness-loss of a medication.
Endoplasmic reticulum
Cellular organelles, biggest intracellular Ca^{2+} store, protein folding sites.
Eukaryotic cell
Any cell containing a cellular nucleus and other membrane-enclosed organelles.
Flavin adenine dinucleotide
Reduction equivalent.
Healthspan
The time an organism is healthy.
Hetero-oligomer
An oligomer composed of at least two different monomers.
Homeostasis
Equilibrium of a condition.
Hybrid cell
A cell formed by fusion of cells with different origin.
In vitro
Experiments performed in cells.
In vivo
Experiments performed in living organisms.
Intermembrane space
Space between outer and inner mitochondrial membrane.
Ischemia
Restriction in blood supply.
Junction
Inter-organelle bridge.
Lifespan
The time an organism lives.
Malignant tumor
A tumor not self-limited in its growth and its ability to invade.
Metabolite
Intermediate or product of metabolism.
Microbiota
Ensemble of microorganisms.
Mitochondria
Cellular organelles, known as cellular power plants and executors of cell death.
Mitochondria-associated endoplasmic reticulum membranes
Specialized endoplasmic reticulum membranes in close proximity to mitochondria.
Mitochondrial biogenesis
Increase of mitochondrial mass and copy number.
Mitochondrial fission
Division of mitochondrial structures.
Mitochondrial fusion
Aggregation of mitochondrial structures.
Mitochondrial matrix
Volume within the inner mitochondrial membrane.
Mitochondrial respiration
A set of metabolic reactions within mitochondria converting stored energy in adenosine triphosphate.
Mitophagy
Programmed degradation of damaged mitochondria.
Mutation
Alteration of the nucleotide sequence of the genome.
Nanocarrier
Nanomaterial used to transport drugs.
Nanoemulsion
A sort of nanocarrier.
Nanomicelle delivery systems
A sort of nanocarrier.
Nicotineamide adenine dinucleotide
Reduction equivalent.
Oxidative decarboxylation
Removal of a carboxylate group by oxidation.
Pharmaceutical formulation
Different medicinal products.
Pharmacokinetics
The process of drug absorption, distribution, metabolism and excretion.
Pharmacophore
Steric and electronic feature of a molecule for interactions.
Phenotype
Observable characteristics.
Placebo
Fake treatment lacking active compound.
Polyphenol
Secondary metabolites of plants.
Reactive oxygen species
Derivatives of oxygen inducing oxidative stress.
Ribosomal ribonucleic acids
Ribonucleic acids at the organelles that serve as sites for protein synthesis.
Tricarboxylic acid cycle
A series of chemical reactions to gain energy.
Tumor suppressor
A gene protecting from cancer development.

SECTION IV

BIOAVAILABILITY AND EFFECTS ON METABOLISM

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CHAPTER

22

Bioavailability of Flavonoids: The Role of Cell Membrane Transporters

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Abbreviations

BCRP	breast cancer-resistant protein
BTL	bilitranslocase
GLUT1	glucose transporter 1
GLUT2	glucose transporter 2
MCT1	monocarboxylate transporter 1
MRP1	multidrug-resistance-related protein 1
MRP2	multidrug-resistance-related protein 2
MRP3	multidrug-resistance-related protein 3
OAT1	organic anion transporter 1
OAT3	organic anion transporter 3
OATPs	organic anion transporting polypeptides
P-gp	P-glycoprotein
SGLT1	sodium/glucose co-transporter 1
SVCT1	sodium-dependent vitamin C transporter 1
SVCT2	sodium-dependent vitamin C transporter 2
URAT1	urate transporter 1
BA	estimated oral bioavailability
C_{max}	maximal plasma concentration (peak) after single dose oral ingestion
T_{max}	time needed to reach C_{max}
$t_{1/2}$	elimination half-life
IC_{50}	concentration required for 50% in vitro inhibition

1 INTRODUCTION

Flavonoids are secondary metabolites of plants, and therefore occur in the human diet. Numerous epidemiological studies have showed an inverse correlation between dietary flavonoid consumption and chronic degenerative diseases, such as cardiovascular diseases, diabetes, osteoporosis, neurodegenerative diseases, and cancers [1,2]; thus flavonoids are increasingly regarded as nutraceuticals. However, the observed limited bioavailability and consequently low plasma concentrations of dietary flavonoids raise questions about the mechanisms by which they attain effective intracellular concentrations in the target tissues. Considering also that flavonoids are, in general, hydrophilic compounds (having low $\log P$ values) with low molecular weight, the translocation of flavonoids through the cell membrane must mainly occur via specific transmembrane transporter proteins, rather than just by diffusion through the lipid portion of the membrane. The transporters must therefore be regarded as important factors in mediating the protective activity of flavonoids in the cardiovascular, renal, hepatic, and central nervous systems, as well as in other tissues, on both the cellular and organ level.

In spite of their biological relevance, currently we have only fragmentary knowledge of the interactions between flavonoids and transporters. Flavonoids comprise over 6000 different chemical moieties found in virtually all plants and fruits [3]. In addition, there is also large variability in their metabolism, e.g., methylation, glucuronidation, sulfation, and other conjugations, which further increases the number of chemically distinct molecules with the potential to act as substrates for the transporters. For these reasons, many flavonoids and their metabolites have not yet been studied with respect to their biological activity and/or pharmacokinetic properties. Moreover, many flavonoids can move across the cell membrane by multiple transporters, a fact that makes

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mechanistic interpretation of flavonoid transport rather a daunting task.

In the following we first describe the pharmacokinetic evidence pointing to the involvement of transporter-mediated flavonoid uptake in cells and/or passage through cellular barriers. Then, we describe the key membrane transporters that, according to current knowledge, are involved in flavonoid absorption and disposition (distribution and elimination). These transporters are important for understanding not only flavonoid bioavailability and bioactivity in themselves, but also flavonoid-drug interactions, so shedding light on the role of the individual's diet in possible interference with the action of drugs.

2 PRINCIPLES OF MEMBRANE TRANSPORT

Translocation across cellular and intracellular membranes plays a crucial role in determining the biological properties of endogenous and exogenous compounds. Translocation through membranes is generally affected by the physicochemical properties of the compound, namely molecular weight, lipophilicity, electronic charge and pK_a, along with hydrogen bonding ability [4]. Even though these factors have a measurable impact on both simple diffusion and carrier-mediated transport [5], there is growing evidence pointing to the predominant role of transporters in membrane transport processes in vivo, with only a marginal contribution from nonspecific passive diffusion [6,7]. Consistent with this, over 10% of all functionally characterized genes encode for transporters or transporter-related proteins, as expected of key players in cellular homeostasis [8]. Indeed, 1300–1500 membrane transporter proteins have been identified in the human genome [9].

Transporters form intermediate complexes with specific compounds (referred to as substrates), accompanied by a coordinated sequence of protein conformational transitions, thus allowing translocation from one side of the membrane to the other. Similarly to what happens with enzymes, transporter-mediated membrane transport is characterized by saturability and inhibition by substrate analogs able to fit into or close to the transport site.

Transporters can be classified in a number of different ways, as shown in Fig. 22.1. Depending on their transport mechanisms, they can be divided into either passive or active transporters. The latter can be functionally classified as primary or secondary/tertiary active transporters, according to their driving force. Finally, they can be further categorized as either influx or efflux transporters, based on the direction of substrate movement across the membrane. In the Transporter Classification Database (TCDB, http://www.tcdb.org), transport proteins have been assembled into roughly 600 families and arranged in five main classes [10]. The TCDB is based on a

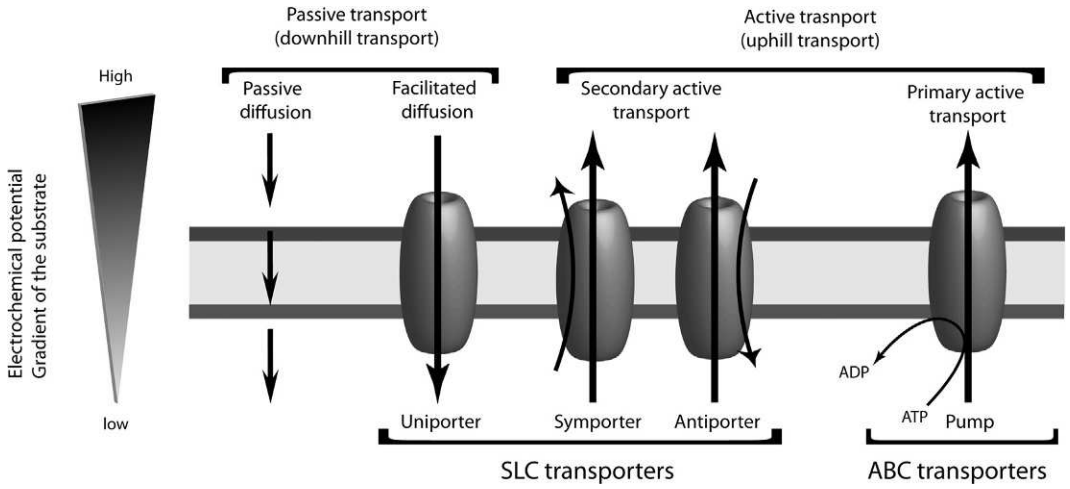


FIG. 22.1 Major routes by which flavonoids and their metabolites may cross the cell membrane. (I) Passive diffusion. This type of transport is possible only if the compounds are small, lipid-soluble, nonionized, and if there is a concentration gradient between the two sides of the membrane (principle of Fick's law). This passage can occur without energy expenditure. (II) Carrier-mediated transport. This involves one or more transporter(s), and an influx or efflux of substances. This kind of transport is saturable and ligand-specific. It can be passive, or primarily/secondarily active. It is mediated (a) by solute carriers (SLC) transporters, or by (b) ATP-binding cassette (ABC) transporters. (A) *SLCs*. According to the transport direction they may be (i) bidirectional (direction of net transport determined by the substrate concentration gradient), or (ii) unidirectional either into or out of the cell. According to the driving force, they may (i) involve an exchange of one substrate for another, or (ii) be driven by an ion gradient. In the latter, the direction of transport is also reversible depending on the electrochemical potentials. SLCs can furthermore be categorized as (i) uniporters (transport of a single molecule); (ii) symporters (transport of two or more different molecules or ions in the same direction); or (iii) antiporters (transport of two or more different molecules or ions in opposite directions). (B) *ABC transporters* function as efflux pumps that remove from the cell or organelle passively penetrating solutes against their electrochemical potentials and chemical gradients.

functional/phylogenetic system of classification formally adopted by the International Union of Biochemistry and Molecular Biology (IUBMB).

Primary active transporters include ATP-binding cassette (ABC) transporters (TC#3.A.1) that utilize the free energy of ATP hydrolysis as their driving force. ABC transporters are found on the extracellular and intracellular membranes of tissue barriers and excretory organs. They function as efflux pumps that move their substrates against an electrochemical gradient [11,12]. Some major members of this group include P-glycoprotein (MDR1, ABCB1), multidrug resistance-associated proteins (MRPs, ABCC), and breast cancer resistance protein (BCRP, ABCG2).

Solute carriers do not utilize any energy source other than the chemiosmotic gradient of the membrane. They mediate a variety of so-called porter mechanisms, like facilitated diffusion and secondary/tertiary active transport, coupled to the symport or antiport of ions or low-molecular-weight compounds, which provide the driving force [13–19]. Included in this group of transporters are members of the organic anion transporting polypeptide (OATP, TC#2.A.60, SLCO) family, the organic anion/cation/zwitterion transporter (OAT/OCT, TC#2.A.1.19, SLC22A) family, and the monocarboxylate transporter family (MCT, TC#2.A.1.13, SLC16A) [10]. Besides these, and the pertaining flavonoid membrane transport, a solute carrier whose gene is still unknown must also be mentioned, i.e., bilitranslocase (BTL, TC#2.A.65.1.1) [20].

Solute carriers are conventionally considered to be uptake transporters. Indeed, they can perform as either mono- or bidirectional transporters, depending on the concentration gradients of the substrate and coupled ions across the membrane. Gradients are often maintained by active transporters of the ABC superfamily [13].

3 INDIRECT EVIDENCE SUPPORTING THE EXISTENCE OF FLAVONOID MEMBRANE TRANSPORTERS

Numerous observations were obtained in complex experimental settings indirectly indicating the necessity for transporter-mediated cellular uptake of flavonoids. In contrast to the direct data on transporter-flavonoid interactions obtained in membrane-based or cell-based assays, these indirect observations can be presented in terms of pharmacokinetics and pharmacodynamics.

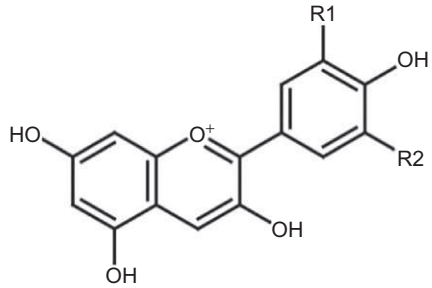
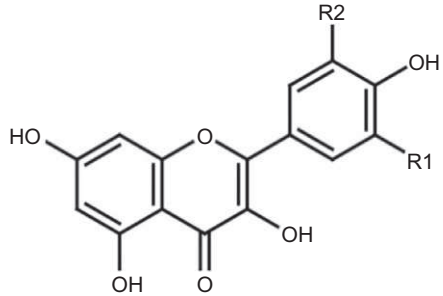
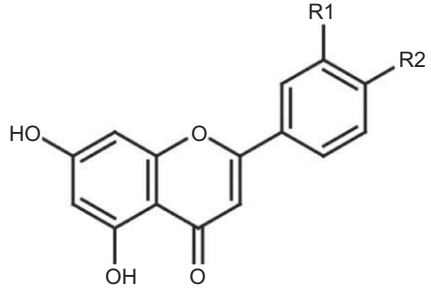
3.1 Pharmacokinetic Perspective

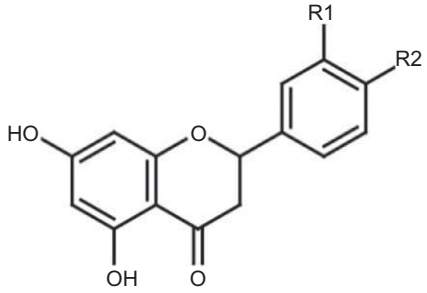
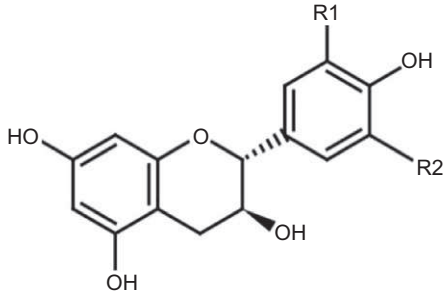
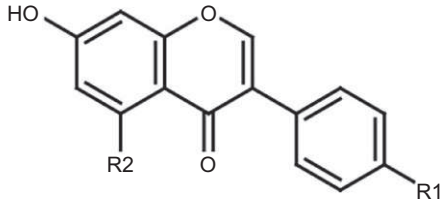
Membrane transporters can directly influence the rate of absorption, distribution, and excretion of flavonoids. First of all, some flavonoids exhibit rapid absorption and rapid appearance in plasma, e.g., T_{max} (time for maximum plasma concentration to appear) for anthocyanins is 15 min [21], while some others can undergo slower absorption with T_{max} up to 11 h [22] (isoflavones); values for the pharmacokinetic properties of the flavonoids subclasses are summarized in Table 22.1. In addition, the absence of flip-flop kinetics for flavonoids, which means that absorption is not much slower than elimination, shows that the uptake rate might be transporter-dependent. For instance, one study showed that quercetin has similar elimination kinetics after peroral or intravenous application [38]. Though some of the less polar flavonoids, e.g., isoflavones and flavones, exhibit concentration-independent permeability coefficients (P_{app}), thereby suggesting passive diffusion pathways [123], other flavonoids may use various patterns of membrane transport. They might be substrates of either influx or efflux transporters, or, even more, they might be involved in a cycling mechanism composed of carrier-mediated influx and efflux.

Another indication of transporter-mediated uptake is the existence of molecular specificity, as shown by the differences in gastrointestinal absorption displayed by flavonoid subclasses. For example, in the anthocyanin subgroup, only the anthocyanin glycosides are initially absorbed in plasma from the gastrointestinal tract, while no aglycones appear [124,125]. Interestingly, there is also a further difference in absorption among anthocyanin glycosides, namely acylated anthocyanins have a lower gastrointestinal absorption efficiency and lower bioavailability than the related non-acylated anthocyanins [126]. In a similar mode, flavanone glycosides display higher absorption than their aglycones, e.g., naringin (naringenin glycoside) has higher plasma values than naringerin aglycone [52]. On the other hand, other flavonoid subclasses are absorbed only as aglycones, namely: (i) flavones, e.g., luteolin glucosides must be first converted to aglycone luteolin before absorption [45]; (ii) isoflavones, e.g., only genistein aglycones are absorbed (not the glucosides) [127]; (iii) flavonols, e.g., isoquercitrin (quercetin 3-*O*-gluoside) is converted to aglycone quercetrin [128]. These differences in gastrointestinal absorption among different flavonoid subclasses, or even among substances from the same group, suggest that transporter-mediated uptake of dietary flavonoids from the gastrointestinal tract is a general principle. Indeed, even aglycones might be efficiently absorbed via transporters, rather than passively.

Moreover, it is noteworthy that when a high concentration of blackberry anthocyanins was injected into the gastric lumen, the percentage of cyanidin 3-glucoside absorption was lower than after administration of a low concentration [125], which can be explained by postulating that high amounts of anthocyanins induced saturation of

TABLE 22.1 Synopsis of Reported Pharmacokinetic Parameters of Flavonoids in Animal and Human Studies, and Their Interactions With Membrane Transporters[a]

Flavonoid subclass	Structure	Preclinical pharmacokinetics Animal studies	Clinical pharmacokinetics Human studies	Interactions with transporters[b,c]
Anthocyanidins (Note: anthocyanins (AC) are the glycosylated form)		C_{max} (monomeric AC) = 0.18 μM [23]–0.84 μM [24] C_{max} (mixture, total AC) = 0.1 μM [25]–3.8 μM [26] T_{max} = 15 min [21]–60 min [27] $t_{1/2}$ = NR[a] BA = 0.49% [24]–1.82% [21]	C_{max} (monomeric AC) = 0.56 nM [28]–5.8 nM [29] C_{max} (mixture, total AC) = 30 nM [27]–0.5 μM [30] T_{max} = 0.5 h [31]–2.2 h [32] $t_{1/2}$ = 1.5 h [31]–6.6 h [32]	BCRP, BTL, P-gp, SVCT1
Flavonols		C_{max} = 0.9 μM [33]–6.8 μM [34] T_{max} = 30 min [35]–2.9 h [36] $t_{1/2}$ = 14.8 h [36]–65.4 h [36] BA = 0.5% [37]–5.3% [38]	C_{max} = 137 nM [39]–7.64 μM [40] T_{max} = 0.68 h [40]–7.5 h [39] $t_{1/2}$ = 2.6 h [41]–28.1 h [42]	BCRP, BTL, GLUT2, MRP1, MRP2, MCT1, OAT1, OATP, P-gp, SGLT1, SVCT1, URAT1
Flavones		C_{max} = 134 nM [43]–50 μM [44] T_{max} = 15 min [45]–3.9 h [44] $t_{1/2}$ = 0.8 h [46]–91.8 h [47] BA = 2.2% [48]	C_{max} = 127 nM [49]–300 nM [50] T_{max} = 0.5 h [51]–7.2 h [49] $t_{1/2}$ = 3.1 h [50]	BCRP, GLUT2, MRP1, MRP2, MRP3, OATPs, P-gp, SVCT1

Flavanones		C_{max} = 21 nM [52]–6 μM [53] T_{max} = 20 [52]–80 min [52] $t_{1/2}$ = 1.3 [53]–2.2 h [53] BA = NR[a]	C_{max} = 60 nM [54]–7.4 μM [55] T_{max} = 2 h [56]–5.8 h [54] $t_{1/2}$ = 1.3 h [53]–3.1 h [55]	BCRP, MCT1, MRP2, MRP3, OATPs, P-gp, SVCT1
Flavan-3-ols (Synonym: catechins)		C_{max} = 0.3 μM [57]–2.7 μM [57] T_{max} = 24 h [58] $t_{1/2}$ = 41 min [59]–4.03 h [57] BA = 0.1% [59]–31.2% [59]	C_{max} = 70 nM [60]–7.35 μM [61] T_{max} = 1.2 h [60]–4.1 h [62] $t_{1/2}$ = 0.42 h [63]–5.7 h [64]	GLUT2, MRP1, MRP2, OATPs, P-gp
Isoflavones (Synonym: phytoestrogens)		C_{max} (rats) = 2.2 μM [65]–20.9 μM [66] C_{max} (cats) = 5 μM [67]–15 μM [67] T_{max} = 10 min [66]–1.5 h [67] $t_{1/2}$ = 8.5 h [65]–17 h [68] BA = 1.4% [68]–30% [68]	C_{max} = 0.17 μM [69]–25.4 μM [22] T_{max} = 2.5 h [22]–11 h [22] $t_{1/2}$ = 3.4 h [70]–10.8 h [71]	BCRP, MRP2, OATP1B1, P-gp, SVCT1, SVCT2

[a] BA, *estimated oral bioavailability;* C_{max}, *maximal plasma concentration (peak) after single dose oral ingestion;* T_{max}, *time needed to reach* C_{max}; $t_{1/2}$, *elimination half-life;* NR, *not reported in the literature.*

[b] *Detailed interactions between membrane transporters and specific flavonoids are presented in Table 22.2, with the corresponding references.*

[c] *Abbreviations used for the mentioned transporters:* BCRP, *breast cancer-resistant protein;* BTL, *bilitranslocase;* GLUT2, *glucose transporter 2;* MRP1, *multidrug-resistance-related protein 1;* MRP2, *multidrug-resistance-related protein 2;* MRP3, *multidrug-resistance-related protein 3;* MCT1, *monocarboxylate transporter 1;* OAT1, *organic anion transporter 1;* OATPs, *organic anion transporting polypeptides;* P-gp, *P-glycoprotein;* SGLT1, *sodium-dependent glucose transporter 1;* SVCT1, *sodium-dependent vitamin C transporter 1;* SVCT2, *sodium-dependent vitamin C transporter 2;* URAT1, *urate transporter 1.*

the membrane transport. Another study confirmed the saturability of anthocyanin uptake through MKN-28 adenocarcinoma stomach cells [129]. Similarly, higher oral doses of quercetin in pigs (500 vs. 50 mg/kg) resulted in a decreased estimated bioavailability for quercetin and its conjugate metabolites (2.6% vs. 8.6%) [37], again suggesting saturability of the transport mechanisms in the gastrointestinal tract.

Gastrointestinal absorbance of flavonoids can be greatly increased in the presence of specific efflux pump inhibitors, as has been shown in the intestinal cell culture model [111]. In a similar way, a flavonoid mixture can have a better bioavailability in comparison to a single flavonoid application, since flavonoids are good efflux inhibitors, e.g., green tea polyphenols inhibited P-glycoprotein activity [107], as discussed in the next section. There are several other recent studies pointing at increased bioavailability of a single flavonoid molecule when ingested in a mixture with other polyphenols. Absorption of flavonol isorhamnetin ingested as a component of *Hippophae rhamnoides* L. (TFH) was significantly higher than the absorption of isorhamnetin ingested in its pure form [92]. The most probable reason for this was the down-regulation of MRP2 expression at mRNA level by other components present in TFH. As a result, MRP2-mediated efflux of isorhamnetin was decreased. Similarly, the absorption properties of isoflavonoid puerarin, the main component of *Pueraria labora*, was increased in a Caco-2 cell model when the Radix Angelicae Dahurica extract was added (an herb-pair frequently used in traditional Chinese medicine) [119]. Since the permeability of puerarin increased in an apical-to-basolateral direction while it decreased in the opposite direction in the presence of P-gp and MRP inhibitors (verapamil, cyclosporin A, MK571), it is presumable that Radix Angelicae Dahurica components might act in the same way by enhancing puerarin uptake.

Greater bioavailability of individual compounds if applied as unpurified extracts rather than alone was found in a study evaluating transport mechanisms of chamaechromone and neochamaejasmin B, bioflavonoids with antimicrobial activity present in the root of *Stellera chamaejasme* L. [130]. Neochamaejasmin B significantly improved the bioavailability of chamaechromone favoring its uptake through inhibition of MRP2 and BCRP in the cells overexpressing these transporters.

The distribution of flavonoids is another important pharmacokinetic parameter that shows the involvement of transporters, especially in explaining the presence of hydrophilic flavonoid molecules, e.g., anthocyanins, in various peripheral tissues. In particular, anthocyanins were detected in the brain, both cortex and cerebellum, in pigs that were fed a blueberry-supplemented diet for 4 weeks [131]. A similar study with oral administration of pelargonidin on rats showed the presence of pelargonidin in the brain [132]. Moreover, in another experiment in rats with ligated stomachs, after only a short incubation (10 min) in the stomach, anthocyanins were present in the brain tissue (~0.5 μM) [133]. The mechanisms remain to be elucidated but the results of these studies indicate that anthocyanins can cross the blood-brain barrier, and can potentially accumulate in the central nervous system. Also, orally administered quercetin accumulated in brain tissue [134]. In addition, anthocyanins were also recovered in various ocular tissues, confirming that intact anthocyanins are capable of passing through the blood-retinal barrier [24]. All these findings are interesting because CNS-targeting drugs are usually more lipophilic (have higher log P values), while anthocyanins and other flavonoids are hydrophilic molecules.

Many distribution studies were performed using single-dose peroral application of radiolabeled flavonoids, and then following their radioactivity in the body. The most widely distributed flavonoid is flavan-3-ol (−)-epigallocatechin gallate (EGCG), which was detected in the gastrointestinal tract, lungs, heart, liver, spleen, pancreas, kidney, brain, bone, bladder, skin, and many other organs in mice, indicating the ability of EGCG and its metabolites to access all tissues [135]. The isoflavone genistein also distributes into reproductive tissues in higher concentrations than in peripheral tissues [65]. Furthermore, genistein also crosses the placental barrier, reaches the fetal tissues [136], and is also present in the milk of lactating animals [137]. Similarly, catechins cross the placenta and reach the fetal organs (~1 nM concentration) 0.5–1 h after the mother received the dose [138].

Differences in the elimination kinetics of flavonoids and their metabolites in urine and/or via bile into feces also suggest the potential involvement of membrane transporters. One example is that of the inhibition of excretion, namely (−)-epigallocatechin (EGC) and (−)-epicatechin (EC) affect the elimination of (−)-epigallocatechin-3-gallate (EGCG) [59]. Anthocyanins exhibit fast excretion, thus their plasma values fall below detection levels after 4 h [139]. On the other hand, isoflavones are eliminated somewhat slower, e.g., 90% of the genistein dose was excreted in 48 h [65].

3.2 Pharmacodynamic Perspective

In general, pharmacodynamics predicts the extent and time course of the observed flavonoid bioactivity in correlation with the local tissue concentration. In fact, tissue-specific flavonoid concentrations are determined by both uptake and efflux transporters within a tissue. Another variable is that transporters are present in varying abundance in different tissues. Thus, plasma concentrations usually differ from the levels in target organs in which the bioactivity is studied.

Flavonoids have pharmacological activity in the very low concentration range, with saturation observed at higher concentration. For example, cellular antioxidant activity (CAA) assay showed that both monomeric cyanidin 3-glucoside [140], and a mixture of 15 bilberry anthocyanins [141] have strong intracellular antioxidant activity in the low concentration range (nM), which correlates with the reported human plasma values (Table 22.1); moreover, apparent saturation was also detected, thereby showing the plausibility of carrier-mediated transport of anthocyanins. Likewise, another study showed that apple juice phenolic extract exhibited stronger antioxidant activity in a cell-based antioxidant assay, as predicted from chemical-based methods that measure just the inhibition of chemical oxidation [142]. At extracellular concentrations in the nM range, passive membrane transport would occur at a rate too low to warrant the possibility of observing a cellular biological response. Therefore, carrier-mediated uptake of flavonoids must be assumed.

Further, in a more complex model of studies such as experiments on isolated organs, the role of membrane transporters can be evident. On the isolated perfused rat liver model, the interaction of genistein with MRP2 transporter was discovered, as genistein was able to inhibit the secretion of anionic MRP2 substrates [143]. Recently a novel approach to transporter inhibition has been introduced into transporter pharmacology, i.e., using specific antibodies for targeting the extracellular domain of the transporter protein, and thereby inhibiting its function. Our research group has successfully tested antibodies against BTL on the model of the isolated rat aorta, where BTL inhibition reduced the vasodilation induced by anthocyanins [144], and also on the model of ischemia-reperfusion injury in rat hearts, where pretreatment with BTL antibodies reduced the cardioprotection of cyanidin 3-glucoside [140]. The advantage of using antibodies against membrane transporters in comparison to chemical inhibitors is their specificity, and the avoidance of inducing secondary (unwanted) pharmacological responses.

From a clinical perspective, looking at peripheral tissues such as brain and retinal tissues, the involvement of membrane transporters can again be invoked. Recently, flavanols, anthocyanins and/or flavanones have been suggested to possess the greatest potential to act on the cognitive processes [145]. In one clinical study, adults with memory decline who consumed grape juice polyphenols had significant improvement in a measure of verbal learning and improved recall [146]. Likewise, in a similar study, participants who consumed grape juice showed reduced semantic interference on memory tasks, and greater activation in the anterior and posterior regions of the right hemisphere, as observed by functional magnetic resonance imaging [147]. Both studies show that polyphenols can enhance neurocognitive function in older adults with mild memory decline, thereby suggesting that flavonoids are not only distributed across the blood-brain barrier (as shown by numerous animal studies), but that they also have marked clinical effects. Similarly, berry anthocyanins can pass the blood-retina barrier, distribute widely in ocular tissues, interact directly with rhodopsin, and modulate visual pigment function, though clinical evidence is still lacking [148].

4 ANATOMICAL DISTRIBUTION OF FLAVONOID-RELATED MEMBRANE TRANSPORTERS

There is interplay between uptake and efflux transporters, which is further dependent on their differential distribution at the cellular, tissue, and organ levels. ABC transporters and solute carriers can be found in the cell membrane of nonpolarized cells, and on either basolateral or apical surfaces of polarized cells. In the latter settings, vectorial transport of solutes is present. The cellular locations of only certain transporters are currently known, while for the majority of the others there still is a gap in our knowledge. Indeed, transporter function can be fully understood only if their cellular and tissue localization is assessed. Even more, no inferences can be drawn from data obtained in a given experimental model, since species-dependent variations in cell sorting or tissue expression can be expected.

In comparison to the number of transporters involved in xenobiotic disposition, only a few transporters are currently known to significantly interact with flavonoids. In principle, transporters that are localized at key gateway organs, such as the stomach [149,150], intestine [151], liver [72], kidney [96,152], and brain [153], can potentially contribute to the epithelial barrier permeability of flavonoids, as shown schematically in Fig. 22.1. However, the exact molecular mechanisms by which flavonoids are transported still remain to be elucidated. On one hand, multiple transporters recognize flavonoids as their substrates and/or modulators, as presented in Table 22.2; on the other, the structural features required of flavonoids to act as substrates and/or modulators for a specific transporter are still poorly known.

4.1 Intestinal Transporters

Flavonoid bioavailability is very low, due to extensive metabolism at the intestinal level and low overall permeability. Isoflavones, followed by catechins, flavanones, and quercetin glucosides are best absorbed. The least absorbable are the proanthocyanidins (also known as condensed tannins), galloylated tea catechins, and

TABLE 22.2 Synopsis of Reported Interactions of Flavonoids With Selected Transporters[a,b]

Flavonoid	BCRP	BTL	GLUT1	GLUT2	MCT1	MRP1	MRP2	MRP3	MRP4	OAT1	OAT3	OAT4	OATP1A2	OATP1B1	OATP1B3	OATP2B1	P-gp	SGLT-1	SVCT1	SVCT2	URAT1	Refs.
Flavones																						
Apigenin	I				I	I	I						I	I	I	I			I			[72–79]
Apigenin sulfate	S								S													[80,81]
Baicalin							S															[82]
Chrysin	I Ind				I		S	S									Ind					[74,83–85]
Chrysin sulfate									S													[81]
Diosmetin					I	I																[74,86]
Luteolin					I	I								I					I			[73,74,78,86]
Orientin																	S					[87]
Oroxylin A-7-*O*-glucuronide							S															[82]
Pongamoside A											S											[88]
Scutellarin	S						S										S					[89]
Spinosin																	S					[90]
Vitexin																	S					[87]
Wogonoside							S															[82]
Flavonols																						
Galangin		S				I																[86,91]
isorhamnetin							S															[92]
Kaempferol	I	S			I	I	I S						I			I	S I St(P)					[74,86,91,93–95]
Morin					I	I				S I				I			I				I	[73,77,86,95–97]
Quercetin	S I Ind	S			I	I	S Ind			S	S		I			I	S I Ind St	S	I	I		[74,78,85,86,93,95,96,98–100]
Quercetin-3′-*O*-sulfate												S										[101]

Rutin						S	S						St			S						[73,98,102]
Tiliroside						S																[94]
Flavanones																						
Hesperitin	S			I		S	S									I						[74,100,103]
Hesperitin sulfate								S														[104]
Liquiritin																S						[104]
Naringenin	I			I	I	S									I	S I Ind		I				[78,95,105]
Naringin	I			I								I				I						[74,103,105,106]
Flavanols																						
Catechin																I						[107]
Epicatechin				S		S						S		S		St(al)						[107–109]
ECG												S	I	S	I							[107,110]
Epigallocatechin						S										I						[107,110]
EGCG				S	S	S						S	I	S	I	N						[107,110,111]
Isoflavones																						
Biochanin A	I			I	I								I			I						[73,74,76,112]
Genistein	S I			I	I	S I			S	S			I			I Ind				I		[73,74,77,96,113–118]
Daidzein	I S				I	S			S	S						S						[112,114,115]
Puerarin						S										S						[119]
Anthocyanins																						
Cyanidin -3-glu	I	S	S													I	S	I				[78,120–122]
Delphinidin	S	S																I				[78,121,122]
Flavolignans																						
Silybin				I	I				S I				I			I						[73,77,95,96]

[a] *Abbreviations used for the type of interaction:* S, *Substrate;* I, *Inhibitor;* Ind, *Inducer;* St, *Stimulant.*

[b] *Abbreviations used for the mentioned transporters:* BCRP, *breast cancer-resistant protein;* BTL, *bilitranslocase;* GLUT1, *glucose transporter 1;* GLUT2, *glucose transporter 2;* MCT1, *monocarboxylate transporter 1;* MRP1, *multidrug-resistance-related protein 1;* MRP2, *multidrug-resistance-related protein 2;* MRP3, *multidrug-resistance-related protein 3;* OAT1, *organic anion transporter 1;* OAT3, *organic anion transporter 3;* OATPs, *organic anion transporting polypeptides;* P-gp, *P-glycoprotein;* SGLT1, *sodium/glucose cotransporter 1;* SVCT1, *sodium-dependent vitamin C transporter 1;* SVCT2, *sodium-dependent vitamin C transporter 2;* URAT1, *urate transporter 1.*

anthocyanins [1]. Though limited in terms of extent, flavonoid absorption across the intestinal epithelium is a fast process, pointing to the involvement of membrane transporters.

To illustrate the complexity of flavonoid passage through the intestinal epithelial barrier, resulting from the interplay of either uptake or efflux transporters (Fig. 22.2), we describe a study of the transport mechanisms of two physicochemically and pharmacologically distinct flavonoids, quercetin (a flavan-3-ol), and naringenin (a flavanone), under various pH conditions across a Caco-2 cell monolayer [154]. The two flavonoids were absorbed by both passive diffusion and a transporter-mediated mechanism, susceptible to inhibition by various inhibitors targeting known membrane transporters. Quercetin permeability in the apical-to-basolateral direction was pH-dependent, suggesting involvement of a pH-sensitive transporter. The use of estrone-3 sulfate, an OATP2B1 substrate and inhibitor, decreased quercetin absorption, thereby confirming that quercetin is an OATP2B1 substrate. Naringenin absorption, on the other hand, was not pH-dependent, neither was it decreased in the presence of estrone-3 sulfate, suggesting that naringenin is not a substrate of the OATP2B1 transporter. Moreover, coincubation with phlorizin, a glycosylated dihydrochalcone, which is transported by the sodium-dependent glucose transporter 1 (SGLT1) [155], increased naringenin permeability, suggesting that it was not an SGLT1 substrate either. Indeed, results obtained by other authors rather suggest that SGLT1 might not transport flavonoids [156]. However, when MK-571, an MRP1 inhibitor, was applied, naringenin absorption was decreased, showing that naringenin is transported via MRP1. Since the permeability of both quercetin and naringenin was higher in the basolateral-to-apical than in the opposite direction, it was reasonable to assume the involvement of a carrier-mediated transport efflux system. Using specific ABC inhibitors, it has been shown that the quercetin efflux is mediated by MRP2 but not by P-gp. Naringenin, on the other hand, was both an MRP2 and a P-gp substrate.

Transport of the flavonol isorhamnetin in Caco-2 cells involves various mechanisms as well [157]. It seems that isorhamentin uses both para- and transcellular pathways and that membrane transport mechanisms mediate both passive diffusion and active transport. The latter paradigm was characterized by using various inhibitors of membrane transporters, both influx and efflux.

Another similar study addressed the metabolism and transport of hesperetin (the aglycone of hesperidin, the

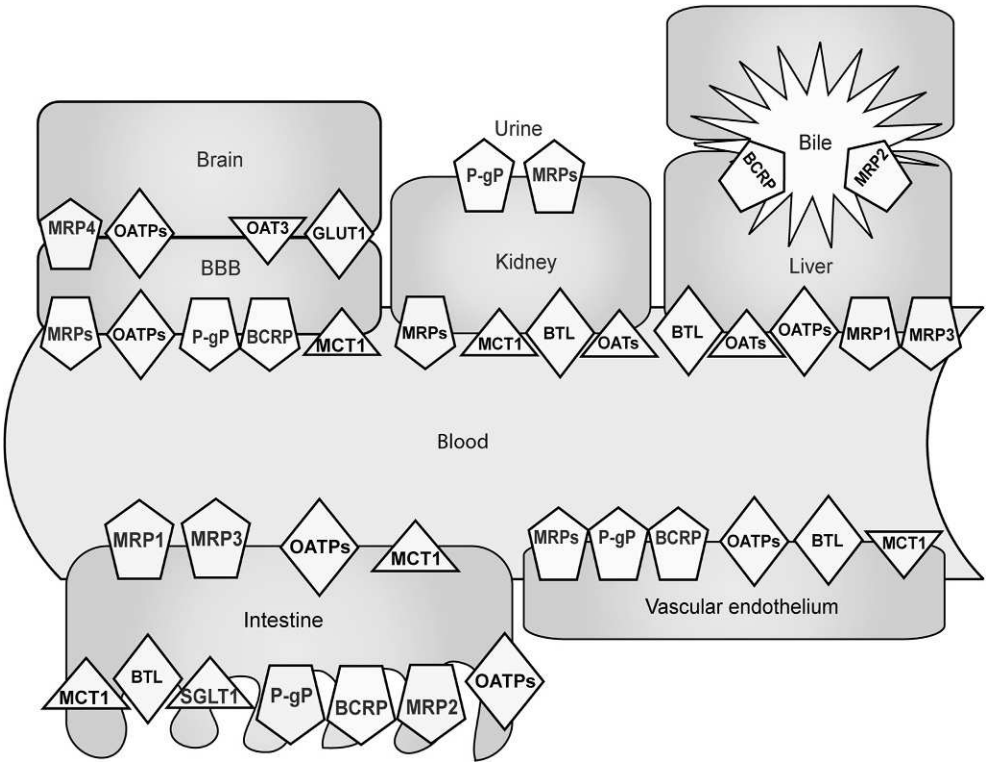


FIG. 22.2 Scheme showing extensive distribution of cell membrane transporters as potential routes for flavonoid cell transport, in both the central compartment and different peripheral tissues (brain, kidney, liver, intestine, and vascular endothelium). ABC transporters are indicated by pentagons (⬟). Solute carriers by triangles (▲) (one direction transport), or diamonds (◆) (bidirectional transport). *BBB*, blood-brain barrier; *BCRP*, breast cancer-resistant protein; *BTL*, bilitranslocase; *MCT1*, monocarboxylate transporter 1; *MRPs*, multidrug-resistance-related proteins; *OATs*, organic anion transporters; *OATPs*, organic anion transporting proteins; *P-gP*, P-glycoprotein; *SGLT1*, sodium/glucose cotransporter 1.

major flavanone present in sweet oranges and orange juice), and its metabolites across the intestinal epithelium, using the same Caco-2 cell monolayer model [158]. Apically applied hesperetin permeated the monolayer and was found in the basolateral compartment. Since this process was not affected by inhibitors of ABC transporters (Cyclosporin A, MK571, PSC-833, GF120918, Ko143), the authors concluded that hesperetin could move transcellularly by passive diffusion [158]. However, this conclusion should be supported by the experimental demonstration that influx transporters were indeed not involved. Moreover, that study showed efflux of hesperetin conjugates (hesperetin 7-*O*-glucuronide and hesperetin 3′-*O*-glucuronide) to the apical side of the transwell monolayer, mediated by BCRP (but neither by P-gp, which was highly expressed in Caco-2 cells, nor by MRP2) [158]. These findings indicate that ABC-mediated hesperetin transport could be one of the main steps limiting hesperetin bioavailability.

A further study indicated that hesperetin 7-*O*-glucuronide and hesperetin 3′-*O*-glucuronide might be low-affinity substrates of MRP2 and MRP3 [159]. Because of its location in the basolateral membrane of intestinal cells, MRP3 could likely play a role in the basolateral transport of hesperetin glucuronides from intestinal cells into the blood [159].

It must be noted that the involvement of apically located influx transporters in flavonoid absorption remains incompletely characterized. Some studies have been undertaken using compounds belonging to certain flavonoid subclasses.

Green tea flavonoids—epicatechin gallate (ECG), epigallocatechin gallate (EGCG), epicatechin (EC) and epigallocatechin (EGC)—have also been the subject of uptake and transepithelial transport studies. ECG and EGCG are able to penetrate the apical membrane of Caco-2 cells and accumulate intracellularly in significant amounts [108,160]. Monocarboxylate transporter (MCT) inhibitors increased the uptake of ECG, thereby suggesting that MCT1 physiologically limits its absorption [108,160]. The subcellular localization of MCT1 is controversial [161], and indeed more studies are needed to interpret these findings.

In more recent work [110], the interactions of flavan-3ols (ECG, EGCG, EC and EGC) with four members of the OATP/SLCO family were elucidated. This study showed that OATP1A2, which is present at the apical membrane of enterocytes, could be involved in the absorption of ECG and EGCG from the intestinal lumen.

Absorption of anthocyanins through Caco-2 cells has been associated with glucose intestinal uptake inhibition, possibly involving the facilitative glucose transporter 2 (GLUT2) [162]. Expression of GLUT2 transporter in Caco-2 cells increased by 60%, if pretreated with anthocyanins, in comparison to the control, as assessed by RT-PCR. In another work, a polyphenol extract from strawberry, rich in pelargonidin-3-*O*-glucoside, was able to influence glucose uptake into the cells, and transport to the basolateral side by inhibiting the activity of GLUT2 [163]. A more recent study showed that SGLT1 and GLUT2 might participate in cyanidin-3-*O*-glucoside absorption in the small intestine [120]. In Caco-2 cells both pharmacological (ploretin, GLUT2 inhibitor; phloridzin, SGLT1 inhibitor) and biological inhibition (siRNA specific for SGLT1 and GLUT2) decreased the absorption of cyanidin-3-*O*-glucoside.

Apart from these well-known transporters, bilitranslocase (BTL) is a high affinity transporter for various dietary anthocyanins [20,91]. BTL is located in the mucus-secreting cells, gastric pit, and the H^+-secreting parietal cells [164]. It is also expressed on the enterocyte brush border [20]. However, the function of BTL in the intestinal absorption of these flavonoids still needs to be investigated.

Intestinal efflux transporters have received more attention and have been studied extensively. In the study analyzing 16 dietary anthocyanidins and anthocyanins [121], all of the tested compounds interacted with BCRP; 7 of them emerged as potential BCRP substrates, and 12 as potential inhibitors. The study suggested that anthocyanins and their aglycones cannot cross the cell membrane passively, and may be actively transported out of intestinal tissue [121].

Although P-glycoprotein was not involved in active quercetin efflux in the Caco-2 cell model [154,155], another study on Bcap37/MDR1 cells (human breast cancer cell line overexpressing P-gp) [93] showed that quercetin, keampferol, and isorhemnetin from *Ginkgo biloba* leaves are substrates for P-gp. The same transport mechanism is reported for liquiritin, active compound of a traditional Chinese pain relief medication [165]. A bidirectional transport permeability test in Caco-2 cell model showed that the absorptive permeability of liquiritin was low while its secretory permeability was much higher. In the presence of verapamil (P-gp inhibitor) the efflux ratio was significantly lower due to an increase of absorptive permeability and decrease of secretory permeability. An interesting in vivo study [166] demonstrated that intestinal absorption of the flavonoid glabridin (the major isoflavone of the root of *Glycyrrhiza glabra*, one of the most commonly used herbal medicines) was significantly higher in P-gp knockout mice than in wild-type mice. Additional experiments with the P-gp inhibitor verapamil and the P-gp substrate digoxin in Caco-2 cells confirmed that glabridin was a P-gp substrate, as well as a potent P-gp inhibitor. We have already mentioned that naringenin has also been reported to be a P-gp substrate [154]. Orientin and vitexin, flavones from the flowers of *Trollius chinensis*, pass the Caco-2 monolayer by passive diffusion but are also transported by efflux transporter P-gp [87].

Numerous studies have demonstrated that MRP2 plays an important role in flavonoid efflux into the intestinal lumen. Some of them have already been mentioned in the previous text. The role of MRP2 in pumping tea flavonoids (EC, ECG and EGCG) back into the lumen has been well described [108,111,160]. Genistein-7-glucoside, the main dietary source of genistein (a natural estrogenic compound belonging to the isoflavones) has also been reported to be a substrate of MRP2 [113], as well as the flavone chrysin [83]. More recently, also tiliroside has been listed as MRP2 substrate. An interesting study evaluated absorption, transport, and metabolism of tiliroside and its related flavonoids (kampferol and kaempferol-3-glucoside) in Caco-2 cells [94]. The transport studies revealed a very low absorption of tiliroside while its efflux was seven-fold higher. On the other hand, keampferol transport was predominantly in the apical to basolateral direction. When tiliroside was incubated with MK571 or rifampicin (MRP2 inhibitors), its transport in apical to basolateral direction significantly increased while basolateral to apical direction decreased. This suggested that MRP2 might play an important role in tiliroside efflux and consequently reduces its absorption. Kaempferol transport was not changed in the presence of two inhibitors. However, coincubation with kaempferol increased tiliroside absorption, suggesting that keampferol is probably not a MRP2 substrate but rather its inhibitor. These findings again point to the importance of interactions between flavonoids found together in foods.

MRP2 figures as an important factor in the study evaluating the absorption, disposition and metabolism of three major *Radix scutellariae* flavonoids (flavones baicalein, wogonin, oroxylin A) [82]. Membrane transporters were studied in relation to their glucuronides formed inside the cell. While the apical to basolateral transport of three conjugates was limited, their basolateral to apical transport was shown to be mediated by membrane transporters. MK751, estrone 3-sulfate and estradiol glucuronide inhibited basolateral to apical transport in Caco-2 cells indicating the role of MRPs and BCRP in it, while P-gp inhibitors had no effect. Additional experiments done in MDCKII cells overexpressing human MRP2 (MDCK/MRP2), MRP3 (MDCK/MRP3) or MDR1 (MDCK/MDR1), showed that all three flavone metabolites were accumulated at the apical side of MDCK/MRP2 cells in which their permeability coefficient were significantly increased in the basolateral to apical direction.

BCRP has also gained much attention recently as an actor in flavonoid transport. It has been demonstrated that BCRP, localized apically in enterocytes, is actively involved in the transport of several anthocyanins and anthocyanidins out of intestinal tissue [121], as well as in the efflux of quercetin glucuronides [167], thereby limiting their intestinal absorption and bioavailability in plasma. Genistein is another natural substrate of BCRP and at the same time a competitive inhibitor of the BCRP-mediated drug efflux [114]. However, an interesting recent study on the pharmacokinetic parameters of chrysin and its metabolites (chrysin-sulfate and chrysin-glucuronide) and the role of BCRp in their excretion, brings out discrepancy between in vivo and in vitro results [80]. After oral administration of chrysin in *wt and Bcrp*$^{-/-}$ *mice* no significant impact on systemic exposure of chrysin conjugates was noticed (no changes in C_{max} and T_{max} were detected for chrysin and chrysin sulfate in plasma). In Caco-2 cells, on the other hand, BCRP inhibitor Ko123 reduced apical excretion and increased basolateral excretion of chrysin-sulfate. This discrepancy is explained with the hypothesis that certain amounts of conjugates probably do not enter the systemic circulation but are excreted into the bile.

It is quite common that a flavonoid molecule appears as a substrate for several different efflux transporters, which, additionally, limits its bioavailability. In situ single-pass intestinal perfusion of flavone scutellarein shows that its absorption, as well as the formation of its conjugate scutellarin, was increased in rat jejunum after perfusion with verapamil (P-gp), probenecid (MRP2), and reserpine (BCRP) [89]. in vitro experiments done in Caco-2 cells confirmed the role of these transporters in the absorption of scutellarein. Studies on rutin absorption and transport into Caco-2 reveal an increase in apical to basolateral transport after transporter inhibitors verapamil, cyclosporine and MK157 are applied, suggesting the role of P-gp, MRP2 and MRP3 in the efflux of rutin across the apical membrane [102]. Isoflavones genistein and daidzein and their microbial metabolites dihydrogenistein and dihydrodaidzein all act as BCRP substrates since estrone-3-sulfate (BCRP inhibitor) increases their permeability coefficients in Caco-2 cell model [115]. However, daidzein is probably transported also by P-gp and MRPs, since their known inhibitors (verapamil and MK571) increased its apical-to-basolateral transport. Interestingly, the transport of genistein, dihydrogenistein and dihydrodaidzein might involve passive diffusion as well.

4.2 Hepatic Transporters

Flavonoids and their metabolites are transported from the circulatory system into the liver, further metabolized and finally exported to the bile. Once in the GI tract, they can return to the liver via the enterohepatic circulation [168,169]. This type of dynamics results, again, from a complex functional interplay of influx and efflux transporters.

Focusing on the transport mechanisms by which flavonoids are taken up into the liver, the first evidence of transporter-mediated uptake of dietary anthocyanins

has been provided by a pioneering study on the interaction of dietary anthocyanins with bilitranslocase [122], further refined by detailed QSAR analyses [170]. This transporter [20,171], known to mediate bilirubin transport into human liver cells [172], has also been show to mediate anthocyanins into HepG2 cells [173].

More recently, a series of flavonoid aglycones have been shown to interact with OATP1B1 [73]. In further work [110], interactions of ECG, EGCG, EC and EGC with four members of the OATP/SLCO family were elucidated. The focus was on OATP1A2 (expressed at the apical membrane of enterocytes), OATP1B2 (expressed both in enterocytes and hepatocytes), and OATP1B1 and OATP1B3 (both expressed in hepatocytes). CHO cells stably transfected with human OATP1B1, OATP1B2, and OATP1B3, and HeLa or HEK-293 cells transiently transfected with OATP1A2 were used. ECG and EGCG did not only alter the function of the four OATPs and transport of their well-known substrates, but were themselves identified as novel substrates for OATP1A2 and OATP1B3 [174].

Another recent study confirms the role of OATs and OATPs in the uptake of quercetin conjugates (glucuronades and sulfates) in HepG2 and HEK293 cells [101]. While the most probable mechanism for glucuronides uptake was deemed to be passive diffusion, sulfates entered the cell in a carrier-dependent way. OAT and OATPs inhibitors significantly reduced the uptake of quercetin-3′-*O*-sulfate in HEPG2 cells. Using HEK293 cells expressing OAT2, OAT4 and OATP4C1, it was found that quercetin-3′-*O*-sulfate uptake increased when OAT4 and OATP4C1 were overexpressed. Similarly, when OATP4C1 was silenced by siRNA in HepG2 cells the uptake of flavonoid was lower by 40%.

Another study characterized the integrated contribution of both influx and efflux transporters to the disposition of the flavone baicalein by the liver [175]. Both OATP1B3 and especially OATP2B1 were shown to contribute to the hepatic uptake of flavonoid conjugate metabolites, whereas BCRP and MRP2 were found responsible for the biliary excretion of the conjugates.

MRP2 is located at the apical side of hepatocytes, being responsible for the biliary excretion of flavonoids such as quercetin. MRP2 transporter shares a 49% identity with MRP1, but is inhibited by flavonoids of different structural type [175]. Indeed, recently it was demonstrated that among 29 flavonoid compounds belonging to the subclasses of flavones and flavonones, only myricetin and robinetin inhibited MRP2 in the physiologically relevant concentration range (having IC_{50} values below 50 μM) [86]. An investigation of the effect of efflux transporters on the disposition of naringenin metabolites in liver vesicles, in the presence of the MRP2-inhibitor MK-571, confirmed the hypothesis that MRP2, together with BCRP1, play important roles in the efflux of naringenin glucuronides [176]. In addition, these transporters can also compensate for each other's functional deficiencies [176]. Moreover, all of the quercetin conjugates activate MRP2 ATPase, albeit with different potencies and efficacies [177]. Another study showed the primary role of MRP2 in the biliary elimination of silymarin flavonolignan conjugates [178]. Indeed, tests done using isolated perfused livers from MRP2-deficient TR-rats showed that MRP2 is the major canalicular transport protein involved in the biliary excretion of silymarin flavonolignan conjugates [178].

Immunoquantification of the expression of ABC transporter proteins has shown that P-gp is most abundant in the liver [179], but the mRNA levels of the same transporters showed that MRP2 is significantly more abundant than P-gp in the same tissue [180]. All P-gp substrates share common chemical properties, such as relative hydrophobicity and a neutral or cationic character under physiological conditions [181]. Some of thoese characteristics are shared by flavonoids, and thus many flavonoid moieties were reported to interact with P-gp [93,182–184]. Using the specific P-gp inhibitor verapamil and the Bcap37/MDR1 cell line which expresses high levels of P-gp, the flavonoids quercetin, kaempferol and isorhemnetin from *Ginkgo biloba* leaves were confirmed as substrates for P-gp [93]. The accumulation of rhodamine 123 in several cells transfected with MDR1 gene was studied in the presence of naturally occurring plant-derived flavonoids. Some of these, such as rotenone, chrysin and epigallocatechin increase the fluorescence activity ratio better than the reference compound verapamil [185]. Others have moderate (cathechin, neohesperidin, naringin, robinin, phloretin), or no effect (dihydroquercetin, dihydrofisetin, dihydrorobinetin, phloridzin, kaempferol, sakuranin, robinetin and dihydrobinetin) [185]. Moreover, MCF-7/ADR cells, which overexpress P-gp, enhanced rhodamine-123 accumulation when treated with quercetin [186]. Naringin, if injected intraperitoneally for 3 consecutive days prior to doxorubicin treatment, is able to both significantly lower P-gp expression and also to increase sensitivity to the drug [187].

The expression of MRP1 in the liver is limited and its contribution to biliary excretion is unclear [188]. Flavonoids with a higher inhibitor effect on MRP1 activity are characterized by the presence of two hydroxyl groups at positions 3′ and 4′ in ring B (that generate the catechol moiety), and the planarity of the molecule. Transport of ^{3}H-LTC4 was measured on membrane fractions from Sf9 insect cells, overexpressing human MRP1 transporter, in the presence of various concentrations of baicalein-7-glucuronide (BG) [189]. BG activated the ATPase activity of MRPs in the physiologically relevant concentration range [189]. Quercetin metabolites of II phase, formed by 4′-*O*-methylation and especially glucuronidation at the 7-hydroxylmoiety,

resulting in, e.g., 7-*O*-glucuronosyl quercetin, significantly increased the potential of quercetin to inhibit MRP1- and MRP2-mediated calcein transport [190].

4.3 Renal Transporters

Urinary excretion of flavonoid conjugates is well documented and varies according to the individual flavonoid aglycone [191–196]. Vectorial transport of hydrophilic compounds across the renal proximal tubules is achieved through cooperative transport by uptake transporters expressed on the basolateral membrane such as OAT1/SLC22A6, OAT3/SLC22A8, and efflux transporters expressed on the brush border membrane such as MRP2/ABCC2, MRP4/ABCC4, P-gp (MDR1/ABCB1), and BCRP/ABCG2 [197,198]. Similarly, the coupled action of uptake transporters and efflux transporters may result in effective elimination of flavonoids and their metabolites from the circulation, and contribute to the rapid elimination of flavonoids in humans. However, the molecular mechanism underlying the possible uptake of flavonoid conjugates into renal tubules via basolateral transporters is still poorly characterized.

In OAT1- or OAT3-overexpressing human embryonic kidney cells, namely the HEK 293 cell line, nine quercetin and isoflavone metabolites that are found in vivo were characterized for their transport activities [199]. The authors found that sulfated conjugates of quercetin and genistein are transported by OAT1, while glucuronide conjugates of daidzein, genistein, glycitein and quercetin appear to be preferential substrates for OAT3. Quercetin-3′-*O*-sulfate was also transported by OAT3, but the transport activity was much lower compared with OAT1 [199]. Moreover, the aglycones quercetin and genistein were also taken up into the OAT1- and OAT3-expressing cells, but the uptake was not significantly different from the control cells, thereby suggesting the potential role of passive diffusion owing to the relative hydrophobicity of aglycones. All in all, glucuronidated and sulfated conjugates of flavonoids are substrates of apical efflux transporters. Quercetin glucuronides are transported by MRP2 [200] and BCRP [167]; while quercetin-3′-*O*-sulfate is transported by MRP2 [201].

The role of Oat3 (predominantly expressed in the kidney) in multiple metabolic and signalling pathways was studied using WT and Oat3-deficent mice as well. The list of natural metabolites that were significantly altered in the plasma of Oat3-deficent mice included a flavone pongamoside A, suggesting the role of Oat3 in its transport [88].

Importantly, in vitro studies also showed that quercetin significantly inhibits the cellular uptake of [3H]p-aminohippurate mediated by human (h) OAT1 and [3H]-estrone sulfate mediated by hOAT3 in MDCK cells [202].

The contribution of BTL in the urinary excretion of dietary anthocyanins has also been described in a study that confirmed the previously known [203] basolateral localization of BTL, and highlighted the different affinity for anthocyanins of either the liver or the kidney homologues of BTL [152].

Primary active efflux transporters, expressed on the apical surface of proximal kidney tubular cells, are important mediators of urinary excretion of flavonoids and their metabolites. The proximal tubular cells line HK-2 was used as a model to study the effect of grapefruit juice (GFJ) and its active principles, the flavonoids kaempferol (KF) and naringenin (NAR), on the tubular P-gp transporter [204]. The study demonstrated that treatment of HK-2 cells for several days with increasing concentrations of GFJ, KF, and NAR decreased both P-gp activity and its expression [204]. Moreover, pretreated cells were also more susceptible to the cytotoxic effects of cyclosporin A and vinblastine, two well-known cytostatic substrates of P-gp [204]. However, even though the concentrations required for flavonoids to produce significant modulation of P-gp activity appear to be achievable after ingestion of food and dietary supplementation (physiologically relevant), the main metabolites of flavonoids (glucuronides and sulfate conjugates) are unable to interact with P-gp since these metabolites are anionic. Thus, systemic inhibition of P-gp by flavonoids or their metabolites may, in general, be insignificant after regular supplementation [205].

Other important membrane transporters, MRP1, MRP4 and MRP5 are present in the basolateral or luminal membrane of kidney proximal tubules. In experiments using inside-out vesicles prepared from red blood cells known to express MRP1, MRP4 and MRP5, six different polyphenols (quercetin, silymarin, daidzein, naringenin, hesperetin and resvetratrol) were used in increasing concentrations to assess if they could affect MRPs-mediated transport [105]. All six polyphenols showed high potencies and comparable IC_{50} values for inhibition of MRP4-mediated cGMP uptake, whereas they had only limited potency against MRP1. However, resistance to quercetin was not observed in MRP1-expressing cells, thereby suggesting that quercetin, or some of its metabolites, are better substrates for MRP4 and MRP5 than MRP1 [105]. On the contrary, silymarin behaves as a typical inhibitor in short term uptake assays, flow cytometry studies and ATPase assays, and it significantly inhibits MRP1 and MRP4 ATPase activities [105].

The role of MRP4 in flavonoid efflux was highlighted in a recent study, which aimed at characterizing hesperitin sulfonation and transport of its sulfated metabolites (H-7-*O*-sulfate, H-3′-*O*-sulfate) using HEK293 cells overexpressing SULT1A3. MRP4 is the predominant efflux transporter of hesperitin sulfates, since both MRP family inhibition by MK-571 and knockdown of MRP4 caused significant reduction of sulfate excretion. On the other

hand, the role of BCRP was negligible (no changes in sulfate excretion where detected when Ko143, BCRP selective inhibitor, was applied) [104].

Similarly, characterization of apigenin and chrysin sulfate transport in HEK293 SULT cells using specific inhibitors (pan-MRP inhibitor, MK-571, and BCRP inhibitor, Ko143) or knock-down cell models (BCRP- and MRP4-knock down by shRNA) showed that MRP4 was responsible for sulfate excretion. Its inhibition/knock-down increased sulphate accumulation. BCRP was not involved [81].

Furthermore, flavonoids represent a class of potent and non-toxic inhibitors of monocarboxylate transporters 1 (MCT1), even if only few (metabolites of quercetin) are transported by MCT1 [74,206]. Among all the flavonoids tested in this study, luteolin (3,4,5,7-tetrahydroxyflavone) was the strongest inhibitor of γ-hydroxybutyrate uptake by MCT1 in transfected MDA-MB231 cells [74]. In human kidney HK-2 cells, MCT1 and MCT2 are considered to be important transporters responsible for GHB renal reabsorption [207].

4.4 Endothelium in the Vascular and Central Nervous System (Blood-Brain Barrier)

The vascular endothelium regulates a wide variety of functions, such as maintenance of vascular smooth muscle tone, host-defense reactions, angiogenesis, maintenance of interstitial tissue fluid homeostasis, and importantly also the control of macromolecule passage into interstitial tissues. Many in vivo studies showed that after oral administration, certain flavonoids pass the blood-brain barrier (BBB) and have various effects on the central nervous system (CNS), even though their retention within brain tissue only ranges from pM to low nM concentrations [131,133,134,208–210].

Features that distinguish the BBB endothelial cell layer from other vascular endothelial cells include: (i) complex, fenestration-less, high-resistance tight junctions, which prevent paracellular transport of small and large water-soluble compounds from blood to the brain; (ii) much lower levels of pinocytosis, limiting transport of substances through the cytoplasm [211]. In addition, a number of specific uptake and efflux transport systems, together with a complex set of metabolic enzymes (including acetylcholinesterases, alkaline phosphatases, gamma-glutamyl transpeptidases, monoamine oxidases), work to actively deliver nutrients to and remove many other metabolites from the brain [212]. These properties make the BBB an active biochemical interface between the circulation and the CNS; therefore, the BBB plays a crucial role in maintaining homeostasis of the CNS by tightly controlling the transendothelial molecular traffic of metabolites, hormones, immune cells, nutrients and drugs/xenobiotics, including flavonoids [213].

Various in vitro cellular models, such as ECV304/C6, bEND5 and RBE4, have been used to study flavonoid transfer across the BBB [214–218]. They provided evidence that certain flavonoids and their major circulating metabolites, i.e. glucuronides and O-methylated derivatives, can access and transverse the endothelial cell layer with some specificity [219,220]. This is dependent in part on the particular flavonoid's lipophilicity and polarity, i.e., less polar O-methylated derivatives seem capable of greater brain uptake than more polar flavonoids and/or sulfated and glucuronidated derivatives [219,220]. However, it is not yet clear whether the primary route by which they cross the BBB is simple diffusion or carrier-mediated transport. Up to now, only a few compounds from each of the flavonoid subclasses were studied, and there is limited knowledge on the effects of flavonoid structure on bioavailability at the level of the brain. Thus, the mechanism by which flavonoids and their circulating metabolites interact with the BBB remains a hot topic in neuroscience research.

Several transporters of different classes have been described on the plasma membranes of both the luminal (blood) and abluminal (brain) sides of the BBB endothelial cells [221–223]. From the perspective of flavonoid permeability, efflux transporters of the ABC superfamily such as P-gp (MDR1/ABCB1), MRP1-5 (ABCC1-5) and BCRP (ABCG2), together with solute carriers such as GLUT1 (SLC2A1), and members of the OATP (SLCO) and OAT/OCT (SLC22A) transporter families are of special interest. The precise location and orientation of these transporters, together with their expression profile and functionality, largely determine the permeability properties of the BBB, and maintain the rate of flavonoid transport from the blood into the brain, or from the brain back into the peripheral blood [213,224].

From the uptake perspective, there is evidence suggesting that transport mediated by GLUT1, OATs and OATP1A2 may affect the distribution of flavonoid glucuronides into the brain, in a similar manner to other glucuronides, such as morphine-glucuronide [225–227]. Indeed, GLUT1 and OATP1A2 are highly expressed on the luminal and abluminal membrane of capillary endothelial cells of the blood–brain barrier [228,229]. In contrast, the localization of OATs is not clear [230].

From the efflux perspective, ATP-binding cassette (ABC) transporters, such as P-gp and BCRP (localized on the luminal membrane of capillary endothelial cells), or the MRPs (found in both membranes), are the main transporters responsible for limiting the entry of lipophilic substances into the CNS [231]. These transporters are extensively expressed on the BBB [232,233], and some studies suggest that they reduce net cellular absorption of flavonoids or their metabolites. For instance, naringenin is primarily exported by P-gp, whereas the BCRP efflux

pump preferentially exports quercetin [216]. The MRP members MRP1, MRP2, MRP4 and MRP5 exhibit overlapping substrate specificity to P-gp and are also able to transport flavonoid metabolites, including their glutathione, glucuronide and sulfate conjugates [200].

Two recent studies underline the possible role of P-gp in flavonoid efflux at BBB. An in vivo study which analyzed pharmacokinetic characteristics of free spinosin in rat brain, blood and bile, showed that spinosin concentration in blood and brain increased when coadministered with cyclospoin A (a well-known P-gp inhibitor), suggesting the role of P-gp in its disposition. Spinosin concentration in bile was reduced during first 40 min after which it exceeded the control group levels. This indicates the role of P-gp as well as other transport mechanisms in the hepatobiliary excretion of spinosin [90].Another study evaluated transport ability and distribution of eight flavonoids (quercetin, rutin, puerarin, hesperidin, genistein, kaempferol, apigenin and isoliquiritigenin) in BBB cell model (rat brain microvessel endothelial cells) and Caco-2 cells. Flavonoid transport was linearly correlated to their concentration pointing at the passive diffusion as the main mechanism of permeation. However, when quercetin (in BBB cell model) and rutin (in Caco-2 cells) were used in combination with verapamil, their Paap values (apparent permeability coefficients) increased, suggesting the role of P-gp in their transport in these cell models [98].

Furthermore, some flavonoids may act as dual inhibitors/modulators of other transporters, even though they are not substrates of those transporters, suggesting an indirect role in neuromodulatory actions. Among these transporters are MCT1 (SLC16A1) [74,207], SVCT2 (SLC23A2) [99], and CNTs (SLC28A) [234].

Interestingly, BTL has also been found in vascular endothelium, and was characterized as a quercetin uptake transporter [235], and recently also as a cyanidin 3-glucoside transporter [140]. The data suggests that BTL is a bidirectional transporter for flavonoids in the endothelium [140]. In this study, cyanidin 3-glucoside was exposed for a short time (1 min) to the human endothelial cell line, and its uptake was measured. Interestingly, after this brief incubation peonidin 3-glucoside, which is formed in the intracellular compartment by the methylation of C3G, was also detected in the extracellular compartment. Up to date, it is not yet known if BTL is also expressed on the BBB.

5 ISSUES RELATED TO THE LOW BIOAVAILABILITY OF FLAVONOIDS

Membrane transporters together with the cell membrane build up a selective barrier, also in regard to flavonoids. Nevertheless, flavonoids are highly bioactive, and have low apparent toxicity, which makes their medicinal use very attractive. Low plasma concentrations are attributed to their poor absorption and extensive cellular uptake into excretory organs [236], followed by metabolism.

Tight regulation of flavonoid plasma concentrations is accomplished by an additional mechanism, i.e., binding to serum albumin [237], which lowers plasma concentrations of unbound bioactive flavonoids, while offering a storage system for delayed release into the plasma. In addition, albumin-bound flavonoids are more stable against oxygen-dependent degradation [238], so prolonging their biological availability and further extending their plasma half-life. These conditions would protect target tissues from a transient, high-level uptake of flavonoids, which might trigger a toxic reaction in the cells. Indeed, prolonged release of flavonoids from plasma proteins would ensure a more constant rate of cellular uptake of flavonoids.

Transporter-regulated uptake of flavonoids into cells is apparently an efficient system for avoiding flavonoid toxicity. An example of flavonoid toxicity has been shown in the myocardial ischemia-reperfusion model, where low concentrations of bilberry anthocyanins (physiologically relevant concentration range) were cardioprotective, while higher concentrations (supra-physiological concentrations) aggravated the ischemic injury [239]. Limited uptake of flavonoids combined with extensive and fast metabolism predicts that cinnamic acids, hydrophobic flavonoids and lignans are the most bioavailable polyphenols in free-living healthy subjects, who consume a regular healthy diet [240].

However, low bioavailability must not be confused with low bioactivity. Recent studies suggest that some of the flavonoid metabolites also have the potential to possess similar pharmacodynamics as the parent compound [241]. Moreover, a given flavonoid with its metabolites could have opposite, additive, or synergistic effects; therefore, the bioactivity observed in in vivo settings depends on the particular phenolic profile. The synergistic bioactivity of multiple flavonoids, as found in food matrices, is also important. For instance, the antiproliferative activity in vascular smooth muscle cells of four red wine polyphenols, i.e., resveratrol, quercetin, ethyl gallate and (+)-catechin, was stronger than the predicted additive activity of each individual substance [242]. In addition, also in anti-cancer research, the two flavonoids, diosmetin and luteolin, exert synergistic cytostatic effects [243]. Synergistic pharmacodynamics has been also observed for flavonoids and drugs, e.g., the antiviral actions of acyclovir were potentiated by 4′-phenylflavone [244]. In general, there are numerous other studies confirming the synergistic potential of flavonoids. This suggests an explanation for some epidemiological observations whereby dietary flavonoids, even if present

in low concentrations (nM range) in plasma, display health-protective properties. On the other hand, this brings about problems for in vitro cell-based assays or in situ isolated organ studies, which must focus not only on using single molecules or mixture of molecules (plant extracts), but importantly also on the mixture of all their metabolites, and in the physiologically relevant concentration range (nM–μM).

Another important pharmacological concept, i.e., hormesis, must be introduced at this point to further explain the paradox of the "low bioavailability, but high bioactivity" of flavonoids. Hormetic dose response is characterized by a biphasic dose-response, e.g., low-dose stimulation and a high-dose inhibition, or vice versa, and can result from either a direct stimulation or through an over compensatory stimulatory response following disruption of homeostasis [245]. Flavonoids also exhibit hormesis, which can be represented by the example of quercetin bioactivity in cell-based studies. The cancer preventive effects of quercetin are observed at low concentrations of approximately 1–40 μM, and are likely to be mediated by quercetin's antioxidant properties, while prooxidant cytotoxic effects are present at concentrations of 40–100 μM [246]. Membrane transporters can therefore play an essential role in determining the concentration levels of flavonoids, treated as xenobiotics, in both plasma and target tissues, and thereby govern hormesis. Flavonoids can be seen in this context as noxious molecules that reach subtoxic levels in the body, and thus induce mild cellular stress responses which can involve activation of various kinases and transcription factors that induce the expression of genes encoding antioxidant enzymes, protein chaperones, metabolic phase-II enzymes, neurotrophic factors, and other cytoprotective proteins [247].

Moreover, low concentrations of flavonoids (in the physiological range) can affect the expression of genes involved in vascular aging. For instance, treatment of cultured human umbilical vein endothelial cells (HUVEC) with ferulic acid, quercetin or resveratrol (in 0.1 μM concentration) resulted in down-regulation (>twofold) of 363 genes and up-regulation (>twofold) of 233 genes, out of 10,000 analyzed genes [248]. Importantly, the mentioned compounds significantly increased expression of the gene encoding endothelial NO synthase (eNOS), while decreasing expression of the potent vasoconstrictor endothelin-1 [248]. To further develop this concept, more research is needed to identify the cellular targets of flavonoids that account for cytoprotection [249], and not those which have in vitro antioxidant activity. For example, EGCG, via activation of the Akt and ERK1/2 signaling pathway, upregulates heme oxygenase-1 expression in endothelial cells [250], leading to protection against oxidative stress [250]. An additional point to be addressed is whether activation of signaling pathways is triggered by binding of flavonoids on membrane receptors, or by binding to intracellular targets following transmembrane transport.

To accurately translate observations from in vitro assays to in vivo studies, and even further to clinical studies, we must properly address the principal underlying mechanisms, starting by mechanistic interpretation of pharmacokinetic studies. This involves studying the membrane transporters, which can be seen as the key-initiating step towards pharmacological responses.

6 TRANSPORTER-BASED FLAVONOID-DRUG INTERACTIONS

In recent years, there has been increasing awareness about the importance of predicting and optimizing food-drug interactions, especially in natural products containing bioactive moieties and drugs with a narrow therapeutic index. In this context, flavonoid-drug interactions are specific since they must be interpreted from the perspective of flavonoid consumption, i.e., either as therapeutics to ameliorate certain chronic medical conditions, or as part of a healthy diet as primary or secondary preventive medicine measures. Potential interactions should be investigated during the drug discovery process rather than at the final stage, when patients who are on concomitant drug therapy already consume a specific diet or nutraceuticals. It must be noted that the majority of interactions described in the literature are based on metabolic enzyme interaction; however, here we describe examples of flavonoid-drug interactions based on the cell membrane transporters.

As a general rule of thumb, flavonoids increase the bioavailability of co-administered drugs, but this activity is in most cases ascribed to the inhibitory effect on metabolizing enzymes of CYP450 isoforms and/or on the efflux transporter P-gp [251]. However, recently the focus of research has moved to the other transporter proteins, but the issue is experimentally demanding. First, the transporter can have multiple binding sites for multiple substrates, which makes interactions with inhibitors and substrates complex, and difficult to predict [177]. Second, coadministration of flavonoids with drugs can lead to conflicting pharmacokinetic responses, as seen in one study where green tea catechins stimulated uptake by the OATP1B3 transporter, and inhibited uptake of estrone-3-sulfate by OATP1A2, OATP1B1, and OATP2B1 transporters [110]. Following, we present some pharmacokinetic examples based on the flavonoid-drug interaction involving membrane transporters.

Coadministration of fexofenadine, which is an H1-antagonist, with fruit juices such as those of grapefruit, orange and apple, reduced its bioavailability, maximum plasma concentration (C_{max}), and plasma exposure

(AUC) due to sharing of the common membrane transporters P-gp and OATP-A [252,253]. Flavonoids inhibited the uptake of fexofenadine by inhibiting the OATP-A transporters [253]. Also, naringin (the main flavanone found in citrus juices) alone decreased fexofenadine's bioavailability [254]. Interestingly, if individuals were pretreated for 7 days with a peroral dose of quercetin, the bioavailability of fexofenadine increased, which can be explained by the inhibition of P-gp-mediated efflux [255].

Likewise, quercetin increased the bioavailability of cyclosporine, an immunosuppressive drug, both in single dose application or by 3-day pretreatment [256]. Also, grapefruit juice increased cyclosporine bioavailability, C_{max} and AUC. Interestingly, if cyclosporine was administered intravenously, grapefruit juice had no influence on C_{max} and AUC, showing that the effects of grapefruit juice are localized in the gastrointestinal tract [257].

Another interaction was found between silymarin and metronidazole, an antiprotozoal medication. In this study, 4-day peroral application of silymarin decreased the bioavailability of metronidazole, as measured by decreased $t_{1/2}$, C_{max} and AUC, through induction of both P-gp transporter and the metabolizing enzyme CYP3A4 [258].

Quercetin's action as a P-gp inhibitor was described in the literature in relation to pharmacokinetic modulation of several drugs. Namely quercetin increased the bioavailability of (i) valsartan [259], an angiotensin II receptor antagonist; (ii) verapamil [260], a Ca-antagonist; (iii) digoxin [261], a cardiac glycoside; (iv) paclitaxel [262], a cancer chemotherapeutic; (v) moxidectin [263], an antiparasitic drug; and other drugs.

Some studies indicated that flavonoids can act as dual inhibitors, namely by inhibiting both P-gp mediated transport and CYP450-mediated metabolism inside the target cell. Naringin acted as such a dual inhibitor, and thus increased the bioavailability of diltiazem 2.2-fold [264], and paclitaxel up to 3-fold [265]. Also, genistein increased the bioavailability of paclitaxel by the proposed dual mechanism [266].

Some animal studies also suggest that flavonoids could affect pharmacokinetics by the involvement of other transporters, such as BCRP, MRPs, and OATs, in addition to the aforementioned P-gp and OATPs. Crysin, a naturally occurring flavone, is an inhibitor of BCRP-mediated transport, and by this mechanism also increased the bioavailability of nitrofurantoin, an antibiotic [267]. However, only high doses were effective, while low doses had no effect [267]. Other flavonoids, as suggested by cell-based uptake studies or intestinal in situ perfusion studies, are recognized as modulators of BCRP-functioning, such as quercetin [167,268], hesperetin [158], genistein [114], and others, but no relevant in vivo pharmacokinetic interaction has been confirmed. Furthermore, some flavonoids have been identified as inhibitors of MRPs- and OATs-mediated transport, but there is a lack of animal and human studies confirming their role in the interaction with drugs.

7 CONCLUSION

Flavonoids are widely consumed, and thus almost ubiquitously present in human and animal tissues. But from the cell-based perspective, flavonoids are xenobiotics, i.e. they are not synthetized by animal cells and thus mammals and other animals do not express flavonoid-specific or flavonoid-selective cell membrane transporters, in contrast to plants [269]. In other words, flavonoids "hitchhike on carriers," so reaching the intracellular compartment by exploiting both the well-known promiscuity of transporters [270] and a necessary degree of similarity to endobiotics [6]. However, even though they have poor bioavailability, and are present in low concentrations in plasma, they exhibit strong bioactivity. It remains to be elucidated if they are bioactive per se due to their tissue accumulation leading to higher concentrations, or whether they trigger adaptive cell responses already in low concentrations, as could be explained from the hormetic perspective on xenobiotics. Either way, the presence of transporters is the minimum requirement needed for some flavonoid molecules to enter the systemic circulation, and later into the cells of body organs.

It can be speculated that many new transporters interacting with flavonoids will be discovered in the near future. Apart from identification of the membrane protein, the next logical step is to determine the characteristics of the transporter, i.e., the mode of transport (uniport, antiport, symport), tissue localization (renal, hepatic, cardiovascular, etc.), localization of the transporter in the cell (apical, basolateral site), potential role of any cosubstrates, and other properties. In general, we are still at an early stage in our understanding of how flavonoids interact with protein membrane transporters, but current rapid advances in the field of transporter pharmacology are promising.

There is another important issue that is not yet well described in the literature, i.e., individual genetic polymorphisms of cell membrane transporters, and their effect on flavonoid pharmacokinetics. These can lead to potential differences in transporter activity, i.e. substrate specificity and transport kinetics, as has been shown for some solute carriers and ABC transporters with clinical relevance for some drugs. Genetic variants in transporters may be a significant determinant of interindividual variability, and thus represent an important issue for individualized therapy/diet approaches [271].

From the clinical perspective, the healthy food paradigm involves consuming a flavonoid-rich diet and/or food products for both asymptomatic and symptomatic

(on drug therapy) individuals. Therefore, all potential flavonoid-drug interactions, which can occur due to the mutual influence with either cell membrane transporters or metabolizing enzymes, must be characterized in order to ensure better food and drug safety for patients, and importantly also to assure appropriate dietary recommendations for individuals on concomitant drug therapy.

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Disclosure Statement

The authors have no conflicts of interest to report.

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CHAPTER

23

Interaction of Polyphenols With the Intestinal and Placental Absorption of Some Bioactive Compounds

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1 INTRODUCTION

In recent years, an increasing interest in studying polyphenols, which constitute the active substances found in many medicinal plants, has been observed. The chief reason for this is their great abundance in the human diet and their probable role in the prevention of various diseases, such as cancer and cardiovascular, neurodegenerative, and inflammatory diseases, associated with oxidative stress [1–6]. Noteworthy is the fact that both consumers and the food industry are also gaining interest in this subject [1–8]. In addition to their antioxidant properties, polyphenols have several other specific biological actions such as modulation of the activity of a wide range of enzymes and cell receptors [2,4], and interference with the activity and expression of several cell membrane transporters.

Both endogenous and xenobiotic compounds must use membrane transporters to efficiently cross biological membranes. So, their absorption, distribution, and elimination, as well as the extent of their biological activity, are largely dependent on cell membrane-located transporters. This is especially true for transport across epithelial cells, which form barriers separating different compartments in the body. In this context, the aim of the present chapter is to review information concerning the putative influence of these compounds upon the intestinal and placental membrane transport of some organic molecules with important biological functions.

The primary function of the intestinal epithelium is to absorb small molecules produced from digestion of food. Additionally, the intestinal epithelium constitutes one of the major routes of entry of xenobiotics (e.g., therapeutic drugs, drugs of abuse, or environmental toxins) into the blood circulation. So, intestinal transporters present both at the luminal-facing apical membrane and at the serosal-facing basolateral membrane of enterocytes will be determinant in promoting/limiting the absorption of substances present in the intestinal lumen.

There is presently a large debate on the ability of certain food components to interfere with the absorption of nutrients and drugs, resulting in alterations of their biological effects [2,9]. Many of these food-food or food-drug interactions can be explained by changes in the cellular uptake or extrusion of molecules. Studies on the interaction of polyphenols with the intestinal absorption of nutrients have been mainly performed by using the Caco-2 cell line or rat intestinal tissue. Caco-2 cells are one of the most widely used cell models to study intestinal epithelial transport [10–12], as these colonic adenocarcinoma-derived cells present an enterocyte-like phenotype [13], forming confluent cell monolayers with functional properties of transporting epithelia [12,14,15].

As to the placenta, it constitutes the main link between the mother and the developing fetus and performs a variety of functions that are essential for the maintenance of pregnancy and fetal development. One of the major functions of the placenta is to mediate transfer of nutrients from the mother to the fetus and to eliminate fetal metabolic waste products. This function is mediated by transporters present both at the maternal-facing brush-border membrane and at the fetal-facing basal membrane of the

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syncytiotrophoblast, a polarized epithelium that constitutes the functional unit of the placenta. The activity of these transporters will largely determine the extent to which compounds will cross the placenta and enter the fetal blood circulation.

Because polyphenolic compounds are obtained from dietary sources, the intestine is obviously expected to constitute a primary target for these compounds. Indeed, a beneficial effect of polyphenolic compounds in intestinal health has been well documented [16–18], and a putative interference of polyphenols with the intestinal absorption of nutrients, drugs and other exogenous compounds has been investigated in recent years. Moreover, polyphenolic compounds are, to a greater or lesser extent, absorbed from the gut lumen into the blood circulation [8,19–21], and so the placenta will be exposed to these compounds, namely through the ingestion of polyphenolic-rich foodstuffs or beverages, such as wine, tea, etc. So, this organ also represents a target for the action of polyphenols, which could also interfere with the placental uptake of nutrients or other bioactive substances from maternal circulation, and this also has been the subject of some investigation in the last few years.

2 EFFECT OF POLYPHENOLS ON THE TRANSPORT OF 1-METHYL-4-PHENYLPYRIDINIUM

A fair amount of data implicates phytochemical compounds in the modulation of the intestinal transport of organic cations [22–25]. Many biologically relevant organic molecules are organic cations, i.e., possess net positive charges at physiological pH. These include several classes of therapeutic drugs (e.g., antihistamines, antiacids, antiarrythmics, antihypertensives, and anticholinergics), but also nutrients such as vitamins (thiamine and riboflavin), bioactive amines (e.g., catecholamines, serotonin and histamine) and environmental toxins [26].

1-Methyl-4-phenylpyridinium (MPP^+) (Fig. 23.1), a positively charged molecule at physiological pH, is widely used as a model organic cation in intestinal uptake studies, since it is not metabolized in vivo [27,28] and is efficiently taken up by the intestinal epithelium [29,30]. MPP^+ is efficiently absorbed by Caco-2 cells in the apical-to-basolateral direction by two transporters belonging to the amphiphilic solute facilitator (ASF) family, the organic cation transporter 1 (OCT1), and the organic cation transporter 3 (OCT3, also known as the extraneuronal monoamine transporter), both of which are expressed in Caco-2 cells [30]. However, other transporters such as the plasmalemmal serotonin transporter (SERT) might also be involved [31].

FIG. 23.1 Chemical structure of 1-methyl-4-phenylpyridinium.

2.1 Effect of Wine on the Intestinal Transport of MPP^+

Wine is a complex drink in which more than 500 different molecules coexist, and where different classes of polyphenols are present: phenolic acids, stilbenes, and flavonoids including flavonols, flavanols, and anthocyanins [1].

The first report on the interactions of polyphenol sources with MPP^+ uptake by Caco-2 cells tested the effect of intact red or white wine in cell monolayers [25]. Although direct treatment of Caco-2 cells with the beverages may be debatable, it is recognized that some wine components may reach the intestinal epithelium intact. On the other hand, treatment with the whole beverage allows detection of interactions between components of the complex matrix that is wine.

The study by Monteiro et al. [25] revealed that red wine induced a concentration-dependent increase in ^3H-MPP^+ uptake into Caco-2 cells, and that this effect was partially abolished by the concomitant incubation of cells with the OCT inhibitor decynium22 [30]. These results suggest the involvement of OCT in the effect of red wine upon ^3H-MPP^+ transport, but also that other routes of ^3H-MPP^+ entry into Caco-2 cells may also be affected by red wine. In contrast, white wine caused only a slight decrease in ^3H-MPP^+ transport. As the two wines tested had approximately the same amount of ethanol (12%, v/v), the differences between their effects were most likely due to their nonalcoholic components, because red wine has about six times more total polyphenols than white wine and is about four times richer in high-molecular-weight polyphenols [25]. Incubation of Caco-2 cells with alcohol-free red wine led to a smaller effect than red wine, and alcohol-free white wine showed a higher inhibitory potency on ^3H-MPP^+ uptake than white wine. Ethanol, on the other hand, inhibited ^3H-MPP^+ uptake in a concentration-dependent manner. So, the authors concluded that ethanol would increase the bioavailability or solubility of the remaining wine components, thus facilitating their effect.

2.2 Effect of Tea on the Intestinal Transport of MPP^+

Tea is a beverage prepared by the infusion of leaves of the plant *Camellia sinensis*. It is largely consumed worldwide, being increasingly related to beneficial health effects [32].

Green and black tea are produced by different processes, which is reflected in the organoleptic properties of these two beverages and evidently on their chemical composition [32]. Green tea usually has a higher amount of polyphenols (mainly the catechins epigallocatechin-3-gallate (EGCG), gallocatechin, epigallocatechin digallates, epicatechin digallates, 3-*O*-methyl epicatechin, epigallocatechin (EGC), catechin gallate and gallocatechin gallate) than black tea. Leaves oxidation during black tea production results in a lower catechin level, since these are converted to theaflavins and thearubigins [33]. Tea may also contain flavonoids from other groups, such as myricetin, quercetin and kaempferol, and xanthines, especially theophylline and caffeine. Xanthines are more abundant in black tea, whereas green tea is especially rich in EGCG (30% of dry weight, compared with only 9% in black tea) [33].

When testing the effects of different concentrations of green and black tea on ^{3}H-MPP^+ transport by Caco-2 cells, green tea (and, less potently, black tea) was able to increase the uptake of ^{3}H-MPP^+, in a concentration-dependent manner [24]. In the presence of the OCT inhibitors decynium22 or corticosterone [30], the stimulatory effect of tea upon ^{3}H-MPP^+ transport into Caco-2 was attenuated, suggesting, similarly to Monteiro et al. [25], that uptake of ^{3}H-MPP^+ by Caco-2 cells appears to be mediated by both OCT and non-OCT routes.

2.3 Effect of Isolated Polyphenols on the Intestinal Transport of MPP^+

Grape seed-extracted procyanidins. Procyanidins belong to the flavan-3-ols group and are polymeric compounds formed of catechin and epicatechin monomers [34]. They are found in several food sources such as tea, apples, and cocoa, being especially abundant in red wine. Faria et al. [35] studied the effect of procyanidin extracts isolated from grape seeds and separated in different fractions according to their size and complexity.

The authors began to test the effect of a 60-min incubation with procyanidins on ^{3}H-MPP^+ uptake by Caco-2 monolayers. At the higher concentration tested (600 mg/mL), all fractions significantly increased ^{3}H-MPP^+ uptake, and the effect increased with the structural complexity of the fractions. High molecular weight procyanidins exist in higher concentrations in red wine and in much lower amounts in white wine. Therefore, the effects reported by Faria et al. [35] are well correlated with those described by Monteiro et al. [25] showing opposing effects of red and white wine on ^{3}H-MPP^+ uptake and strengthen the involvement of procyanidins in the effect of wine.

Experiments with different incubation periods revealed that this effect was also time-dependent. Since procyanidins, as strong reducing and therefore antioxidant agents, are readily oxidized due to their o-dihydroxyl groups [36], it was speculated that, as the incubation time increases, oxidation of these compounds could change the state of their interaction with the transporters involved in ^{3}H-MPP^+ uptake. Cell exposure to the oxidized procyanidins confirmed this hypothesis. Indeed, oxidized procyanidins tested for 3 min increased ^{3}H-MPP^+ uptake to levels similar to the ones found with nonoxidized procyanidins tested for 60 min. Furthermore, a 60-min incubation with oxidized procyanidins resulted in a significantly more pronounced stimulation of ^{3}H-MPP^+ uptake. The involvement of intra- and extracellular redox state was confirmed by testing the effect of both oxidant and reducing agents on the transport [22,37]. Redox state may interfere with the transport by the following mechanisms: (a) changes in transporter affinity for the substrate; (b) changes in interaction of the transporter with the cytoskeleton, with influence in the number of membrane-located transporters; (c) regulation at the gene expression level [38].

It is possible that the oxidation level of L-Cys residues can affect the occurrence of phosphorylation and dephosphorylation reactions. This attractive hypothesis may indeed constitute the mechanism underlying the effects of procyanidins, because intestinal OCT has been shown to be regulated by phosphorylation and dephosphorylation mechanisms [39–43], and polyphenols are known regulators of intracellular kinases and phosphatases [2].

Anthocyanins and derivative pigments. Anthocyanins are present in flowers, fruits, and other vegetables, being an important group of plant pigments [8,44]. They exist in high amounts in blueberries (*Vaccinium myrtillus*), having been related to their health-promoting properties [45]. Although growing scientific evidence of anthocyanin bioavailability is being gathered [19,44], few studies have been devoted to the investigation of their possible interference with the intestinal absorption of other substrates.

Faria et al. [46] isolated anthocyanins from blueberries (extract I) and used it to synthesize anthocyanin pyruvic acid adducts (extract II) and vinylpryranoanthocyanin-catechins (portisins, extract III). The ability of these extracts to influence ^{3}H-MPP^+ apical intestinal uptake was then studied [23] by incubating Caco-2 cells with 100 μg/mL of each extract. Only one of the extracts (extract II) had a significant effect, decreasing ^{3}H-MPP^+ uptake in a concentration-dependent manner. As there are no reports on the ability of OCT to transport

anthocyanins or their derivatives, the possibility of an allosteric regulation or interference with the regulation of these transporters should not be excluded. Since molecular weight increases from extract I to III, size was thought not to be relevant to explain why only extract II showed an effect. On the other hand, it was advanced that the presence of a carboxyl group on the D ring of extract II components might play an important role and this was further explored by testing the effect of phenolic acids on the transport (see the following).

Phenolic acids. The effect of four phenolic acids on the intestinal transport of MPP^+ was tested by Faria et al. [23]. Whereas gallic acid had no effect on the transport, p-coumaric, caffeic, ferulic, and tannic acids induced a decrease in cellular ^{3}H-MPP^+ uptake. The authors suggested that the presence of a vinylphenolic group, with a probable higher electronic conjugation, was likely to play a role. They also advanced the hypothesis that the effects observed resulted from ionic interactions between ^{3}H-MPP^+ and the dissociated acid moieties.

As opposed to the other phenolic acids, the structurally more complex compound tannic acid increased ^{3}H-MPP^+ uptake by Caco-2 cells. Because this effect more closely resembles the one obtained with more complex flavonoids, such as procyanidins, molecular size was suggested to contribute to the effects.

Other polyphenols. The effect of some monomeric polyphenols (quercetin, myricetin, catechin, and resveratrol) on MPP^+ uptake by Caco-2 cells has also been investigated. Resveratrol did not affect ^{3}H-MPP^+ uptake, whereas all other compounds decreased it, quercetin having the strongest effect [25].

The green tea polyphenol EGCG (2 mM) significantly increased ^{3}H-MPP^+ uptake, leading to the hypothesis that the difference between black and green tea in relation to ^{3}H-MPP^+ uptake could be related to their different amounts of EGCG (see the preceding). Procyanidin dimers and trimers can also be found in both teas, although green tea has almost double the amount found in black tea [47]. When tested upon ^{3}H-MPP^+ transport, a procyanidin mixture (containing B1, B2 and B3 dimers and C1 trimers found in tea) stimulated this uptake [24].

3 EFFECT OF POLYPHENOLS ON THE TRANSPORT OF FOLATES

Folate is a generic term used to designate a family of naturally occurring B-group vitamins (vitamin B_9) structurally derived from folic acid (pteroylglutamate, FA). FA is the fully oxidized, most stable, and synthetic form of the vitamin used in supplements and in food fortification.

Folates are responsible for the intracellular transfer of one-carbon moieties and are thus critical for the synthesis of purines and thymidylate, and the generation of S-adenosyl-methionine, a major methyl donor for DNA methylation processes. As such, folates are essential compounds for nucleic acid synthesis and thus for cell division, and they play a determinant role in epigenetic regulation of gene expression. Considering that fetal programming of adult disease may result, at least in part, from epigenetic changes occurring during fetal development, folates may be implicated in fetal programming [48,49].

Humans and other mammals cannot synthesize folate, being dependent on the intake of this vitamin from the diet. The gut thus plays a critical role in regulating body folate homeostasis and understanding the mechanism of intestinal folate absorption process is of significant nutritional importance. Similarly, placental folate transport, and its modulation, represents a key step in fetal development and human biology.

This section will deal with the effect of polyphenols on the transport of folates through the intestinal and the placental epithelium.

3.1 Effect of Polyphenols on the Intestinal Transport of Folates

The effect of polyphenols upon folate intestinal transport has been studied by Lemos et al. using two main approaches: the study of polyphenols in the context of a drink [50,51] and the study of isolated polyphenols [51]. Using the first approach, Lemos et al. investigated the effect of red wine upon the intestinal absorption of ^{3}H-FA, by testing its effect upon rat jejunal mucosal-to-serosal P_{app} to ^{3}H-FA. Red wine was tested both chronically in vivo (21-day consumption of a 12% (v/v) ethanol-containing red wine) and acutely in vitro (tissue exposure to red wine diluted 1:5) [50]. In this work, neither chronic nor acute red wine changed the mucosal-to-serosal P_{app} to ^{3}H-FA; ethanol was also devoid of effect. One of the causes of FA deficiency observed in chronic alcoholism is thought to be a reduction in the intestinal absorption of this vitamin [52,53]. However, the effect of alcoholism upon the intestinal absorption of FA should not be inferred from the effect of ethanol ingestion alone, because many alcoholic drinks contain polyphenolic bioactive compounds; so, study of polyphenolic compounds in the context of an alcoholic drink might be especially interesting.

The effect of some isolated polyphenols and of polyphenol-rich drinks (red and white wine, several beers, tea, and orange juice) upon ^{3}H-FA or ^{3}H-methotrexate (^{3}H-MTX; an antifolate) intestinal uptake was also studied by Lemos et al., using Caco-2 cells as a model of the intestinal epithelial barrier [51].

All the tested beers (lager, stout, and alcohol-free beer), green tea, black tea, and orange juice significantly

inhibited ^{3}H-FA and ^{3}H-MTX apical uptake. Moreover, contrary to what had been observed with mucosal-to-serosal P_{app} to ^{3}H-FA across rat jejunum [50], both red and white wine significantly inhibited the apical uptake of ^{3}H-FA, red wine being more potent than white wine, and the same degree of inhibition was obtained with alcohol-free wines. On the other hand, ethanol in the same concentration as that present in the red and white wine did also reduce ^{3}H-FA apical uptake, but much less potently [51].

Because [1] ethanol had a much more discrete effect than the alcoholic drinks (wines and beers) upon ^{3}H-FA uptake, and [2] alcohol-free drinks (red and white wine and beer) had almost the same effect as alcohol-containing wine and beer, it was concluded that other components of these beverages, most likely polyphenols, must play a role in their inhibitory effect upon ^{3}H-FA uptake. Indeed, analyses of the effect of some isolated polyphenolic compounds known to be present in wines, beers, and/or teas, in these same cells, confirmed this suspicion [51]. When tested acutely, myricetin, EGCG, and isoxanthohumol concentration-dependently inhibited ^{3}H-FA uptake (IC_{50} values of 12.5, 7.7, and 35.7 μM, respectively). Myricetin and EGCG also had a concentration-dependent inhibitory effect upon ^{3}H-MTX uptake (IC_{50} values of 10.6 and 10.1 μM, respectively). Other polyphenolic compounds (xanthohumol, resveratrol, quercetin, and kaempferol) were found to moderately (20%–50%) inhibit the uptake of ^{3}H-FA and/or ^{3}H-MTX, but only when tested in a high (100 μM) concentration. Moreover, a long-term (2-days) exposure of the cells to isoxanthohumol resulted in inhibition of ^{3}H-FA uptake; the other polyphenols were devoid of effect [51].

Interestingly enough, many compounds that reduced the uptake of ^{3}H-FA and ^{3}H-MTX by Caco-2 cells (the stilbene resveratrol and the flavonols quercetin, myricetin, kaempferol) are present in wines, where they are present in concentrations ranging from 0.2 to approximately 20 mg/L [1]. So, it is possible that the phenolic compounds present in wines are, at least in part, responsible for the inhibitory effect of these beverages upon the intestinal uptake of ^{3}H-FA. This hypothesis is supported by the fact that red wine, which has a much higher content of phenolic compounds than white wine [51], also showed a more potent inhibitory effect than white wine.

Similarly, because beer constitutes the main dietary source of xanthohumol and of other prenylflavonoids like isoxanthohumol [54], and both xanthohumol and isoxanthohumol inhibited ^{3}H-FA uptake, these compounds may be responsible for the observed inhibitory effect of beers on the intestinal uptake of this vitamin.

The effect of green tea might be explained by its high content of EGCG [55], which was one of the most potent inhibitors of the uptake of both ^{3}H-FA and ^{3}H-MTX. Other phenolic compounds like myricetin, quercetin, and kaempferol can also be found in teas [33] and may also contribute to inhibition of ^{3}H-FA and ^{3}H-MTX uptake. In agreement with the results from Lemos et al. [51], green and black tea extracts, as well as two catechins present in green tea (EGCG and ECG), were also found to inhibit FA uptake by Caco-2 cells [56].

The difference in the effect of red wine in relation to ^{3}H-FA uptake in Caco-2 cells (inhibition) [51] and rat jejunum (no effect) [50] can be explained by two important differences. First is the different duration of the treatment with red wine: a 48-h in vitro exposure of Caco-2 cells vs. a 21-day in vivo ingestion. Second is the concentration of red wine in direct contact with the cells or the tissue (higher in the experiments with Caco-2 cells). Finally, it should be noted that the process of digestion significantly alters the composition of red wine, and that this digestion was not assumed in the study by Lemos et al. [51].

The observation that ^{3}H-FA and ^{3}H-MTX uptake in Caco-2 cells were similarly modulated by most of the beverages and phenolic compounds suggests that these compounds share the same transport system in these cells. The proton-coupled folate transporter (solute carrier family 46, member 1 (SLC46A1)) is currently accepted as the major folate transport system at the intestinal level [57], and it has been shown to be an important target of some of the tested polyphenols. Furumiya et al. [58] have recently shown that myricetin is indeed a noncompetitive inhibitor of PCFT-mediated ^{3}H-folate or ^{3}H-MTX uptake. Myricetin was shown to reduce both V_{max} and K_m of ^{3}H-folate transport both in Caco-2 cells and in MDCKII cells stably expressing PCFT. In two subsequent works by the same group, myricetin-induced inhibition of PCFT was found to be persistent as it was observed even after this compound had been washed out from the cell medium [59]. This sustained inhibition of PCFT was observed to increase with increasing myricetin concentrations [60]. So, myricetin is believed to act directly on PCFT, although the exact mechanism of interaction remains to be elucidated.

In addition to myricetin, EGCG was also found to act upon PCFT, although recent literature is not coherent regarding the mechanism of inhibition. Furumiya et al. [58] describe EGCG as a noncompetitive inhibitor of PCFT, similarly to what they had observed with myricetin. This conclusion arises from the observation that EGCG (100 μM) inhibits ^{3}H-folate (and ^{3}H-MTX) uptake with the same efficiency under low (5 nM) or high (5 mM) unlabeled folate conditions.

Kissei et al., however, describe EGCG as a competitive inhibitor of PFCT through Dixon plot analysis, with a K_i of 9.15 μM [61]. The different observations from Furumiya et al. [58] and Kissei et al. [61] may arise from different conditions used in the assays. Furumiya et al. have used EGCG at 100 μM while Dixon plot analysis

performed by Kissei et al. were based on EGCG concentrations ranging from 0 to 10 μM. In addition, the tritiated substrate used by Furumyia et al. is called folate, and we cannot ascertain whether it corresponds to the oxidized form of ^{3}H-FA used by Kissei et al.

In conclusion, the results obtained by Lemos et al. [51] suggest that the effect of several polyphenolic-rich beverages (red and white wine, beer, black and green tea, and orange juice), significantly decreasing FA and MTX uptake, can be justified, at least in part, by their phenolic compounds.

From the studies reviewed here, it can be concluded that dietary habits, especially those related to the consumption of polyphenol-containing beverages or phenolic compounds, can modulate the intestinal uptake of both ^{3}H-FA and ^{3}H-MTX. Importantly, they may reduce the therapeutic efficacy of oral MTX. These results also suggest that, in human alcoholism, FA deficiency can result, at least partially, from a decrease in its intestinal absorption. This subject has been extensively studied by Hamid et al., [62–64] who demonstrated that chronic alcohol ingestion in rats deregulates folate transport mechanisms at the intestinal level.

Polyphenols were very recently found to prevent the inhibitory effect of oxidative stress upon ^{3}H-FA apical uptake by Caco-2 cells [65]. This suggests that polyphenols may act differently depending on the intestinal epithelial redox status: under normal conditions, they may directly target folate transport systems, impairing their activity, whereas under a prooxidant environment, their antioxidant properties counteract the oxidative stimuli, eventually overcoming their local/direct action upon the epithelium.

3.2 Effect of Polyphenols on the Placental Transport of Folates

Folates are crucial for fetal development and growth. Indeed, not only low maternal folate status is associated with increased risks of low birth weight, preterm delivery, and neural tube defects (NTDs) [66], but also supplementation of maternal diet with FA decreases the incidence of low-birth weight newborns [67] and NTDs [68,69]. Additionally, folates are recognized as important players in fetal programming, as they enable the production of S-adenosyl-methionine, a major substrate of DNA methyltransferases that perform DNA methylation [70].

Several dietary polyphenols (catechin, chrysin, epicatechin, EGCG, isoxanthohumol, myricetin, quercetin, resveratrol, rutin, and xanthohumol) were tested by Keating et al. [71] as modulators of ^{3}H-FA uptake by a placental cell model (BeWo cells).

In the short-term (26 min), epicatechin and isoxanthohumol inhibited ^{3}H-FA uptake, with a similar maximum effect (around 30% inhibition). Isoxanthohumol acted as a competitive inhibitor, and epicatechin caused an increase in both K_m and V_{max}. The authors hypothesized that epicatechin binding to an allosteric site of the transporter induces an alteration in the conformation of the active site, this causing a reduction in the affinity for the substrate (increasing K_m) and an increase in the transporter's capacity (increasing V_{max}) for high concentrations of the substrate.

In the long-term (48 h), ^{3}H-FA apical uptake by BeWo cells was significantly increased by exposure to xanthohumol, its derivative isoxanthohumol, and quercetin. At physiological pH, ^{3}H-FA apical uptake by BeWo cells seems to involve both reduced folate carrier (RFC) and folate receptor (FR) α [72]. However, the increase in ^{3}H-FA uptake caused by a long-term exposure of BeWo cells to the referred polyphenols was not accompanied by a change in RFC or FRα mRNA levels. So, the effect of polyphenols does not seem to result from a modulation of the gene expression of these transporters. Instead, it may be a result of a direct interaction of the polyphenols with the transporter(s), with a consequent change in the activity of the latter [71].

Interestingly, the short-term effects of epicatechin and the long-term effects of xanthohumol, isoxanthohumol, and quercetin were found to be specific, since these compounds did not affect (at least in the same direction) the uptake of either ^{3}H-thiamine or ^{14}C-alanine by BeWo cells.

Because short- and long-term treatments with these dietary bioactive compounds did not produce parallel results, care should be taken when speculating about chronic effects from acute effects and vice versa [71].

As a whole, it is concluded that polyphenols have the ability to interfere with folate uptake at the intestinal and the placental levels. This may be particularly interesting if we consider that many polyphenols are considered as chemopreventive epigenome-targeting agents [73]. For example, EGCG has been shown to inhibit DNA-methylation in vitro [74]. So, it is likely that by interfering with the bioavailability of folate (for the adult and for the fetus), one major methyl-nutrient, polyphenols may indeed impact genome methylation with consequences for health programming.

4 EFFECT OF POLYPHENOLS ON THE TRANSPORT OF THIAMINE

Thiamine (vitamin B_1) is a complex water-soluble micronutrient, essential for normal cell functions and growth. This vitamin is essential for glycolysis, the tricarboxylic acid (TCA) cycle, branched-chain amino acid metabolism, and nucleotide metabolism. It is required by animal cells as the precursor of thiamine

pyrophosphate, the coenzyme of the indispensable carbohydrate enzyme transketolase and the dehydrogenase complexes for pyruvate, α-ketoglutarate and branched-chain keto acids. Humans and other mammals cannot synthesize thiamine and thus must obtain this vitamin from diet. The gut is thus critical for body thiamine homeostasis and understanding the mechanism of intestinal thiamine absorption process is of significant nutritional importance. In fact, thiamine plasma concentration is regulated mainly by intestinal absorption and renal excretion. More recently, there is evidence that thiamine availability is modulated by or acquired from the dense and diverse microbial communities present in the gut. In addition, it is a challenge to know that diet, namely polyphenols, modulate microbiota composition and metabolism. Thus, it is possible to speculate that polyphenols are able to interfere in thiamine bioavailability since these compounds are microbiota modulators (prebiotic effect) [75]. More studies will be needed to clarify the effect of gut bacteria on thiamine transport.

It is well known that this vitamin is also crucial for fetal growth. Thus, placental transport, and its modulation, represents a key step in fetal development and human biology.

Chemically, thiamine is a hydrosoluble organic cation with a high molecular weight. At concentrations lower than 2 μM, thiamin is absorbed by the intestinal mucosa mainly through active transport that precedes intracellular phosphorylation and dephosphorylation of this vitamin [76]. At these low concentrations, entry at the luminal side occurs largely through exchange with H^+ and very little through enzymatic transphosphorylation to thiamin monophosphate (TMP), by intestinal alkaline phosphatase present in the apical membrane of the enterocyte. Cellular crossing is associated with intracellular enzymatic phosphorylation to thiamin pyrophosphate (TPP) and dephosphorylation of TPP to TMP and thiamine. At higher concentrations of thiamine, simple passive diffusion prevails [77].

The expression of the thiamine transporter ThTr1 (SLC19A1) is very high in skeletal muscle, heart, and placenta and almost absent in the intestine, kidney, and brain [78]. These results are inconsistent with those obtained from functional studies showing a significantly higher thiamine uptake in the intestine and kidney than in skeletal muscle [79,80]. This seems to suggest the involvement of other intestinal transporter(s) in thiamine intestinal absorption. In agreement with this, Lemos et al. showed that apical uptake of thiamine into Caco-2 seems to involve not only ThTr1 and ThTr2, which is a second thiamine transporter belonging to the same SLC19 family, but also members of the OCT family of transporters (most probably OCT1 and/or OCT3), thus sharing the same transporters with several other organic cations at the small intestinal level [81].

4.1 Effect of Polyphenols on the Intestinal Transport of Thiamine

Isolated phenolic compounds seem to have no direct effect on thiamine transport modulation in Caco-2 cells [82]. However, nonalcoholic beverages such as green and black tea, as well as alcohol-free beer and alcohol-free red and white wines, were able to inhibit ^{3}H-thiamine transport into Caco-2 cells, when tested acutely. In these same cells, the effect of some alcoholic beverages (red and white wines and lager and stout beer) was tested, and the results were very curious. Only lager and stout beers inhibited transport; red and white wines had no effect. In agreement with these results, the inhibitory effect of alcohol-free wines was abolished when ethanol (in the same concentration as that found in intact wines) was added. So, the biological activity of phenolic compounds, as modulators of thiamine uptake, depends on the presence or absence of ethanol. In agreement with this conclusion, a recent study showed a crucial contribution of ethanol to phenolic bioavailability and/or bioactivity [22].

An important point relates to the effect of xanthohumol. Although xanthohumol had no effect upon ^{3}H-thiamine uptake into Caco-2 cells, lager and stout beers (which are both rich in this compound) inhibited its uptake. Additionally, stout beer, a chalcone-enriched beer, was more potent than lager beer. So, it is possible that xanthohumol and isoxanthohumol can explain, at least in part, the inhibitory effect found with the beers, as it happens with placental thiamine uptake [83]. Once again, these results show that the original matrix is important for the biological activity of polyphenols.

Curiously, results obtained in rat jejunum showed that chronic (21 days) ethanol ingestion inhibited ^{3}H-thiamine intestinal absorption but that, interestingly enough, red wine containing the same concentration of ethanol, had no effect [50]. These results led the authors to conclude that the nonalcoholic matrix of red wine, which is very rich in polyphenols, was able to counteract the inhibitory effect of ethanol upon thiamine uptake. Of note, this effect was only verified after a chronic ingestion; acute and direct exposure of the rat jejunum to red wine did not antagonize the inhibitory effect of ethanol. As to the apparent contradiction between the acute effect of red wine upon thiamine uptake by rat jejunum and Caco-2 cells, two important reasons might explain this discrepancy: (i) different species were studied (rat vs. a human cell line); (ii) different parameters were analyzed (rat intestinal absorption vs. uptake in Caco-2 cells).

Thus, isolated polyphenols had no effect on intestinal thiamine transport; however, polyphenols in a food matrix seem to be able to modulate absorption of this vitamin.

4.2 Effect of Polyphenols on the Placental Transport of Thiamine

As mentioned before, thiamine is crucial during pregnancy for normal growth and development of the fetus. As the need for this vitamin increases during pregnancy, modulation of thiamine transport through trophoblast epithelia constitutes a key point in fetal development. Indeed, the association between alcoholic abuse and thiamine deficiency is well known [84,85], and thus it is possible that deficiency of this vitamin during pregnancy contributes, at least in part, to the developmental abnormalities observed in the fetal alcohol syndrome.

Considering this, Keating et al. [83] studied the short- and long-term effects of several phenolic compounds upon the apical uptake of ^{3}H-thiamine by BeWo cells. In the short-term, no effect of xanthohumol, isoxanthohumol, catechin, epicatechin, resveratrol, quercetin, myricetin, EGCG, rutin, and chrysin was found. In the long-term (48 h), treatment with xanthohumol or isoxanthohumol (chalcones found especially in beer) significantly decreased ^{3}H-thiamine uptake by these cells; other compounds had no effect. Moreover, the inhibitory effect of xanthohumol and isoxanthohumol was not related to changes in mRNA levels for ThTr1 or ThTr2. Human SERT was previously suggested to be involved in the uptake of thiamine by these cells [83]. However, these compounds had no effect on human SERT mRNA levels as well. So, these effects are most probably not related to changes at the transcriptional level. Considering that human SERT seems to be involved in thiamine uptake by BeWo cells, and that soya affects SERT activity [83], it would be interesting to determine the effect of isoflavones (soya-present polyphenols) upon thiamine placental transport. Indeed, Ito et al. [86] described an effect of beverages such as St John's wort (which is rich in phenolic compounds) on SERT activity that explains, at least in part, its psychopharmacological effects.

The mechanisms involved in polyphenol modulation of thiamine placental uptake remain unknown. However, as is known, SERT is inhibited by phosphorylation pathways [87], and several phenolic compounds interact with the activity of kinases and phosphatases [80,88]. Thus, it is possible that some phenolic compounds could interfere with transport activities through an indirect effect upon, for instance, phosphorylation and dephosphorylation mechanisms.

5 EFFECT OF POLYPHENOLS ON THE TRANSPORT OF GLUCOSE

5.1 Effect of Polyphenols on the Intestinal Transport of Glucose

Maintenance of glucose homeostasis is of crucial importance to human physiology, and failure of this mechanism can result in metabolic syndrome, a multifactorial condition leading to accelerated atherosclerosis and increased risk for diabetes, major cardiovascular events, and a high mortality rate.

Growing evidence indicates that various dietary polyphenols may influence glucose homeostasis at many levels. In animal models, and in a few human studies carried out so far, polyphenols and polyphenol-rich foods or beverages have attenuated postprandial glycemic responses and fasting hyperglycemia and improved acute insulin secretion and insulin sensitivity [89,90]. Several distinct mechanisms appear to contribute to this effect, including inhibition of carbohydrate digestion, stimulation of insulin secretion from pancreatic β-cells, modulation of glucose release from the liver, and activation of insulin receptors and of glucose uptake in insulin-sensitive tissues [90]. Additionally, inhibition of intestinal absorption of glucose by polyphenol-rich foods or drinks and by individual polyphenols has also been reported, both in vitro, by using intestinal epithelial cell lines or intestinal fragments (Tables 23.1 and 23.2) and in vivo, either in animal models (Table 23.3) and in humans (Table 23.4).

According to the "classical model of sugar absorption," luminal glucose is actively taken up by the enterocytes by an apically located high-affinity and Na^{+}-dependent Na^{+}/glucose cotransporter 1 (SGLT1) [102,103]. As shown in Table 23.5, several polyphenols and polyphenol-rich extracts were shown to inhibit SGLT1-mediated intestinal glucose uptake. In this context, a special remark should be made concerning phloridzin, the glucoside of the polyphenol phloretin, that has long been used as a specific inhibitor of SGLT1 [104,105], and has more recently been demonstrated to be an SGLT1 substrate [106]. Moreover, some polyphenols are able to interfere with the effect of other conditions on SGLT1-mediated transport, as

TABLE 23.1 Polyphenol-Rich Plants or Extracts With Inhibitory Effect on Glucose Absorption Measured In Vitro.

Acerola extract	[131]
Chamomile and green tea	[132]
Green tea, chokeberry and honeysuckle extracts	[133]
Apple, strawberry extracts	[134]
Pulp pf carob fruit	[135]
Blueberry and pomegranate juices	[136]
Green tea decoction	[137]
Grape Juice	[138]
Apple extract	[139]
Berry extract	[140]
Herbal extract	[141]

TABLE 23.2 Polyphenols With Inhibitory Effect on Glucose Absorption In Vitro

Anthocyanins	
Cyanidin-3-α-*O*-rhamnoside	[131]
Delphinidin	[142]
Pelargonidin-3-α-*O*-rhamnoside	[131]
Catechins	
Catechin	[91,92]
Epigallocatechin	[92]
Epicatechingallate	[92]
EGCG	[92,93,143]
Dihydrochalcones	
Phloridzin	[136,139]
Phloretin	[136]
Flavonols, Flavones, Flavanones	
Naringenin	[144]
Quercetin	[92]
Quercetin-3-glucoside	[94]
Quercetin-4-glucoside	[94]
Myricetin	[92]
Kaempferol	[136,139]
Apigenin	[92]
Phenolic acids	
Caffeic acid	[91]
Chlorogenic acid	[91,139,145]
Ferrulic acid	[91]
Tannic acid	[91]

TABLE 23.3 Effect of Polyphenols and Polyphenol-Rich Extracts on Intestinal Glucose Absorption in Rodents, In Vivo

Acerola extract	Mice	Inhibition of intestinal glucose absorption	[131]
Apple extract; phloridzin	Mice	Glycemic response after glucose load	[139]
Carob fruit pulp	Rats	Glycemic response after glucose load	[135]
Gingko biloba extract	Rats	Inhibition of Intestinal glucose absorption	[146]
Green tea decoction	Rats	Glycemic response after glucose load; inhibition of SGLT1, stimulation of GLUT2	[137]
Helichrysum and grapefruit extracts	Rats	Glycemic response after maltose or starch load	[147]
Multiflorin A	Mice	Inhibition of intestinal glucose absorption	[148]
Naringin, hesperidin	db/db mice	Reduction in glycemia	[149]
Naringenin, EGCG, phloridzin, phloretin, naringenin-7-glucoside, isoquercetin	Rats	Inhibition of intestinal glucose absorption	[150]
Nerium indicum leaf extract, chlorogenic acid	Rats	Inhibition of intestinal glucose absorption	[145]
Quercetin	Type II diabetics mice and rats	Inhibition of intestinal glucose absorption	[95–97]
Quercetin	Type I diabetic mice and rats	Inhibition of intestinal glucose absorption	[98–101]
Syzygium polyanthum leaf extract	Rats	Inhibition of intestinal glucose absorption	[151]
Tea extract	Rats	Inhibition of intestinal glucose absorption	[152]

described in a recent study, where EGCG was found to reverse the marked decrease in intestinal mucosal SGLT1 gene expression induced by high-fat diet in mice [117].

A recent study suggested that inhibitors of SGLT1, such as phloridzin, bind to SGLTs with two synergistic forces: sugar binding to the glucose site, and binding of the aglycone, that affects the binding affinity of the entire inhibitor [118]. This is in accordance with the fact that not only glucosides, but also polyphenolic aglycones, were found to be potent SGLT1 inhibitors (Table 23.5). Nevertheless, some controversy remains concerning the type of inhibition involved. For instance, Kobayashi et al. [93] proposed a competitive inhibition mechanism for ECG in relation to SGLT1, although ECG itself was concluded not to be transported via SGLT1; however, inhibition of SGLT1 by (+)-catechin, ECG, and EGCG was later suggested to be of noncompetitive nature [107]. Also, according to some authors, quercetin glucosides such as quercetin-4-*O*-glucoside (the major dietary form of quercetin) are absorbed within the intestine by the active glucose transporter SGLT1, thus being SGLT1 substrates [108–110]. However, other studies have concluded that

TABLE 23.4 Effect of Polyphenol-Rich Foods or Extracts on Intestinal Glucose Absorption in Humans, In Vivo

Apple juice	Healthy subjects	Decrease in glycemic response after glucose load	[153]
Apple extract	Healthy subjects	Decrease in glycemic response after glucose load	[139]
Berries purée	Healthy subjects	Decrease in glycemic response after sucrose load	[154]
Blackcurrant and lingonberry nectars	Healthy subjects	Decrease in glycemic response after glucose and fructose load	[155]
Chlorogenic acid	Healthy subjects	Decrease in glycemic response after glucose load	[156]
Chlorogenic acid	Overweight subjects	Decrease in glycemic response after glucose load	[157]
Chlorogenic acid-enriched instant coffee	Healthy subjects	Decrease in glycemic response after sucrose load	[158]
Delphinidin-Rich Maqui Berry Extract (Delphinol)	Prediabetic subjects	Decrease in glycemic response after glucose load	[159]
Red wine, tannic acid	Type II diabetics	Glycemic response after a meal	[160]
Sugar cane extract	Healthy subjects	Decrease in glycemic response after a high-glycemic index meal	[161]

TABLE 23.5 Polyphenol-Rich Foods and Polyphenols With Inhibitory Effect Upon SGLT1- and GLUT2-Mediated Intestinal Absorption of Glucose

SGLT1		
	Phloridzin	[92,104–106]
	Quercetin-3-*O*-rhamnoside, phloridzin, 5-caffeoylquinic acid, pelargonidin-3-*O*-glucoside	[134]
	Apple extract and phloridzin	[139]
	Green tea decoction; EGCG+EGC	[137]
	Berry extract	[140]
	Herbal extract	[141]
	Tea extract	[152]
	Yerba Maté extract	[162]
	(+)-catechin, ECG, EGCG	[91,93,107,143]
	Quercetin-3-*O*-glucoside, Quercetin-4-*O*-glucoside	[94,108–110,163]
	Pentagalloyl glucose	[164]
	Emodin, chrysophanol	[165]
	Kaempferol-3-*O*-α-rhamnoside	[166]
	Luteolin, naringenin, pelargonidine	[111]
	Arbutin	[112,113]
	Caffeic, ferulic and chlorogenic acid	[91]
GLUT2		
	Quercetin-3-*O*-rhamnoside, phloridzin, 5-caffeoylquinic acid, pelargonidin-3-*O*-glucoside	[134]
	Green tea	[132]
	Phloretin	[104,114,167,168]
	Berry extract	[140]
	Herbal extract	[141]
	Quercetin, apigenin, chrysin	[115]
	ECG, quercetin-3-*O*-glucoside, fisetin, gossipin	[114]
	Quercetin, myricetin	[92]
	Quercetin, isoquercitrin, fisetin, myricetin, quercetin-3-*O*-glucoside	[116]
	Quercetin	[95]

neither quercetin nor any of its glycosylated derivatives are transported by SGLT1 [111].

Besides SGLT1, apical GLUT2 (a facilitative Na^+-independent glucose transporter) also appears to be involved in the intestinal absorption of glucose [103]. Interestingly, animal studies suggest that luminal-facing GLUT2 is responsible for a large proportion of small intestinal glucose uptake when high concentrations of luminal glucose are present (e.g., associated with the ingestion of a carbohydrate-rich meal). Being a major pathway of sugar absorption, GLUT2 is therefore an attractive target of potential agents [103]. Interestingly enough, some polyphenols were also found to interact with this transporter (Table 23.5). For instance, our group concluded that anthocyanins such as malvidin-3-*O*-glucose are transported via GLUT2 and increase GLUT2 expression at the human intestinal epithelial level [22].

Quercetin, present in high amounts in onions and capers, is a potent inhibitor of GLUT2-mediated uptake of glucose [94,95,114–116]. The effect of quercetin upon glucose uptake seems to be GLUT2-specific, because it does not interact with either SGLT1 or GLUT5 [95,116], although it does not appear to be a GLUT2 substrate [95,116]. Because quercetin acts as a potent luminal inhibitor of sugar absorption independent of its own transport, it shows promise as a new pharmacological agent in the treatment of the diabetes epidemic disease [116]. Importantly, in animal models of type I diabetes, administration of quercetin reduced fasting hyperglycemia [98–101]. Moreover, quercetin was also effective in controlling blood glucose levels in animal models of type II diabetes [95–97]. However, other effects of quercetin, such as inhibition of intestinal α-glucosidase, must also be considered [100,119].

A comparative study on the effect of different classes of dietary polyphenols upon Na^+-dependent (SGLT1-mediated) and Na^+-independent (GLUT2-mediated) intestinal uptake of glucose was done by using human intestinal epithelial Caco-2 cells [92]. Aglycones (such as quercetin, apigenin, and myricetin) inhibited GLUT2-mediated uptake and nonglycosylated dietary polyphenols (EGCG, EGC, and ECG) were effective against both GLUT2 and SGLT1. Additionally, dietary glycosides (such as naringin, rutin, and arbutin) and phenolic acids (such as caffeic, ferulic, and chlorogenic acids) were ineffective against both GLUT2- and SGLT1-mediated uptake of glucose [92]. Of importance, these results stand in contrast with findings from others. For instance, the glycoside arbutin was shown to be transported by SGLT1 in hamster tissue [112] and in *Xenopus* oocytes [113], and the lack of effect of phenolic acids upon both Na^+-dependent and Na^+-independent glucose uptake [92] is in contrast with the study by Welsch et al. [91] showing that caffeic, ferulic, and chlorogenic acids caused an inhibition of Na^+-dependent glucose uptake by rat brush-border membrane vesicles. Although these discrepancies may be related to factors such as distinct inhibitor to substrate concentrations or different experimental models, knowledge on the interaction of distinct polyphenols with glucose transporters is still far from concluded.

In conclusion, in vitro and in vivo evidence suggests that there is the potential for a variety of classes of dietary polyphenols to affect SGLT1- and GLUT2-mediated intestinal glucose transport in vivo. Of importance, polyphenols are potent enough to act in the gut where concentrations can reach millimolar levels [120]. So, these data suggest that foods and unsweetened beverages rich in these dietary polyphenols might provide a convenient dietary mechanism for regulating the rate of intestinal sugar absorption, an important factor in the management of diabetes, and in the long term might offer some protection against development of the metabolic syndrome or type II diabetes.

5.2 Effect on the Placental Transport of Glucose

Glucose is essential for the developing fetus, serving as the primary source of energy for metabolism and growth of the feto-placental unit. Because the fetus cannot synthesize it in the amounts required for its optimal development, supply of glucose from maternal blood into fetal circulation represents a major determinant of fetal growth and development [121,122]. Glucose supply to the fetus is mediated by members of the GLUT family of transporters [123–125], GLUT1 being the predominant glucose transporter expressed at the placental level [126,127].

The effect of dietary polyphenols upon the placental transport of glucose was investigated in a few studies only. In BeWo cells (a human term trophoblast cell line), several polyphenolic compounds were found to affect the apical uptake of ^{3}H-deoxy-D-glucose (^{3}H-DG), a glucose analogue that is efficiently transported by GLUT family members. In the short-term (26 min), resveratrol, quercetin, chrysin, EGCG, and xanthohumol reduced ^{3}H-DG uptake. Quercetin and xanthohumol seemed to act as noncompetitive inhibitors, whereas EGCG acted as an uncompetitive inhibitor. On the other hand, rutin, catechin, and epicatechin increased ^{3}H-DG uptake. Interestingly enough, catechin and epicatechin together decreased ^{3}H-DG uptake, contrary to their effect alone (increase), but the association of epicatechin and xanthohumol counterbalanced each one's isolated effect. In the long-term (48 h), rutin and myricetin increased the apical uptake of ^{3}H-DG, both isolated and in combination [128].

Polyphenolic compounds are also able to interfere with glucose uptake by first-trimester human trophoblasts. As shown by Correia-Branco et al. [129], uptake of ^{3}H-DG by a human first-trimester extravilous trophoblast cell line (HTR-8/SVneo) is inhibited by phloridzin, phloretin, quercetin, EGCG, resveratrol, and xanthohumol. Xanthohumol was found to be the most potent of the tested polyphenols, behaving as a noncompetitive inhibitor of ^{3}H-DG uptake. Extravillous trophoblasts are actively involved in placentation, and xanthohumol showed a negative effect upon placentation-related processes (cell proliferation, viability, and migration). Interestingly enough, the antiproliferative and cytotoxic effect of xanthohumol was mimicked by low extracellular glucose and reversed by high extracellular glucose, showing that its negative effect upon placentation-related processes is related to glucose cellular deprivation.

Finally, resveratrol was found to abolish the decrease in GLUT1 expression and glucose uptake mediated by the reactive oxygen species inducer hypoxanthine-xanthine oxidase in human placental tissue [130].

6 CONCLUSIONS

Phenolic compounds, numerous and ubiquitous in the plant kingdom, are particularly abundant in health-promoting foods.

The studies reviewed in this chapter raise a concern about possible changes in the bioavailability of bioactive compounds upon concomitant ingestion of polyphenols. Important conclusions concerning polyphenolic effects on transport systems can be drawn: (i) different classes of polyphenols affect transport of these bioactive compounds at the intestinal epithelia and at the placenta; (ii) different compounds belonging to the same phenolic family often possess opposite effects upon transport of a given molecule; (iii) short- and long-term exposures to polyphenols does not produce parallel results; (iv) combinations of distinct polyphenols, as happens in natural foods, may give very distinct effects from the expected ones, taking into account the effect of each of these compounds alone, and (v) ethanol is able to modify the effect of polyphenols upon transport mechanisms. So, care should be taken when extrapolating results obtained with isolated polyphenols to dietary recommendations and when drawing conclusions for alcoholic beverages from results obtained with ethanol or polyphenols alone.

Additional experiments are necessary to clarify the effects of these compounds on transport systems at the blood-brain epithelial barrier, as several beneficial effects of polyphenols on neuronal functions, particularly on appetite control and cognition, have been described. Moreover, application of more advanced experimental models, such as recombinant cell lines and genetically engineered mice, would certainly help in finding more definitive answers on identifying the effects of polyphenols on transport mechanisms. Therefore, intensive research is needed before a beneficial effect of polyphenol supplementation can be predicted. It is also necessary to clarify, not only the dietary effect of polyphenols, but also their toxicity to cells or organs when they are supplied as a dietary supplement.

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CHAPTER

24

Analyzing Ingredients in Dietary Supplements and Their Metabolites

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1 INTRODUCTION

Epidemiological studies demonstrate that consumption of dietary fruits and vegetables can be beneficial in protecting against chronic diseases, such as cancer and cardiovascular disease. In addition to micronutrients, plant-based foods contain a vast array of nonnutrient phytochemicals with potential pharmacological benefits, and one of the major groups of these phytochemicals is flavonoids. Flavonoids are polyphenolic compounds composed of a three-ring structure (C6–C3–C6) with various substitutions such as hydroxylation, methoxylation, or glycosylation, resulting in high structurally diverse secondary metabolites. These are termed flavones, flavonols, flavanones, and flavanols depending upon the presence of a carbonyl carbon at C4, an OH group at C3, a saturated single bond in between C2 and C3 and a combination of no carbonyl at C4 with an OH group at C3, respectively [1]. Isoflavones differ from flavonoids with respect to the attachment of ring B to ring C at C3, instead of the C2 position. Flavonoids in some plants are polymerized into tannins. For example, the condensed tannins are polymers of flavan-3-ols, e.g., catechins [2]. Condensed tannins or proanthocyanidins are found in grape seed, cocoa, and many other foods. Our laboratory has identified an oligomeric series of catechin/epicatechin/gallo (epi)catechin units up to nonamers in grape seed extract [3]. Another class of tannins, called hydrolyzable tannins, consist of gallic acid or ellagic acid to which a nonaromatic polyol, such as a sugar or quinic acid, is esterified [4]. Biologically active hydrolyzable tannins are found in many foods, including pomegranate [5].

Total intake of flavonoids varies from country to country. For example, consumption of total flavonoids in the United States is about 20 mg/day, whereas it is >70 mg/day in Holland [6]. To explore linkages between flavonoid ingestion and health benefits, information on the intake of particular subtypes of flavonoids may be more important than total flavonoid intake [7]. Health benefit claims of any bioactive compounds not only depend on what is consumed, but also on the form and quantity that reach the target sites of interest.

Even though flavonoids are consumed in substantial amounts, most physicians do not receive formal education regarding the safety and efficacy of herbal therapies, and concern is growing about the potential health risks and adverse effects caused by impurities in, and variability of, polyphenolic contents of the products and by potential drug-supplement interactions [8,9]. In research, a lack of consistency in quality and variability of dietary botanicals makes the interpretation of their roles in health and disease difficult. While consumers are increasingly buying herbal products and dietary supplements online, information related to content variability and safety is largely lacking. It is, therefore, critical to quantitatively assess and document the entire spectrum of all active ingredients present in any botanical preparation, and to assess degradation of, and impurities in, the consumer products.

This review considers the updated use and analyses of commonly used polyphenol-based, dietary supplements and the discrepancies between industry labeling and the exact content of individual phytochemicals present in a given dietary supplement.

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2 ANTHOCYANINS

Anthocyanins are a group of polar flavonoids that are abundant in many colored fruits and vegetables (bright red-orange to blue-violet colors). Six different anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin; Fig. 24.1) are ubiquitously found in nature, and the most common is cyanidin. Their ingestion provides a number of physiological benefits, including antioxidation [10,11]. They are consumed at a rate up to ninefold higher than other flavonoids [12]. Their content in some diets is very high; for instance, servings of 200 g of aubergine or black grapes can provide up to 1500 mg of anthocyanins [13]. Blueberries, pomegranates, and strawberries are the most common sources of anthocyanins, and there has been growing consumer interest in using anthocyanin-containing dietary supplements such as bilberry extract, which has been approved as a pharmaceutical product in many countries [14]. However, the composition, amounts, and distribution of anthocyanins in blueberry appears to differ based on the cultivar, cultivation conditions, and the areas of production [15]. Different cultivars of blueberries include highbush blueberry and rabbiteye blueberry in Japan, Iowbush blueberry in North America, and bilberry in central and northern Europe [14]. Owing to its higher anthocyanin content compared to others, bilberry extract is used in preparation of anthocyanin-based supplements.

Stability of anthocyanins (conversion of anthocyanin to anthocyanidin) is another critical consideration in use and quality control of anthocyanin-based dietary supplements. Anthocyanins mostly exist in the glycosidic forms in fruits and vegetables. They can be hydrolyzed to anthocyanidins by thermal treatment and glycosidases, and their stability is also affected by pH, temperature, light, and oxygen. Therefore, they are optimally stored at cool temperatures and in dark environments. The anthocyanin's colors are pH-dependent. At pH 3 or lower, they exist as flavylium cations that are orange or red [16]. Anthocyanins are relatively more stable than their aglycone forms. Bilberry extract dietary supplements containing anthocyanins but not anthocyanidins may be useful for skin diseases involving pruritic symptoms [17]. This underlies the importance of profiling ingredients in dietary supplements, preferably using sensitive and specific analytical techniques of LC-MS and LC-MS/MS. For quality control evaluation of botanicals, it is important to choose appropriate marker compounds, characteristic of the botanical of interest, but not necessarily the active component.

Measurement of anthocyanins. Anthocyanin-containing tablets, capsules and other commercial forms are crushed and usually extracted with hydrochloric acid or formic

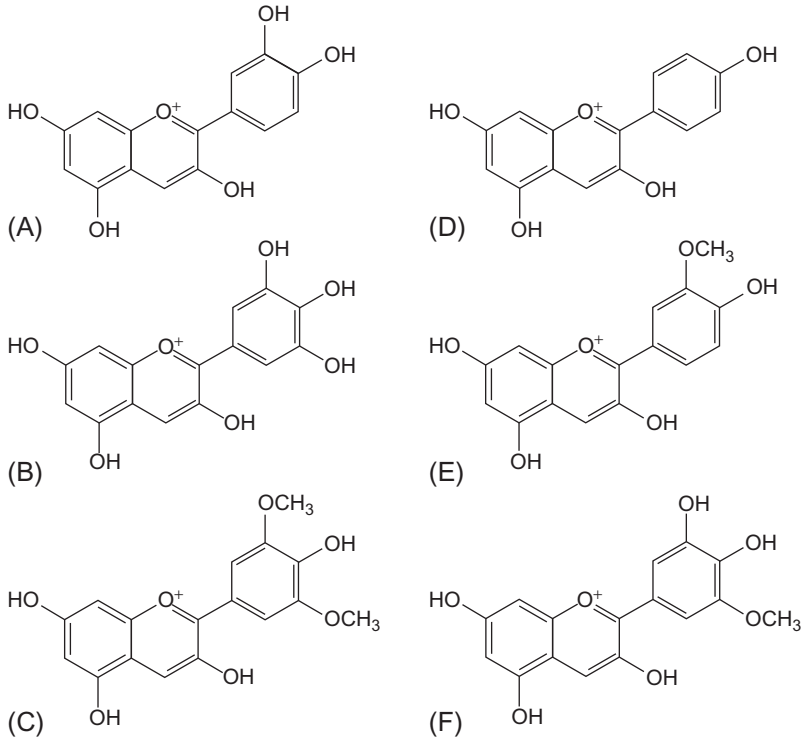

FIG. 24.1 Structures of six common anthocyanins. They have a common A-ring and a heterocyclic ring that contains a positively charged oxygen atom that leads to absorption in the visual range. The differences between the anthocyanins depend on substituents in the B-ring. (A) Cyanidin, (B) delphinidin, (C) malvidin, (D) pelargonidin, (E) peonidin, and (F) petunidin.

acid below at 4°C to maintain their flavylium form. Felgines et al. extracted urinary anthocyanins, acidifying 1/60 volume of 12 mol/L HCl at room temperature [18]. The mixture was further separated by passing over a solid phase extraction cartridge. It has been reported that solvents containing 1% of 12 N HCl can cause the partial hydrolysis of some acetylated anthocyanins during extraction, despite their higher efficiency in extracting total anthocyanins [19]. Solid-phase extraction (SPE) on reverse-phase C18 cartridges or Sephadex can be used for extraction of anthocyaninins [20]. A cation-exchange/reversed phase combination SPE has also been used to selectively extract anthocyanins [21]. For spectrophotometric analysis, the extracted solution is measured from 200 to 600 nm using a UV/vis spectrophotometer. Anthocyanin content is expressed as the equivalent of delphinidin. There is an extensive review by da Costa et al., describing methodologies for the measurement of anthocyanins [22]. For identification and quantification of individual anthocyanins, HPLC and LC-MS can be used. UV/Vis is widely used for HPLC analysis of anthocyanins, since they have a unique absorption at around 520 nm in the visual region, which makes them quite distinct from other flavonoids in terms of their absorption maximum [16].

3 FLAVANOLS

Flavanols or flavan-3-ols represent the most common flavonoids consumed in the American diet [23]. Tea, chocolate, grapes, apples, and red wine are some of the prominent sources of these polyphenols. Presence of flavan-3-ols in foods affects quality, particularly tannins' astringency property. Catechins such as catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) (Fig. 24.2) and proanthocyanidins (Fig. 24.3) are important functional ingredients in many commercially available beverages, whole and processed food and dietary supplements. For instance, EGCG (Fig. 24.2D) is considered as the major polyphenol antioxidant in green tea.

Seeram et al. investigated whether label claims on green tea dietary supplements correlate with actual phytochemical levels and antioxidant capacity [24]. These studies concluded that product levels in the label information were inconsistent with measured phytochemical contents. Other studies also showed that there is a large variation in flavanol content in dietary supplements. In this regard, Manning et al. used HPLC to investigate the catechin content of seven commercial tea products [25]. The measured catechin content ranged

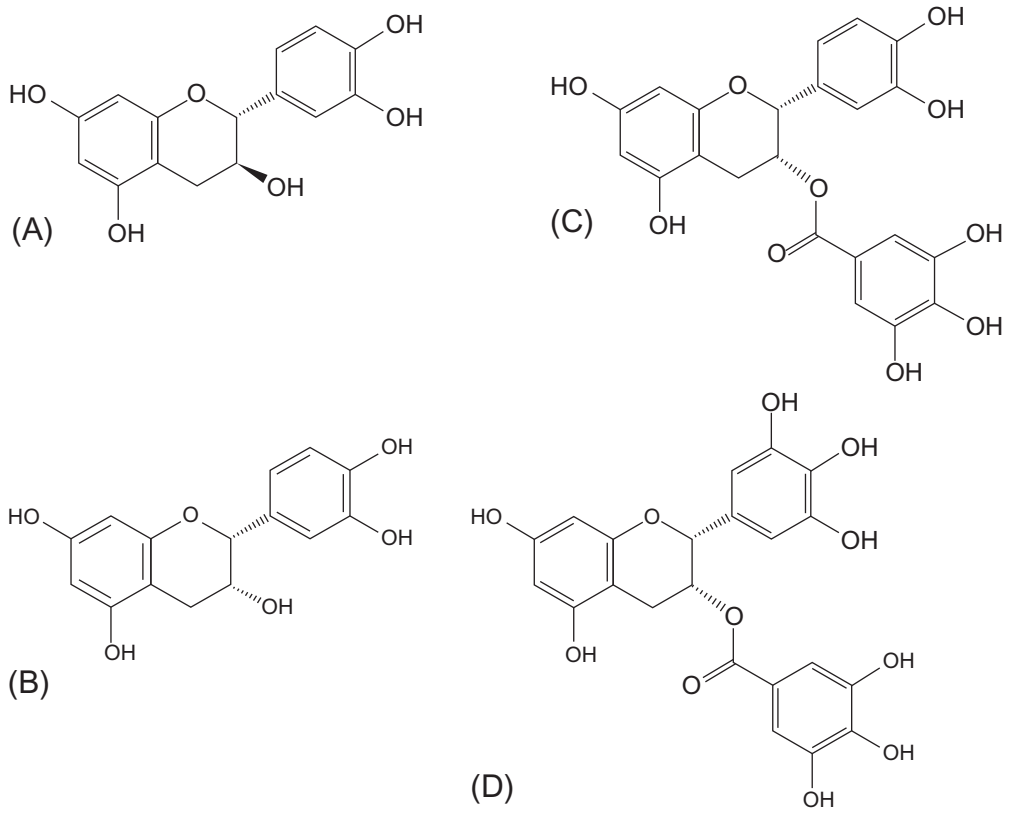

FIG. 24.2 Structures of flavan-3-ols. These are green tea flavanols—flavan-3-ols that have a fully saturated heterocyclic ring with a hydroxyl substituent at C3. This makes C3 a chiral center resulting in two isomers (A, catechin and B, epicatcechin). The other two green tea components contain gallic acid components—(C) epicatchin-3-gallate and (D) epigallocatechin-3-gallate.

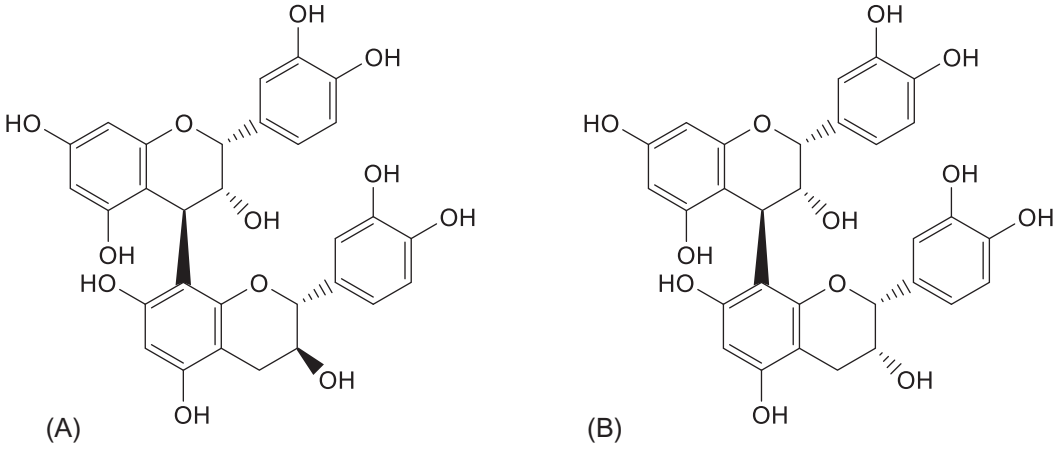

FIG. 24.3 Structures of proanthocyanins. These are oligomers of catechins. Two dimers are shown—proanthocyanin B1 (A) and proanthocynanin B2 (B). However, higher oligomers are typically found in grapeseed extract.

from 9%–48% of the claimed content. All values were significantly lower than the associated claim ($P < 0.05$), indicating poor quality control in the dietary supplements and/or herbal industry. A large variation in the flavanol content was also found in the catechin content of 18 teas and a green tea extract supplement investigated by Henning et al. [26]. These results underscore the importance of reliable labeling information and good quality control for the strength, quality, and purity of dietary supplements.

Catechins and caffeine can be extracted by various methods such as boiling and filtering, liquid-liquid extraction (LLE), and solid phase extraction (SPE). Methanol:water (1:1 v/v) has also been used to dissolve catechins after crushing tablets or capsules [24]. Catechins can be measured at UV 278 nm. Our laboratory has developed sensitive and reproducible LC-multiple reaction monitoring (MRM) methods for quantitative analysis of tea catechins (C, EC, EGC, ECG, and EGCG; Fig. 24.2). Stability of EGCG is one of the major concerns in quantitative analysis of green tea products, since EGCG is stable at acidic pH (2.0–5.5) and is autooxidized at neutral pH to form dimeric structures [27].

4 FLAVONES AND FLAVONOLS

Flavones and flavonols are widely distributed in plants as *O*-glycosides. Flavonols such as quercetin (Fig. 24.4B), myricitin (Fig. 24.4C) and rutin have an OH group at C3, whereas the flavones, (e.g., apigenin, luteolin, and baicalein; Fig. 24.5) have a hydrogen in that position. Popular herbal medicines such as St. John's wort (SJW) contain various active phytochemicals including rutin, isoquercetrin (quercetin-3-*O*-glucoside), and quercetin. Retail SJW lacks any strict controls relative to appropriate safety or efficacy, as evidenced by the very large variation in major compounds that was found among 12 different SJW products [28]. In this study, quercetrin and isoquercitrin showed 6.4- and 28.8-fold variations, respectively, across these SJW products. *Ginkgo biloba* extract is another popular medicinal plant extract which contains flavonols such as quercetin (Fig. 24.4B), kaempferol (Fig. 24.4A), isorhamnetin (Fig. 24.4D), etc. *G. biloba* commercial products are also reported to show large variations in the rutin and quercetin content [29]. Flavones and flavonols are extracted from herbs, vegetables and fruits by LLE and SPE after lyophilization [30].

5 ISOFLAVONES

Isoflavones are considered to be phytoestrogens and are distributed mostly in legume-based dietary supplements. Soybeans, red clover, and kudzu are the main sources of isoflavones, which differ from flavones in regard to the position of the B-ring. In flavones, the B-ring is in the 2-position, but in isoflavone it is in the 3-position (Fig. 24.6). Soy foods/supplements typically contain 12 isoflavones, namely genistein (Fig. 24.6A), daidzein (Fig. 24.7A) and glycitein (Fig. 24.7B), their glucosides genistin, daidzin, and glycitin, respectively, and their malonyl and acetyl esters. The malonylglucosides, 6″-*O*-malonylgenistin, 6″-*O*-malonyldaidzin, and 6″-*O*-malonylglycitin, are the predominant isoflavone glucosides in soybeans; however, these water-soluble isoflavones are heat-labile and are readily converted to acetylglucosides (6″-*O*-acetylgenistin, 6″-*O*-acetyldaidzin and 6″-*O*-acetylglycitin; Fig. 24.8). The isoflavone glucoside conjugates are easily altered during extraction, processing, and cooking. However, the total concentration of isoflavones does not change.

Over the past three decades, isoflavones have been the subject of active investigation due to their roles in the prevention of chronic diseases, including several types of

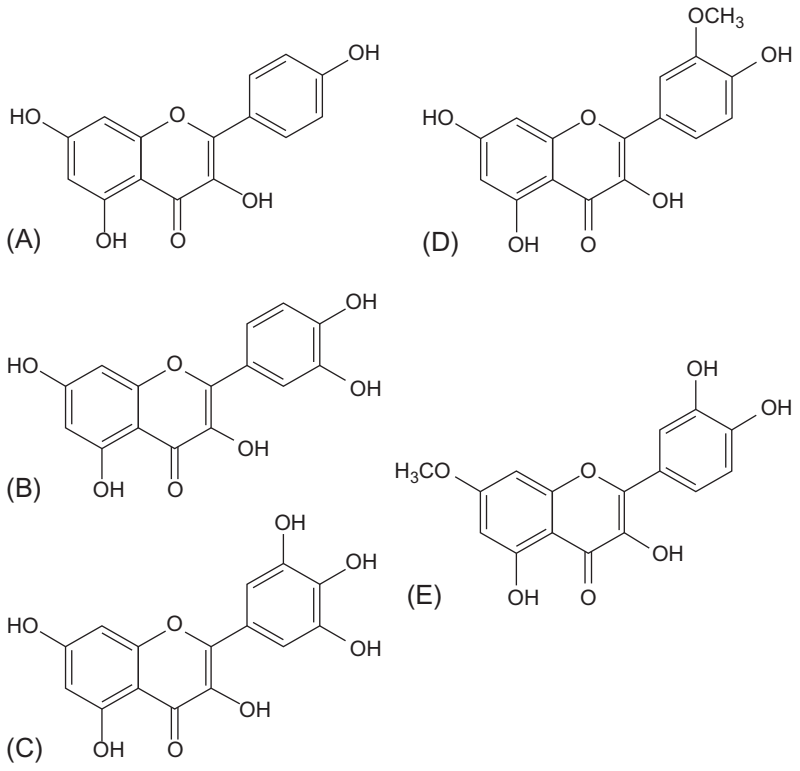

FIG. 24.4 Structures of common flavon-3-ols. The differences in them lie in the number of hydroxyl groups in the B-ring—kaempferol (A), quercetin (B) and myricitin (C). Rhamnetin (D) and isorhamnetin (E) contain methoxy groups in the B- and A-rings, respectively.

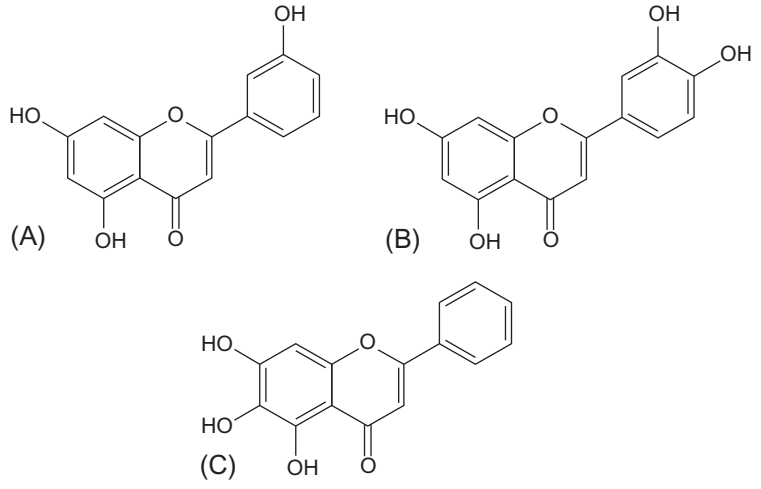

FIG. 24.5 Structures of common flavones. Those flavonoids do not have substituents in the heterocyclic ring. Thereby, apigenin (A) and luteolin (B) are the flavone equivalents of kaempferol and quercetin. Baicalein (C) is unusual in that there are three hydroxyl substituents in the A-ring and none in the B-ring.

cancers [31,32]. Isoflavones are structurally similar to 17β-estradiol and have been the subject of intense research regarding their effects on human health as agonists of the ERβ receptor.

Kudzu root (Radix pueraria) from the vine *Pueraria lobota* is the richest source of isoflavones of the commonly used botanicals, and it is used in traditional Chinese medicines. Its root extract has become commercially available in Western dietary supplements. The isoflavones in kudzu root are puerarin (daidzein-8-*C*-glucoside; Fig. 24.9B), an isomer of daidzin (daidzein-7-*O*-glucoside; Fig. 24.9A), daidzein diglucosides and formononetin (Fig. 24.7B). Kudzu root extracts and their components are known to have antioxidant, antidipsotropic, and other pharmacological effects [33–37]. Many of the "soy isoflavones" sold for women's health are predominantly

kudzu-derived, containing much more C-glucosides than *O*-glucosides. The uptake, metabolism, and biological activities of these two glucosides are very different. In our previous studies, we demonstrated that puerarin is absorbed as the intact glucoside and effectively blunts the rise in blood glucose concentration in response to a glucose load in male C57 BL/6J ob/ob [37]. Our laboratory has also shown that the compound, which was assumed to be daidzin in kudzu-based dietary supplements (KDS), is in fact puerarin [38]. In some kudzu-based dietary supplements, the manufacturers' labels typically do not state that puerarin is one of the phytochemicals in KDS [38]. Although daidzin and puerarin are isomeric and both found in kudzu root, they can be easily distinguished by LC-MS/MS [38]. Neutral losses of 162 and 120 da are characteristics of *O*- and *C*-glucosides of daizein, respectively in their MS/MS fragmentations.

Interestingly, Clarke at al. used LC-MS/MS to analyze the isoflavone content of 35 dietary supplements on sale in the UK, Canada, and Italy, and found that 11 did not match label claims, and the content of total isoflavones ranged from <1 to 53 mg per dose [39].

Red clover, which is consumed by cattle and sheep, is another source for isoflavones. It contains glycosides of 4′-*O*-methylated daidzein (formononetin; Fig. 24.7B) and genistein (biochanin A) (Fig. 24.7D). Phytoestrogen-based dietary supplements are often a blend of soy, red clover, and kudzu. Delmonte et al. developed a 90-min HPLC method using an acetonitrile/water gradient with photodiode array (200–400 nm) for simultaneous detection and quantification of isoflavones from soy, red clover, and kudzu-based dietary supplements [40]. They extracted the samples at room temperature with acetonitrile/water (1:1 v/v) and analyzed before and after hydrolyzing isoflavone glycosides by acid or basic digestion.

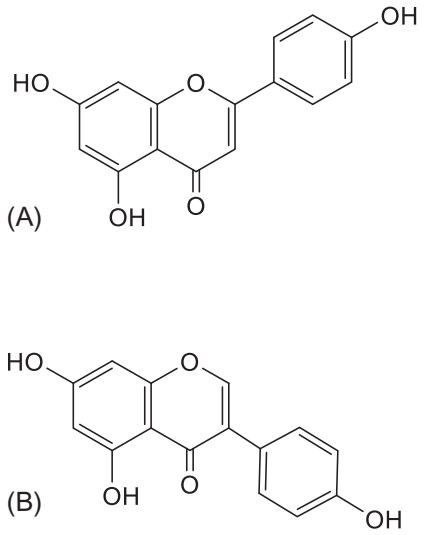

FIG. 24.6 Differences in structure between flavones and isoflavones. Whereas the B-ring in flavones is in the 2-position (e.g., apigenin, A), for isoflavones it is in the 3-position (e.g., genistein, B).

6 EXTRACTION OF ISOFLAVONES

Isoflavones found in nonfermented foods are predominantly in their conjugated (glycoside) forms. The identification and quantification of these compounds depend upon the extraction/purification procedures and conditions used in each method. Extraction of soy isoflavones with 80% aqueous methanol at room temperature is one of the preferred methods as the extraction is temperature-sensitive [41]. Studies have shown that 6″-*O*-malonylglucoside conjugates of isoflavones are prone to both heat-induced decarboxylation and deesterification (Fig. 24.8). The isoflavone glucoside conjugates are easily altered during extraction, processing, and cooking; however, the total concentration of isoflavones does not change. Temperature during extraction of isoflavones may change the composition of isoflavone conjugates; even room temperature storage for extended periods can cause a significant alteration [41].

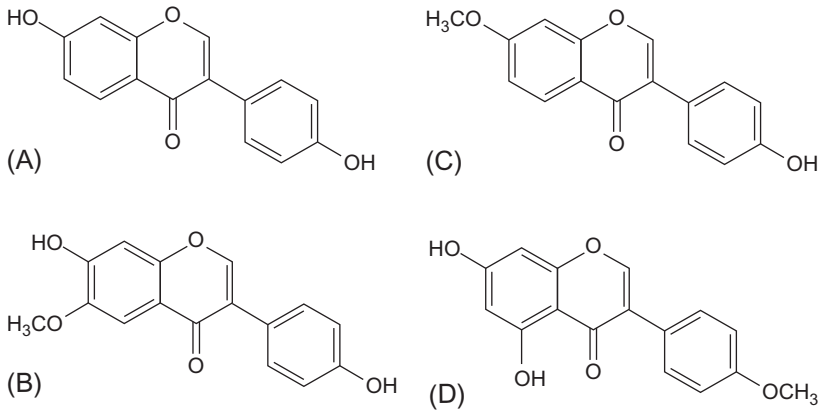

FIG. 24.7 Common isoflavone aglucones in dietary supplements. Daidzein (A) and glycitein (C) in soy and kudzu, and formononetin (B) and biochanin A (D) in red clover.

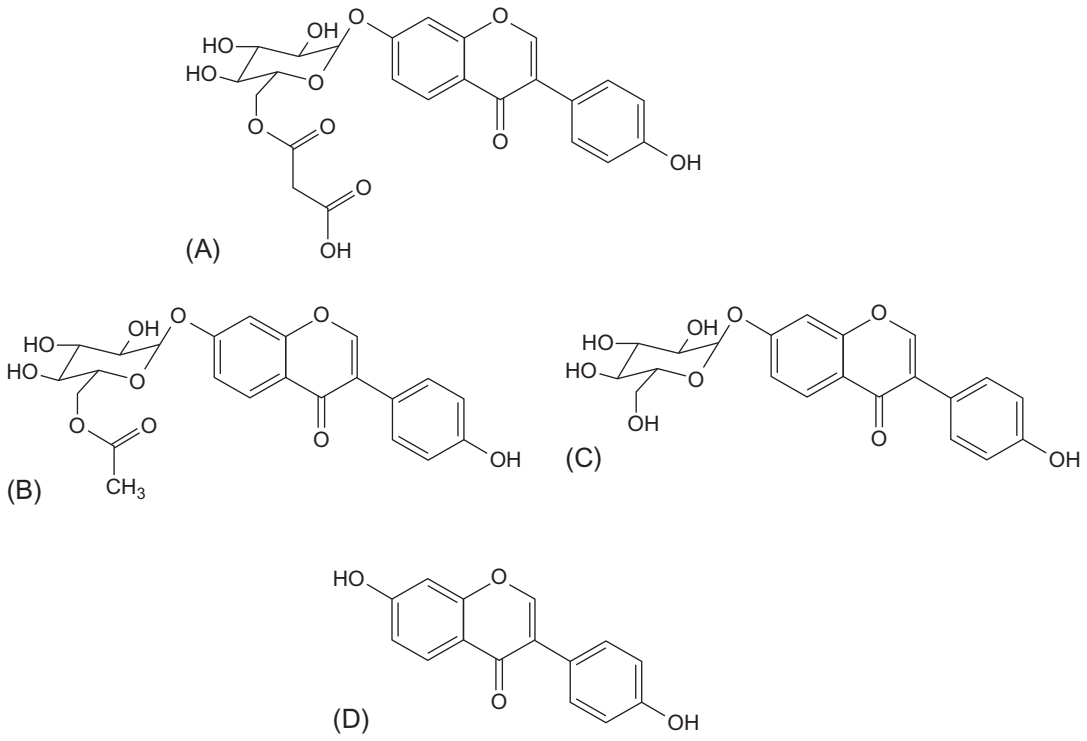

FIG. 24.8 Changes in the chemistry of 6″-malonyl-7′-β-glucosyldaidzein, a major soybean component, during food processing. Dry heating of 6″-malonyl-7′-β-glucosyldaidzein (A) leads to decarboxylation to 6″-acetyl-7′-β-glucosyldaidzein (B). Heated, aqueous extraction of soybeans to make soy milk and tofu, removes the malonyl group to make daidzin (C). Fermentation can also lead to hydrolysis of the glucosyl group (D).

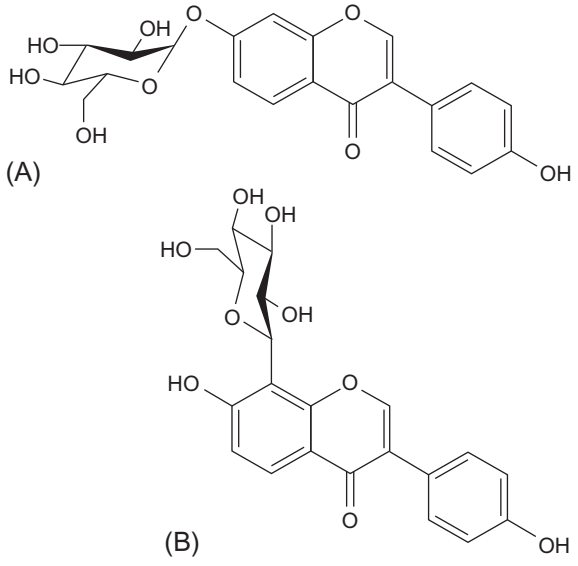

FIG. 24.9 *O*- and *C*-glucosides of daidzein. In daidzin (A), the link between daidzein and glucose is a C—O—C bond, whereas in puerarin (B), it is a C—C bond.

Recently, a comparative study on different extraction techniques—microwave-assisted, ultrasound-assisted, conventional solvent extraction, and adding ionic liquid before extraction—was conducted to optimize kudzu isoflavone extraction from *Pueraria lobota* (Mocan et al.) [42]. Results obtained from these studies indicated that puerarin, daidzin, and genistein are best extracted by conventional extraction with 1:5 (w/v) water:methanol as a solvent for 30 min, while daidzein is best extracted by microwave-assisted extraction using ethanol as extracting solvent with 1:5 solid:liquid ratio. In our previous studies, we demonstrated that ratio of kudzu dietary supplement powder and extracting solvent of 15 mg in 5 mL 80% aqueous methanol is required for efficient extraction of puerarin [38]. Further, a mixture of three solvents, namely ethanol:water:dimethyl sulfoxide in a 70:25:5 v/v/v ratio, can efficiently extract total isoflavonoids from different matrices [42]. These studies show that the ratio of botanical material to extracting solvent plays an important role in efficient extraction of phytochemicals.

It is increasingly clear that the chemical composition of isoflavones (aglycone vs. *C*- or *O*-glucosides) can have significant effects on the bioavailability and, hence, the biological activities in vivo [43]. The ratio of botanicals/dietary supplements to extracting solvents should be optimized for efficient extraction. In our previous studies, 15 mg of kudzu powder was efficiently extracted with 5 mL of 80% methanol in water [38].

In many laboratories, HPLC with C_{18} reverse-phase columns is used for isoflavone analysis. The isoflavones are monitored using a photodiode array in the UV range (260–280 nm), or with electrochemical detection (+500 mV). HPLC combined with mass spectrometry

(MS) or LC-MS has been used for isoflavone analysis with enhanced sensitivity and specificity. There are several reports of using LC-MS techniques to determine isoflavones in herbs and food items [44,45].

7 IN VIVO METABOLISM OF ISOFLAVONES

Isoflavones are found predominantly as glucosides in most food items. Isoflavone glycosides, as well as other glycosidic flavonoids, undergo hydrolysis to their aglucone forms in the small intestine by enzymes in the enterocyte and in large intestine by bacterial action. The aglucones are readily absorbed [46,47]; however, sulfotransferases and UDP-glucuronyltransferases are present in enterocytes, thereby converting a large proportion of the aglucones to phase II metabolites before they enter the bloodstream, and on being taken up by the liver, they may undergo secondary phase II metabolism to double conjugates. The nature of the conjugates varies among humans, mice, and rats [48]. These phase II metabolites are secreted by the liver via bile into the duodenum, and they must be hydrolyzed to be reabsorbed. However, this requires bacterial enzymes and occurs after they enter the large intestine. The bacteria there also cause additional reactions including reduction and heterocyclic ring opening, generating dihydrogenistein from genistein, and dihydrodaidzein (Fig. 24.10B), *O*-desmethylangolensin (Fig. 24.10C) and S(-)-equol (Fig. 24.10D) from daidzein. In addition, further metabolism produces 4-ethyl phenol (Fig. 24.10E) and 2-(4-hydroxyphenyl)-propionic acid (Fig. 24.10F). Each of these metabolites can also form phase II metabolites (sulfonates and β-glucuronides).

Barnes and his coworkers developed an LC-MS/MS method using the MRM mode to analyze deconjugated isoflavones in plasma [49]. Subsequently, a 2-min LC-MS/MS method operating in MRM mode was developed that allows for the characterization and simultaneous quantification of 11 phytoestrogen metabolites and chrysin (as the internal standard) with mass transitions *m/z* 241/119 (equol), 253/132 (daidzein), 255/149 (dihydrodaidzein), 257/108 (*O*-desmethylangolesin), 269/133 (genistein), 283/184 (glycitein), 267/191 (formononetin), 289/109 (biochanin A), 267/91 (coumestrol), enterodiol (301/253), and enterolactone (297/253) [50]. This method facilitates ultrafast analysis without sacrificing chromatographic resolution over a range of 1–5000 ng/mL in human serum (with the exception of dihydrodaidzein, whose lower limit of quantification is 2 ng/mL). The separation was carried out on a Synergi 2.5 micron (50 × 2.0 mm, i.d.) column with water and acetonitrile (both containing 10 mM ammonium acetate) as the mobile phase under gradient conditions, at a flow rate of 0.75 mL/min. Similarly, the isoflavonoid metabolites can be analyzed by LC-MRM-MS without deconjugation using the mass transitions *m/z* 321/241 (equol sulfate), 417/241 (equol β-glucuronide), 333/253 (daidzein sulfate), 429/253 (daidzein β-glucuronide), 335/255 (dihydrodaidzein sulfate), 431/255 (dihydrodaidzein β-glucuronide), 337/257 (*O*-desmethylangolensin sulfate), 433/257 (*O*-desmethylangolensin β-glucuronide),

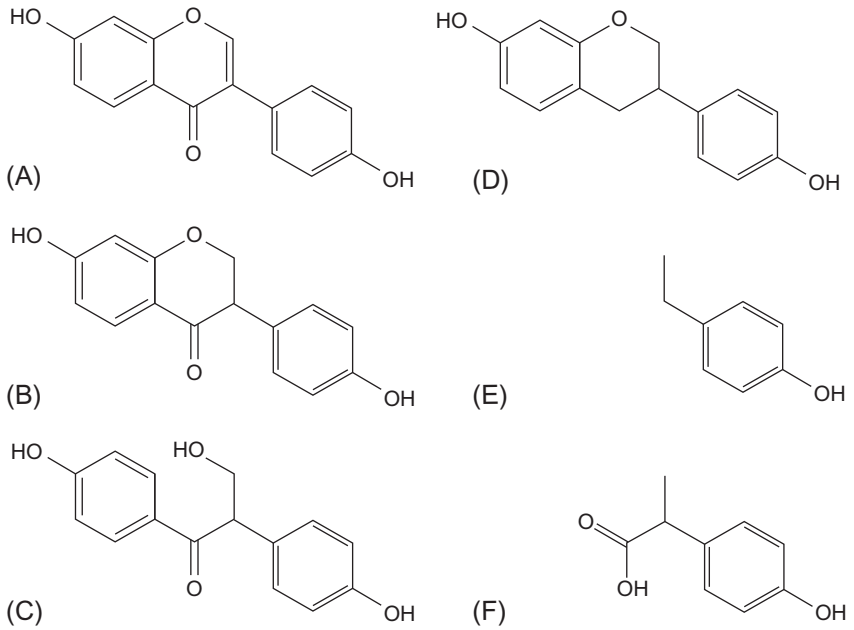

FIG. 24.10 Bacterial metabolites of daidzein. (A) Daidzein, (B) dihydrodaidzein, (C) *O*-desmethylangolensin, (D) S(−)-equol, (E) 4-ethylphenol and (F) 2-(4-hydroxyphenyl)propionic acid.

349/269 (genistein sulfate) and 445/269 (genistein β-glucuronide). This approach was used to analyze these conjugates in domestic cats and cheetahs and demonstrated that, while the cat family cannot form β-glucuronides, they can undergo extensive sulfonation [51,52].

8 CONCLUSIONS

Reliable qualitative and quantitative information on active ingredients that are present in botanical products sold as dietary supplements in markets is critically important to assess potential health risks and benefits associated with their intake. Therefore, estimating the levels of bioactive components before and after ingestion and their access to different compartments of the body (pharmacokinetics) using sensitive and selective analytical methods is required to interpret the outcomes of any investigation to use botanical products as complementary and alternative medicines. Information inconsistency on phytochemical content in a single botanical product using different analytical methods can pose a considerable challenge, but without clear, ongoing data on the reliability and consistency of the various botanicals in research and retail products, the interpretation of the results of basic and clinical studies will be compromised, and recommendations for their clinical will lack a data-driven foundation.

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CHAPTER

25

Metabolism of Dietary Polyphenols by Human Gut Microbiota and Their Health Benefits

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1 INTRODUCTION

1.1 Polyphenols

Polyphenols are a wide range of natural compounds found in plants, which, in the past few years, have received much interest from researchers and food-related industries for their health benefits. Under their chemical composition, polyphenols are compounds having one or more hydroxyl groups attached to a benzene ring [1]. These secondary metabolites are involved in defense mechanisms against ultraviolet radiation or certain pathogens. According to several epidemiological and food intervention studies, it has been suggested that there are strong associations between the consumption of polyphenol-rich foods and beverages and the prevention of many degenerative diseases, such as cardiovascular and neurological diseases, as well as cancer and aging [2]. The health effects of polyphenols can be determined by the amount consumed and their bioavailability. In recent years, polyphenols and their metabolites from the gut microbiota as well as cellular metabolism have garnered attention because of the beneficial health effects. Therefore, this review will focus on the metabolism of different structural classes of polyphenols by the intestinal microbiome and it's impact on human health. The structural, metabolic, and individual diversity in the colonic microbiota, including the polymorphisms of the drug metabolizing enzymes, leads to enormous variations. This poses a necessity for much greater understanding of all the mechanisms of the health benefits of polyphenols. Such understanding offers an opportunity for further research and development of strategies and new products.

Dietary phenolics are one of the most widely distributed classes of natural products in plants [3]. Currently, more than 8000 phenolic structures are known [4]. More than 6000 flavonoids have been discovered [5] and, in recent years, several more compounds have been identified. However, elucidating the mechanisms of phenolics, their metabolites and health benefits is challenging, as they have a wide range of polarities and molecular weights [6].

Polyphenols are categorized into several classes based on the number and presence of phenol rings [7]. Polyphenols are mainly classified into flavonoids, phenolic acids, stilbenes, and lignans (Fig. 25.1) depending on their carbon skeleton [8]. Polymers and oligomers of flavonoids are known as tannins and are classified into two groups: condensed tannins (proanthocyanidins or procyanidins) and hydrolyzable tannins [1]. Flavonoids are metabolites that are constructed of 15 carbon compounds accompanied by two benzene rings, A and B, which are joined by a linear three-carbon chain. They vary in colors ranging from white to yellow [9]. They are further divided into six subclasses as flavonols, flavones, flavanones,

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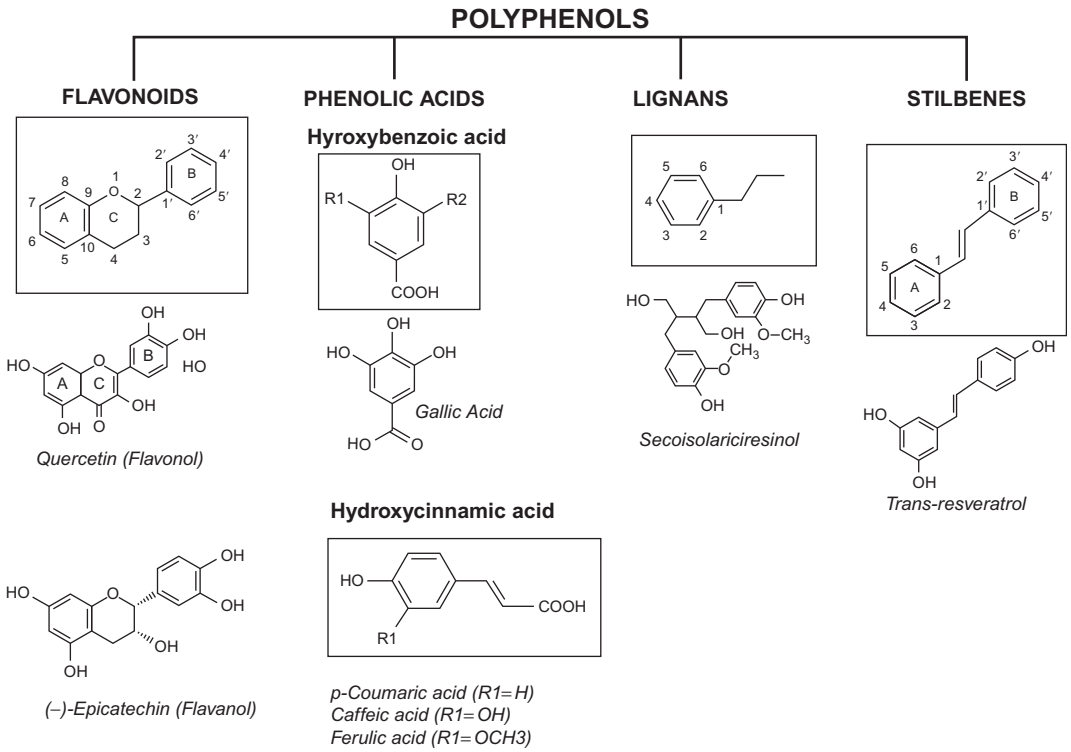

FIG. 25.1 Basic structures of polyphenols and selected examples.

isoflavones, anthocyanidins, and flavanols [7]. Flavonols are the most commonly occurring group of flavonoids in plants, which are consumable as food [9]. Compounds such as quercetin are important flavonoids found in the diet. This metabolite is attributed to being one of the most ubiquitous of flavonols, including others such as kaempferol, myricetin, and isorhamnetin. Quercetin is found to be present at higher concentrations in fruits and vegetables and especially in onions [10]. Besides onions, other good sources of quercetin are apples and tea [11]. Metabolites such as apigenin and luteolin [9] constitute as major representative for flavones, which are found in many herbs and vegetables like celery, parsley, and artichokes [12]. Flavanols are a popular group of flavonoids, which include catechins and procyanidins, found in high concentrations in grapes, red wine, cocoa, and tea. Flavan-3-ols are categorized as the most common flavonoids consumed, which includes simple monomers such as (+)-catechin and (−)-epicatechin as well as oligomeric and polymeric proanthocyanidins, which are also categorized as condensed tannins. Fruits and vegetables such as grapes, blackberries and apricots are found to have high amounts of flavan-3-ols. Other plant products such as green tea [9] are found to contain this metabolite. About 80% of flavonoids occur as glycosides in plants [13]. However, flavan-3-ols are an exception, as these metabolites are always found to be present in the diet in a nonglycosylated form [14]. The major aglycones of flavanones commonly found in the diet are naringenin in grapefruit, eriodictyol in lemons, and hesperetin in oranges [15]. These aglycones are also found in tomatoes and certain aromatic plants such as mint [7].

Anthocyanins, an important group of flavonoids, are categorized by means of their bioactive mechanisms. They occur as hydrosoluble plant pigments found in fruits and flowers and provide colors such as red, blue, and purple [9]. An estimate of more than 300 different anthocyanin compounds has been identified in plants [16]. The aglyconic forms of anthocyanins are regarded as anthocyanidins. Common anthocyanidins like cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin are ubiquitously distributed and of dietary importance [9].

Isoflavones, another major classification of phenolic compounds include genistein, an active ingredient found in soy. Isoflavones are specifically found in leguminous species [17] and play a role as phytoestrogens [18]. Studies have shown that regular consumption reduces the vulnerability to diseases such as osteoporosis and arteriosclerosis and certain cancers [19].

Phenolic acids are the major polyphenolic constituents found in food and greatly represented by gallic acid, p-hydroxybenzoic acid, and vanillic acid. They form specialized components of lignins and hydrolyzable tannins.

Phenolic acids are categorized into two distinctive groups: hydroxybenzoic acids (gallic acid, vanillic acid, salysilic acid) and hydroxycinnamic acids (caffeic acid, ferulic acid, and p-coumaric acids) [2].

Stilbenes are compounds produced due to disease, injury, and stress in plants, and have gained attention because of their biological activities. The major constituent of stilbene is resveratrol (3,5,4u-trihdroxystilbene), which can be present in *trans* and *cis* configurations as aglycones, glucosides, monomers, and polymers [1]. They can be found in grapes, peanuts, berries, red cabbage, and spinach [20].

2 DIETARY INTAKE OF POLYPHENOLS

Several studies have been conducted in daily polyphenol consumption and their health benefits. Due to the high structural diversity in polyphenols, elucidating the exact levels of polyphenols in the daily intake and the total polyphenol intake is challenging. However, estimation studies show that the total daily intake of plant phenols is about 150–1000 mg [2,21]. Hourly intervention studies in the French population indicate that fruit and vegetable intake accounts for about 28% of daily polyphenol intake, with minimal differences in gender [22].

Major sources of polyphenols include beverages such as fruit juice, red wine, coffee, tea, and cocoa. Other sources also include vegetables, dry legumes, and cereals [2]. A glass of red wine or a cup of tea or coffee reportedly contain about 100 mg polyphenols [23], which makes an important contribution to the total intake. Chocolate has been found to be a very rich source of polyphenols [24]. The most abundant flavonoids present in the diet are flavanols, anthocyanins, and their oxidation products. Phenolic acids account for one-third of the total intake, followed by the flavonoids, which account for the remaining two-thirds [2]. Common dietary polyphenols consumed frequently in food are provided in Fig. 25.2.

3 ABSORPTION, METABOLISM, AND BIOAVAILABILITY OF POLYPHENOLS

The absorption of different classes of polyphenols results in substantial variations [22]. The dietary polyphenol complexes have limited bioavailability with low circulating levels in plasma [25]. Different classes of polyphenols follow specific kinetics for absorption; isoflavones and phenol acids such as caffeine acid and gallic acid are the most absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides [26]. The least absorbed polyphenols are proanthocyanidins, the galloylated tea catechins, and the anthocyanins [27]; this is due to the large molecular weight of the phenolic molecules.

The absorption of polyphenols in the small intestine is low, resulting around 10%–20% [28]. Aglyconic forms of dietary polyphenols can be absorbed from the small intestine; however, the presence of most polyphenols is in the form of esters, glycosides, or polymers and cannot be absorbed directly. Hence, they must be hydrolyzed by intestinal enzymes or by the gut microbiota. A notable

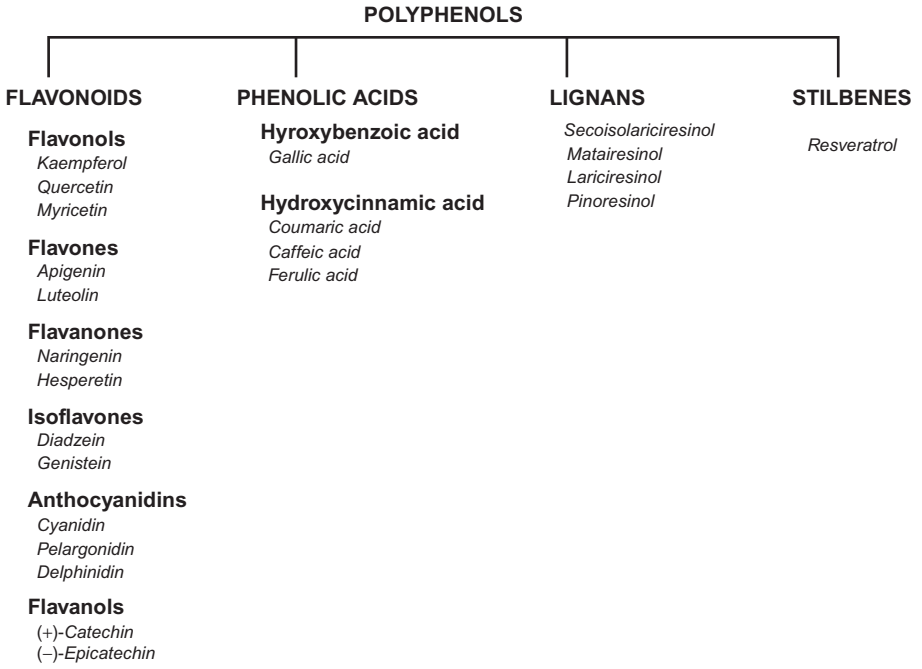

FIG. 25.2 Most common polyphenols consumed in food.

example includes the conversion of quercetin-3-*O*-rhamnoglucoside and quercetin-3-*O*-rhamnoside. Hydrolyzation of these metabolites by endogenous human enzymes may not result in the desired absorbable product; however, this can be achieved through hydrolyzation by gut microflora to the readily available from quercetin. The gut microflora are equipped with organisms such as *Bacteroides distasonis* (a-rhamnosidase and b-glucosidase), *B. uniformis* (b-glucosidase), and *B. ovatus* (b-glucosidase) [2,29].

As the small intestine provides poor absorbability, the infested polyphenols, entering the large intestine, will encounter colonic microflora. Furthermore, the amount of the polyphenol ingested can determine the primary site of metabolism of (1)-catechin. Large doses of phenolic substances are metabolized in the liver whereas small dosed are catalysed by the intestinal mucosa, with the liver playing a secondary role to further modify the polyphenol conjugates from the small intestine [2]. The poor bioavailability of some polyphenols has led to poor clinical trial outcomes, attributed to their intake and their bioavailability. The conversion into phytosomes has improved efficacy without compromising safety. The phytosome technology leads to the creation of intermolecular bonding between individual polyphenol molecules and one or more molecules of the phospholipid, phosphatidylcholine [30].

4 METABOLISM OF POLYPHENOLS BY THE GUT MICROBIOTA

The colon contains various microbial populations of obligate and facultative anaerobes, which aid in the degradation of undigested food [31] in the human GI tract, from stomach to jejunum, ileum and colon in an increasing gradient [32]. An estimate of 1000–1200 species [33] has been reported to reside in a healthy adult gut ecosystem. About 90%–99% of these microorganisms belong to the bacterial phyla Firmicutes, (most dominant species) and Bacteroidetes [33]. Other species of microorganisms include Actinobacteria [34], Cyanobacteria, Fusobacteria, Lentisphaerae, Proteobacteria, Spyrochaetes and Verrucomicrobia [35–40]. The intestinal microbiota is heterogeneous [41]. The mammalian gut tract shows continuous growth in the number of bacterial cells; in the stomach and duodenum about 101–103 bacteria/g are reported and progressing to 104–107 bacteria/g in the jejunum and ileum, culminating in 1011–1012 cells per gram in the colon [42].

Furthermore, a variation in the microbial profile is noticed between the sample sites; the small intestine is observed to be enriched in the Bacilli class of Firmicutes and Actinobacteria, whereas in the colon Bacteroidetes and the Lachnospiraceae family of Firmicutes are more prevalent [38]. The total number of these microorganisms is denoted to be around 10^{12} microorganisms/cm^3, and this microbiota holds increased catalytic and hydrolytic potential [2]. The degradation of flavonoids using enzymes made by the colonic microflora results in a huge array of new metabolites. Furthermore, deconjugation of the polyphenols occurs inside the colon. A major region of the polyphenols persists in the colon, where the microbiota produce metabolites that undergo further metabolism upon entering systemic circulation [25]. The absorption of these polyphenols in the small intestine is in reduced levels. Therefore, it is most likely that the ingested polyphenols will be transported to the large intestine and interact with the colonic microflora [43]. As the fecal excretion of polyphenols is relatively low, a major sector of the ingested polyphenols are transformed or degraded in the gut [44]. In general, the microbiota can perform deglycosylation, dehydroxylation, and demethylation reactions and metabolize polyphenols into small fragments [25] (Fig. 25.3).

Specific classes of polyphenols, such as flavonols, isoflavones, flavones, and anthocyanins, are usually glycosylated with glucose or rhamnose [2]. Therefore, the primary step in degradation of polyphenols involves the release of aglycones and oligomers by the microbial glycosidases and esterases, which aids in the enhancement of their absorption [44]. The formed aglycones are hydrolyzed into various aromatic acids by the microflora. These structures are cleaved by the opening of the heterocycle at various points and at regions where flavonols produce hydroxy phenylacetic acids [8]. Glycosidase activities may occur in food or in the cells present in the gastrointestinal mucosa. They can also be released by the colon microflora [2]. The microbial enzymes, glucuronidases and sulfatases, deconstruct phase II metabolites extracted via the bile, and this enables their reuptake [4]. Conjugation of phenolic hydroxyl groups by sulfate can occur through arylsulfotransferase originated from human intestinal bacteria [45]. Several microbial transformations of the polyphenolic core result in various metabolites that may differ in biological activities [44].

A relatively small number of metabolites are formed in the colon when a varied group of natural polyphenols are considered. Polyphenolic microbial metabolites can be identified from in vivo body fluids or from in vitro fecal incubations. A good relationship is found between the metabolites of flavonoids detected from human body fluids and with those detected from microbial incubations in vitro [45].

The consumption of a variety of phenolics results in the observation of specific metabolites in biological fluids or tissues. The primary derivation of glycine conjugate of benzoic acid, hippuric acid, is from plant phenolics and aromatic amino acids through the mechanisms of intestinal bacteria. The specific level of hippuric acid in the urine is expected to be at increased levels for the individuals

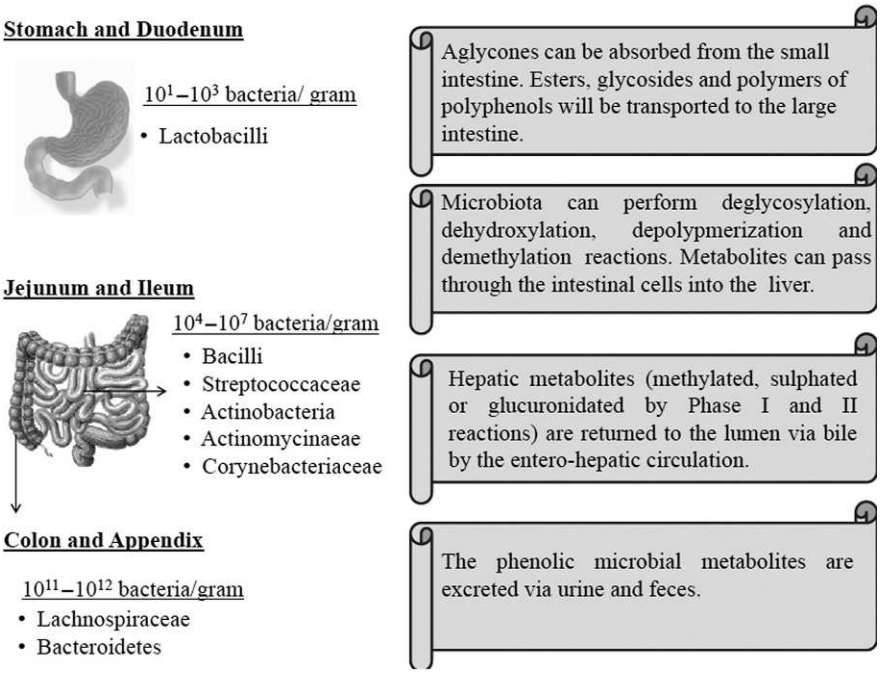

FIG. 25.3 Microbial metabolism of phenolics in the gastrointestinal system. Microbiota is indicated as bacteria/gram of contents.

who consume rich polyphenolic diets [43]. According to Rastmanesh [46], *Firmicutes* possess a disproportionate number of glycan-degrading enzymes when compared to *Bacteroidetes* [47]. It can be hypothesized that intake of different polyphenols might reconstruct the gut microbiota constituents differently. In order to support this hypothesis, a recent study by Manichanh et al. [48] indicated that indigenous gut microbial composition is more plastic than previously anticipated. In animal models such as rat, the transplantation of bacteria has produced a significant increase in the microbial diversity of the recipients. However, the performed transplantation following antibiotic intake resulted in the combined reshaping effects of the individual treatments (including reduced diversity from antibiotic treatment alone). Studies showed that reducing the recipient bacterial load by antibiotic intake prior to transplantation did not increase the donor phylotypes, even when some dominant lineages were transferred successfully. The effects were observed following 1 month of treatment and persisted after 3 months [48].

4.1 The Microbial Metabolism of Flavonoids

The colonic bioconversion of polyphenols is the most studied for flavonoids. It is highly variable because of the differences in bioconversion of flavonoids and substitution patterns, including the dietary aspect of ingested polyphenols, which can moderate polyphenol microbiota interaction [25,49,50]. Several flavonoids undergo a ring-fission process, which results in the degradation of the C-ring; hydroxylated aromatic compounds result from the A-ring and phenolic acids result from the B-ring. The modified structures are achieved through cleavages of functional groups (dehydroxylation, demethylation, and decarboxylation) [46]. Entero-hepatic circulation and colonic metabolism are significant in the metabolism of flavonoids. Flavanols, flavanones, and flavones are all cleaved on the C-ring and are converted to phenylpropionic acid derivatives [45].

Flavones and flavanones result in the formation of hydroxyphenylpropionic acids; flavanols mainly produce phenylvalerolactones and hydroxyphenylpropionic acids [8] and phenylpropionic acids may undergo further metabolism to benzoic acids [43]. The conjugates produced are 3,4-dihydrophenylacetic acid, 3-hydroxyphenylacetic acid, and homovanillic acid by major enzymes of the microflora in the large intestine [43]. The primary metabolite of quercetin is 2-(3,4-dihydroxyphenyl) acetic acid, and that of myricetin is 2-(3,5-dihydroxyphenyl) acetic acid [45]. Furthermore, reactions such as dehydroxylation and methylation occur in the liver following the microbial metabolisms. Hydroxyphenylacetic acids and hydroxyphenylpropionic acids are further metabolized to derivatives of benzoic acid [8].

Microorganisms such as the bacteria within the human microbiota, with the capacity to catabolize flavan-3-ols, have not being completely explored due to the growth-inhibitory effects of proanthocyanidins and the structural features of flavan-3-ols as complex nonplanar molecules [51].

The bacteria *Enterococcus casseliflavus* uses the sugar moiety of quercetin-3-*O*-glucoside to provide formate, acetate, and lactate. However, it does not involve in

further metabolization of the aglycone. Furthermore, *Eubacterium ramulus* present in the human colon involves the transformation of Quercetin-3-*O*-glucoside to 3,4-dihydroxyphenylacetic acid, acetate and butyrate [2].

Trimers of flavanol are processed to phenylpropionic acids by ring-fission and benzoic acid by b-oxidation with the mechanisms of the human microflora [45]. About 11 metabolites were identified in human urine following the intake of (+)-catechin, the major metabolites being 3-(3-hydroxyphenyl) propionic acid and 5-(3′-hydroxyphenyl)-γ-valerolactone-3′-O-β-D-glucuronide [52].

Urinary excretion of microbe-derived phenolic acid after high intake of flavan-3-ols rich foods has shown significant changes. Several nutritional and feeding studies have demonstrated that mono- and di-hydroxylated phenylpropionic and phenylacetic acids, in combination with hydroxyhippuric acids, were major urinary microbial phenolic acids obtained from flavan-3-ol intake [51]. Bacteria with the exclusive capacity to catabolize other types of flavonoid compounds, which mainly includes flavonols and flavones, have been described. These bacteria in general belong to the Clostridium and Eubacterium groups [16]. The biological properties of microbial-derived metabolites of flavan-3-ols are still ambigious [51].

When a condensed form of catechins enters the colon, they are transformed to phenolic acids only to a reduced extent. Condensed catechins such as proanthocyanidins (polymeric flavan-3-ols) are degraded by human colonic microbiota, resulting in the formation of several derivatives of phenylvaleric, phenylpropionic, phenylacetic, and benzoic acids. These are formed through various patterns of hydroxylation, and the total yield is dependent significantly on the degree of polymerization [45,53]. Degradation of flavanonnaringenin and other polyphenols by *E. ramulus* involves the enzymes calchone isomerase [54] and phloretin hydrolase [55]. Furthermore, dehydroxylation and demethylation of both intermediates may occur at various points [44].

Minimal amounts of the isoflavones genistein and daidzein are excreted via urine and observed in vitro anaerobic incubation with human feces. Genistein is reduced to dihydrogenistein and further to 60-hydroxy-*O*-demethylangolensin [56]. Analysis of biological samples after soy consumption showed the presence of metabolites such as 30,40,7-trihydroxyisoflavanone, 40,7,8-trihydroxyisoflavanone, and 40,6,7- trihydroxyisoflavanone [45]. The fate of metabolites of colonic microflora is renal excretion; however, the level to which these compounds absorb into cells and tissues is unknown [38]. For example, bacteria strains of *Streptococcus intermedius* ssp., *Ruminococcus productus* spp., and *Bacteroides ovatus* spp. convert daidzein to equol in in vitro conditions [8]. Furthermore, cyanidin is converted to protocatechuic acid (3,4-dihydroxybenzoic acid), pelargonidin to 4-hydroxybenzoic acid, peonidin to vanillic acid (3-methoxy-4-hydroxybenzoic acid), and malvidin to syringic acid (3,4-dimethoxybenzoic acid), and by the microflora [45,57]. Microbial and spontaneous decomposition of anthocyanins occurs in the intestine, which results in formation of degraded products [45]. Studies investigating the metabolism of colonic microbiota on anthocyanins are scarce [16]. The detected product of colonic microbiota is protocatechuic acid (3,4-dihydroxybenzoic acid) [58]. Other animal research using pig gut microbiota in an in vitro study includes metabolites such as phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde), phloroglucinol acid (2,4,6-trihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), and 3-O-methylgallic acid, this dependent on their chemical structure of anthocyanins [57]. There is a need for metabolite study as, although anthocyanins are partly fragmented to phenolic acids, a large portion of metabolites still remain unclear [16].

4.2 The Microbial Metabolism of Phenolic Acids

Phenolic acids are structured during ring-fission reactions of flavonoids and may be released from the food matrix. Intestinal esterases of the intestinal tissues and of the microbiota play a significant role in the uptake of esterified acid [45]. Hydroxycinnamic acids are structures bound to plant cell walls that require the release by enzymes such as esterases of the colonic microflora. The colonic microflora is the major site for chlorogenic acid metabolism [2].

Several studies have reported that when caffeic acid and its esters (chlorogenic acid and caftaric acid), are incubated with human fecal microbiota, deesterification reactions occur rapidly and the by-product formed is 3-(3-hydroxyphenyl) propionic acid. The microbial transformations of chlorogenic and ferulic acids to 3-(3-hydroxyphenyl) propionic acid are also noted [45]. Degradation of procyanidin polymers into low molecular-weight phenolic acids was also observed by a human colonic microflora grown in vitro and anaerobically [59]. Clinical trial studies show that, following a chlorogenic acid supplementation, several hydroxylated cinnamic acids, benzoic acid, and hippuric acids are present in the urine and plasma [45]. The daily consumption of green and black tea was observed to increase the excretion of hippuric acid in human urine [60]. A study conducted by Plumb suggested that the cleaving of chlorogenic acid into caþeic acid and quinic acid in the body is processed by an esterase of the colonic microflora. Following the hydrolysis of chlorogenic acid, the caþeic acid may undergo several possible chemical and structural transformations by the gut microflora [61].

Metabolites such as ellagitannins are large molecules with limited bioavailability. The hydrolysis to ellagic acid results in conversion to two lactones, urolithins A and B, 3,8-dihydroxy-6Hdibenzopyran-6-one and its monohydroxylated analog [62].

4.3 The Microbial Metabolism of Lignans

Lignans are ubiquitous in the human diet and their most important dietary sources are whole-grain cereals and legumes. They can also be found in various fruits and vegetables [63], the highest concentration being found in flaxseed. Most common lignans consumed with the diet are secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol [64]. They are metabolized into the mammalian lignans enterodiol and enterolactone by human gut microbiota [65]. Due to their estrogen agonist and antagonist properties, lignans are recognized as phytoestrogens [63,66].

4.4 The Microbial Metabolism of Stilbenes

Stilbenes are generally found only in low quantities in our diet. Resveratrol, a well-known molecule of this group, is found in small quantities in wine, grapes, peanuts, and cranberries. It has been reported to have anticarcinogenic, antioxidative, phytoestrogenic, and cardioprotective activities [67]. In a study carried out with cell models, it was reported that trans-resveratrol-3-*O*-glucuronide and trans-resveratrol-3-sulfate are the most abundant metabolites of resveratrol.

The in vitro conversion products procured from the metabolism of trans-piceid by intestinal microflora, a major stilbenoid compound in red wine, were studied and identified in rats. After incubating with gut microbiota, three metabolites, namely resveratrol, dihydropiceid, and dihydroresveratrol, were detected [68].

5 ENZYMES IN THE METABOLISM OF POLYPHENOLS

Microbial metabolites absorbed from the colon after conversion are also subjected to metabolism by the enzymes of the gastrointestinal system and the liver. During and after uptake through the intestinal epithelium, the majority of these microbial metabolites undergo phase I/8ik,II metabolism and are detected as such in urine, serum, and biopsies [69]. They can be hydrolyzed by cytosolic b-glucosidase (EC 3.2.1.1), found in many tissues like the intestine and liver, or by the enzyme lactase phlorizin hydrolase (EC 3.2.1.108), which is found only in the small intestine and has been suggested recently [2].

Polyphenols can be conjugated postdeconjugation as well. Polymorphic catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) enzyme, which methylates polyphenols at the 39 position, occurs in a wide range of tissues [2]. Cytochrome P450 demethylates flavonols specifically at the 49 position [70]. UDP glucuronosyl transferase (EC 2.4.1.17) catalyzes the conjugation of polyphenols to glucuronic acid. It is situated in the endoplasmic reticulum and exists as a large family of related enzymes. UDP glucuronosyl transferase, which shows polymorphism and glucuronidation of polyphenols, which is predominantly by the UGT1A family where the liver has the greatest capacity for glucuronidation [2].

Phenol sulfotransferases (EC 2.8.2.1) are a small but widely distributed group of cytosolic enzymes. Some of them have a high activity on catechol groups of polyphenols, while others are inhibited by polyphenols [2]. In addition, some glutathione transferases, cytochrome P450s, and epoxide hydrolases show genetic polymorphisms and are generally thought to play a minor role in polyphenol metabolism [71].

6 MODULATION OF GUT MICROBIOTA BY POLYPHENOLS

There are only a few studies investigating the impact of polyphenols on the composition and activity of the gut microbial community. In the last few years, it has been noted that some polyphenols in certain concentrations may be involved in influencing gut microbial populations. The growth of some of these bacterial groups can be inhibited, whereas others can be activated [44]. Particularly, flavonoids entering the large intestine and their colonic catabolites may influence the microflora by selecting probiotic bacteria or inhibiting the proliferation of cancerous cells [72]. It is reported that consumption of flavanol-rich foods might exert probiotic actions with positive health effects [73]. For instance, (+)-catechin was shown to inhibit growth of *Clostridium histolyticum* significantly, whereas it enhanced the growth of members of the *Clostridium coccoides-Eubacterium rectale* group and *Escherichia coli* significantly, while growth of *Bifidobacterium* and *Lactobacillus spp*. remained relatively unaffected [73].

In a study with rats, there was a significant decrease in *Clostridium leptum* clusters while the Bacteroides group increased significantly when given a polyphenol-rich diet [74]. In another rat study carried out with red wine polyphenols, it was reported that they had significantly decreased the levels of Clostridium spp. and increased the levels of Lactobacillus spp. [75]. Massot-Cladera et al. reported that a cocoa-enriched diet significantly decreased the proportion of *Bacteroides, Clostridium* and *Staphylococcus* genera in the feces of cocoa-fed rats. This

was associated with changes in the TLR pattern, which could be responsible for the changes observed in the intestinal immune system [76].

Polyphenols also have various bioactivities, the most pronounced one being antioxidant activity. They exhibit notable antibacterial and antifungal activities, as shown in many studies in recent years. Different mechanisms of action have also been proposed for the antibacterial actions of polyphenols [44]. In a recent study that investigated the effect of dietary polyphenols on the human gut microbiota, the effects of a moderate intake of red wine implicated that the daily consumption of polyphenols significantly caused an increase in the populations of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and *Blautia coccoides-Eubacterium rectale* groups in male volunteers. This showed that red wine consumption can significantly modulate the growth of select gut microbiota in humans, suggesting possible probiotic benefits [77].

Gut bacteria are known to act on polyphenols and produce metabolites with different physiological significance [78]. There is also evidence that polyphenols, in turn, may influence colonic microflora [79]. Dietary phenolic compounds are also often transformed by gut microbiota in a two-way phenolic-microbiota interaction [16]. Comparative analysis of five previously sequenced human gut *Bacteroidetes* demonstrated that each genome contains a large repertoire of genes involved in acquisition and metabolism of polysaccharides. This repertoire includes (a) up to hundreds of glycoside hydrolases and polysaccharide lyases; (b) myriad paralogs of SusC and SusD, which are outer membrane proteins involved in recognition and import of specific carbohydrate structures [80]; and (c) a large array of environmental sensors and regulators [81]. These genes are assembled in similarly organized, selectively regulated polysaccharide utilization loci PULs that encode the functions necessary to detect, bind, degrade, and import carbohydrate species encountered in the gut habitat, either from the diet or from host glycans associated with mucus on the surfaces of epithelial cells [47,82–84]. Metabolism of polyphenols by microbiota involves the cleavage of glycosidic linkages and breakdown of the polyphenols heterocycle [85]. Glycans, which are the products of glycosidic cleavage, are required for the establishment and survival of these organisms in the colonic ecosystem and also provide immunomodulatory properties to the host. They are necessary for the survival of the microbiota as a nutrient foundation [47]. An important point here is that the comparison of *Eubacterium rectale* and *E. eligens* (which belong to *Clostridium* Cluster XIVa, one of the most common gut *Firmicute* clades) and 25 other gut *Firmicutes* and *Bacteroidetes* indicate that the *Firmicute*s possess smaller genomes and a disproportionately smaller number of glycan-degrading enzymes [47]. As a metabolic organ, the cellular composition of the human gut microbiota is determined by a dynamic process of selection and competition. Prevalence of the *Bacteroidetes* community following regular wine or vinegar ingestion or through consuming polyphenol-rich fruits and green tea may be a mechanism by which polyphenols exert their weight lowering effect due to the presence of more glycan-degrading enzymes. Mounting direct and indirect evidence suggests that polyphenol/phenolics intake support weight loss [86–95]. Polyphenols may modulate microbiota balance through their growth-promoting effects on *Bacteroides*, while phenolic compounds may exert their effect through their growth-suppressing effects on *Firmicutes*. Phenolic compounds alter gut microbiota, and consequently alter the *Bacteroides*/*Firmicutes* balance [96–98]. For example, tea phenolics and their aromatic metabolites significantly repress the growth of certain pathogenic bacteria such as *Clostridium perfringens*, *Clostridium difficile*, and *Bacteroides* by different degrees, which was in favor of the *Bacteroides* community [97,99].

7 HEALTH BENEFITS OF POLYPHENOLS AND THEIR MICROBIAL METABOLITES

The health benefits of polyphenols are largely dependent on their intake profiles and bioavailability, which varies. It has been suggested that, although present abundantly in the diet, proanthocyanidins are poorly absorbed or not absorbed at all, thus limiting their actions to the intestine. For anthocyanins, however, monomeric flavonol, flavone, and flavanol intakes are relatively low, and plasma concentrations rarely exceed 1 μmol/L^2 where flavanones and isoflavones are the flavonoids that may reach 5 μmol/L^2 of plasma concentration due to their better bioavailability profiles. Therefore, when they are subjected to research, it is important to consider the real physiological concentrations of polyphenols. To date, a number of studies have indicated that, when consumed in certain amounts, polyphenols have important benefits for human health because of their bioactivities, such as antioxidant [100], chemopreventive [101], antihypertensive properties, and so on [24,102]. The metabolite concentrations of polyphenols are anticipated to be low in the circulation, but are reportedly locally high enough to affect signal pathways controlling cell division and programmed cell death (apoptosis), which is disturbed in cancer [45].

Polyphenols are widely known to act as antioxidants and have been suggested by Halliwell et al. [103], to have protective effects along the digestive tract from mouth to colon. Regulating redox balance by nutraceuticals that are rich in polyphenols is an important strategy for better health [104].

The dietary antioxidants resveratrol, genistein, and S-equol, an isoflavone produced from the gut biotransformation of soy daidzein, have been observed to be effective in reducing skin aging. The primary mechanism of action of these antioxidants was found to activate estrogen receptor β, which in turn enhanced the expression of antioxidant enzymes and inhibited the expression of the transcription factor that regulated keratinocyte cell proliferation and migration, rather than the widely accepted free radical scavenging activities [105]. Interestingly, our ongoing in-house study of a polyphenol mixture has proved to exert stronger regulatory action on nuclear receptor factor in subjects with impending metabolic syndrome, as compared to an omega 3-rich sturgeon extract (Caviarlieri, Swisscap, Switzerland) which in our prior work had shown some promising data [106]. In retrospective, upon analyzing the stored fecal DNA sequencing (NextGenomics, Prato, Italy), we found out that while this sturgeon compound was not affecting the microbiota, our polyphenol compound (in progress) could significantly modify gut microbiota gene sequencing. Ongoing work is now confirming that such changes are also affecting inflammatory cytokines (San Babila Clinic protocol no. 21/2017).

In order to determine the role of ellagitannins and ellagic acid as dietary pro-phytoestrogens, colonic microflora metabolites Urolithins A and B and other phytoestrogens (genistein, daidzein, resveratrol, and enterolactone) were assayed to evaluate their capacity to induce cell proliferation on the estrogen-sensitive human breast cancer MCF-7 cells as well as the ability to bind to alpha and beta estrogen receptors. Both urolithins A and B showed estrogenic activity in a dose-dependent manner, without any antiproliferative or toxic effects [66].

The intestinal microbiome is closely involved in the host's health through various mechanisms role [107]. Besides synthesis of essential vitamins K and B [108] in the gastrointestinal tract, it is immensely important in stimulating the immune system [108] as well as nervous systems [109]. Ellagic acid is suggested to induce apoptosis in colorectal adenocarcinoma cell lines [110], suggesting that phenolic acid metabolites exert local effects on epithelium of the colon. Polyphenols and their metabolites can modulate the activity of a wide range of enzymes and cell receptors [111]. It was suggested that phenolic acid metabolites inhibited cyclooxygenase-2 (COX-2) enzyme and affected COX-2 protein levels in colonic HT-20 cell line, where COX-2 plays a major role in the regulation of inflammation [112]. Isoflavone intake and equal production are associated positively with bone health and prevention of osteoporosis. Colonic microflora metabolites of the polyphenols may even have more activity than the original molecules. For instance, equol produced from soya daidzein appears to have phytoestrogenic properties even greater than those of the original isoflavone [113]. There is an emerging consensus that the gut microbiome plays a crucial role in the potential health benefits of polyphenols [114]. Some microbial metabolites such as hydroxyphenylacetic acids have been suggested to inhibit platelet aggregation [115]. The health benefits of flavonoids are due to their potential ability to act as free radical scavengers and provide antioxidant protection [116]. However; it has been seen that interactions with membrane lipids and proteins also display antioxidant effects. For instance, larger and nonabsorbable procyanidins regulate cell signaling through cell membrane proteins and lipid interaction, and they can induce changes in the membrane biophysics by modulating oxidant production [116]. Polyphenols and their metabolites can even interact with the enzymes, transcription factors and call receptors. Therefore, their internalization is not necessary to observe a biological activity [1]. Human intestinal bacteria metabolites of rutin and quercetin, 3,4-dihydroxyphenylacetic acid and 4-hydroxyl-phenylacetic acid have been shown to possess a more effective antiplatelet aggregation activity than rutin and quercetin [115]. Furthermore, 2,4,6-trihydroxybenzaldehyde and quercetin were more cytotoxic against tumor cell lines than rutin [38].

Recently, polyphenols have also found their application in the food industry as weight control food components due to their various biological activities. A high polyphenol and selectively restricted probiotic diet (HPLP diet) has also been proposed as a regimen to maintain normal body weight [46]. The main characteristics of Rastmanesh's HPLP diet is to include large amounts of fruits, vegetables, and polyphenol-rich herbs such as green tea, oriyganum vulgare, and thymus vulgaris and ziziphora tenuior L.

8 CONCLUSIONS

In recent years there has been a growing interest in the microbial metabolites of polyphenols and their biological activities. However it is a difficult area to study because of all the variations in the structure of polyphenols, human gut microbial diversity, and the individual differences in metabolism related to enzyme polymorphisms, differences in food constituents, and polyphenol consumption patterns. The composition of one's diet is also believed to exert strong environmental pressure that can influence the gut microenvironment. New techniques are being developed for studying the impact of polyphenols on human gut microbiota. Epigenotypic or changes in microbiome composition (phylotypic) studies are applied extensively. Emerging evidence suggests that studying the diet modifiable gut microbiome is a promising area of exploration for chronic disease control and prevention. Functional microbiome approaches for

investigation of the effects of polyphenols on the human gut microbiota, including metabolic analyses, metatranscriptomics, and shotgun meta-proteomics (Kemperman [44]), are important methodologies that could reveal the relationship between polyphenols and the gut microbiome. Knowledge of the composition of the intestinal and colonic microbiota would be important for our understanding of how the balance of these microbes is possibly influenced by polyphenol/phenolics profiles and the environment. Different two-way polyphenol profile-microbiota interactions would be thus inevitably possible due to (i) different bacterial diversity along the intestinal tract, (ii) different amounts of polyphenols/phenolics metabolized and/or entered into the small intestine, and (iii) compound degrading capabilities of different microbiotas on polyphenol/polyphenolics.

A competition exists between the inhibitory effect of polyphenols/phenolics on microbial activity and the ability to convert them by the microbiota [117]. Generally flavonols, isoflavones, and glycosides were found to exhibit low antibacterial activity, while phenolic acids were intermediate. The flavanone and flavanol tested also displayed high antibacterial activity. There proved to be no noticeable relationship between structure and activity, apart from glycosylation correlating with low antibacterial activity. This correlation is probably due to limited significance in vivo, because the human intestinal microflora has ample ability to deglycosylate flavonoid glycosides, thereby activating them [115].

Further research into the interaction between phenolic compounds and other intestinal bacteria, as well as their health consequences, is required. We suggest future studies to utilize clone libraries techniques, which can help enable identification of the composition of the microbiota. The microbial community structure can be analyzed via fingerprinting techniques and dot blot hybridization, or fluorescent in situ hybridization for abundance of particular taxa in management of obesity in the future. Indeed, in vitro studies may have contributed to recent disputes on the casual relation between *Lactobacillus* sp. and obesity [118,119].

This review was composed so as to draw attention to the importance that polyphenol metabolism has in the gut and its impact on human health. Integrative approaches and metabolomic studies have proved to be promising in potentially diagnosing infection, particularly gastrointestinal diseases [120]. There remains a lack of evidence on specific metabolites and their corresponding biological activities, which demands further research. New techniques combined with in vitro and in vivo studies will help reveal more about this field and fill the gap between polyphenol metabolites and their biological activities related to human health.

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Conflicts of Interests

No potential conflicts of interest were disclosed.

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CHAPTER

26

Bioavailability and Biochemistry of Quercetin and Applications to Health and Diseases

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Acronyms and Abbreviations

ALA	α-linolenic acid
AUC	area under the curve
AUCi.v.	area under the curve of an intravenous administered substance
AUCoral	area under the curve of an ingested substance
BCRP1	breast cancer resistance protein 1
BH3	Bcl-2 homology domain 3
CHNQu	chloronaphtoquinoneQu
C_{max}	maximum concentration
COMT	catechol-*O*-methyltransferase
COX-2	cyclooxygenase-2
CRP	C-reactive protein
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
HDL	high-density lipoprotein
HO-1	heme oxygenase 1
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
LPH	lactase phloridzin hydrolase
LPS	lipopolysaccharide
MAFQu	monoacetylferuloyl-Qu
MDCK cells	Madin-Darby canin kidney cells
MDR	multidrug resistance
miR-155	microRNA-155
MMP-2	matrix metalloproteinases-2
MRP2	multidrug resistance associated-protein 2
NF-kB	nuclear factor-k-gene binding
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
$NO^{\bullet}$	nitric oxide radical
$O_2^{\bullet -}$	superoxide radical
OATP	organic anion transporting polypeptide
$^{\bullet}OH$	hydroxyl radical
$ONOO^-$	peroxynitrite
PEG 200	poly(ethylene glycol) 200
Pgp	P-glycoprotein
POM	pivaloxymethyl
Qu	quercetin
ROS	reactive oxygen species
SAR	structure-activity relationship
SGLUT1	sodium-glucose transporter 1
SULT	sulfotransferase enzyme
TNF-α	tumor necrosis factor-α
UGT	5′-diphospho-glucuronosyltransferases

1 CHEMICAL FEATURES OF QUERCETIN

Quercetin (Qu) is one of the most abundant dietary polyphenols, belonging to the widely heterogeneous class of flavonoids. In particular, it is classified as a flavonol and it is ubiquitously present in our diet, mostly in vegetables, fruits, and beverages such as red wine and tea [1]. From a chemical point of view, Qu is characterized by the 2-phenylchromen-4-one having a 3,5,7,3′,4′-pentahydroxy group (Fig. 26.1). The chemical stability of Qu depends on oxygen concentration, pH value, temperature, concentration of other antioxidants, as well as the presence of metal ions [2]. Qu undergoes many chemical changes, in particular oxidation [3].

Qu possesses the ideal structural chemistry for free radical-scavenging activities, and it has long been shown to have potent antioxidant properties in vitro [4]. In general, the antioxidant activity of flavonoids is highly correlated to the number of the hydroxyl groups (OH) [5], and in particular to their position in the flavonoid skeleton [6]. Many studies and efforts have attempted to establish a structure-activity relationship (SAR) for the antioxidant effects of Qu. The structural arrangements responsible for the greatest antioxidant activity are: (1) the *ortho* 3′,4′-dihydroxy moiety in the **B** ring (catechol); (2) the 2,3-double bond in combination with both the 4-keto group and the 3-OH in the **C** ring, for electron delocalization, as long as the catechol is also present; (3) the *meta* 5,7-dihydroxy moiety in the **A** ring. The absence of the catechol moiety as well as substitutions by methoxy groups significantly affect the antioxidant potency of Qu [7]. Among flavonoids, Qu is the most potent scavenger for

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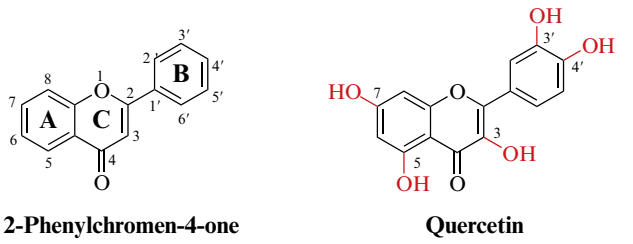

FIG. 26.1 Chemical structures of 2-phenylchromen-4-one and quercetin. The basic structure consists of the fused aromatic **A** ring with the heterocyclic **C** ring, and the hanging phenyl **B** ring attached through its C1′- position to the C2-position of the **C** ring (numbered from the pyran oxygen). According to the oxidation level of the central **C** ring as well as to variation in the number and arrangement of the hydroxyl groups and the extent of glycosylation of these groups, flavonoids are classified into various subclasses.

reactive oxygen species (ROS), including hydroxyl ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO\cdot$) radicals and peroxynitrite ($ONOO^-$) [8–10]. In particular, the catechol moiety remains important for the peroxynitrite-scavenging activity of Qu, while the free 3-OH in the **C** ring is crucial [11]. It represents the reactive center, positively influenced by electron-donating groups at the C5- and/or C7-position. Its reactivity is further enhanced by the 4-keto group and the heterocyclic oxygen at position C1 [12]. Furthermore, Qu has a propensity for metal chelation; in particular, it can react with metal ions such as iron, forming Qu-metal complexes. This property, attributed to the OH groups, first 3-OH followed by 5-OH and catechol, supports the antioxidant role of Qu in terms of inhibiting transition metal-catalyzed free radical formation [13].

In addition to the oxidation process, ROS are also involved in an inflammatory response by activation of transfer factors such as nuclear factor-k-gene binding (NF-kB), which in turn induces the production of tumor necrosis factor-α (TNF-α) cytokine. The interaction of natural antioxidants, especially Qu, with ROS implicated in inflammation has prompted several studies on its antiinflammatory effects in vitro [14]. Qu also exhibits inhibitory effects on cyclooxygenase-2 (COX-2), enzyme with a key role in inflammation and its associated diseases, by catalyzing the synthesis of prostaglandin E2. The inhibition depends on both number and position of OH groups. Again, the free 3-OH is decisive for its antiinflammatory property, since substitutions on the C3-position reduce the inhibitory effect of Qu on COX-2 [15]. The presence of both 5-OH and 7-OH is correlated with higher inhibition of COX-2 [16]. The SAR for the antioxidant and antiinflammatory properties of Qu is shown in Fig. 26.2.

In the past two decades, there have been several studies on the different bioactivities of Qu which imply that Qu, present in food products, has a protective effect against various diseases. Nonetheless, the real mechanisms and actual active molecules involved in these bioactivities are not completely clear yet. The mechanisms observed in in vitro studies cannot be easily translated into an explanation of the effects observed in vivo. In fact, in order to understand the bioactivities of dietary Qu, it is important to take into account that most of the in vitro studies focus on Qu as aglycone, while it is commonly found in nature as glycosides. Furthermore, it is important to explore its fate in the organism since Qu is extensively modified by enzymes in the gut and liver once consumed.

2 BIOAVAILABILITY, ABSORPTION AND METABOLISM OF QUERCETIN

The earliest research on dietary Qu suggested a very poor oral bioavailability, that limited the complete explanation of its effects in vivo [17]. Bioavailability is defined as the fraction of an orally administered substance that is absorbed and available for physiologic/pharmacologic activity [18]. It is classified as absolute bioavailability, which is calculated as the area under the plasma concentration-time curve of an ingested substance

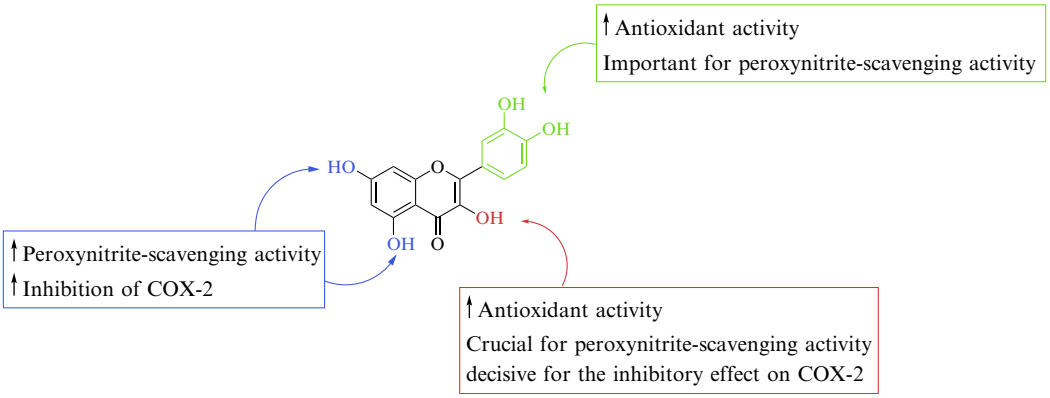

FIG. 26.2 SAR of the antioxidant and antiinflammatory properties of quercetin.

(AUCoral) relative to its intravenous administration (AUCi.v.); and relative bioavailability, which is simpler but less accurate, and is calculated as AUCoral [19].

In its glycosylated form, one or more OH groups of Qu are replaced by different types of sugar groups, which can affect the pharmacokinetic profile of Qu absorption, influencing its bioavailability [20]. The main derivatives are Qu-*O*-glycosides with at least one *O*-glycosidic bond. Frequent glycosylation sites are the 3- and 7-OH groups, and less commonly the 4-keto group. When glycosylated, Qu is conjugated with different monosaccharides, including glucose, galactose and rhamnose, and disaccharides such as rutinose [1,2,20]. The most frequent glycosides present in apple and onions are Qu-3-*O*-arabinoside, 3-*O*-galactoside, 3-*O*-glucoside, and 3-*O*-rhamnoside. The dietary source affects the bioavailability, thus it has been observed how the consumption of onion led to faster absorption, higher concentration, and greater bioavailability as compared to apple [21]. Qu, in the crystalline form, is less soluble at body temperature, limiting its bioaccessibility. Absolute bioavailability of Qu was 16% in rats and its maximum concentration (C_{max}) was 2.01 μM following administration of Qu suspended in aqueous solution. Administration of Qu aglycone dissolved in an ethanol and PEG 200 solution increased its absolute bioavailability to 27.5% and C_{max} to 3.44 μM [22]. Qu is absorbed in the gastrointestinal tract, probably by passive diffusion associated to a secondary organic anion transporting polypeptide (OATP) [23]. Glycosylated forms of Qu are not absorbed in the stomach [24]. In particular, Qu-3-*O*-glucoside can pass the enterocyte by the sodium-dependent SGLUT1 transporter in the small intestine. Then, it is attached by the lactase phloridzin hydrolase (LPH), which catalyzes the deglycosylation and enables the aglycone to pass the enterocyte via passive diffusion [25]. Other Qu glycosides may not be metabolized in the small intestine but fermented by colonic microbiota in a heterocyclic ring cleavage [26]. In general, bioavailability of Qu depends on food matrix and nature of sugar moiety [27]. In vitro studies demonstrated how the coincubation of Qu with vitamin C can limit its oxidation favoring a major bioavailability [28]. These data were also confirmed in vivo with healthy adults ingesting 1095 mg of Qu. It has been demonstrated how the plasma endotoxin (surrogate marker of intestinal permeability) was correlated to the Qu total C_{max} [29]. The in vivo bioavailability of Qu is enhanced if fed with oils [30]. The oil/water partition coefficient favors partition into oil ($\log P = 1.8$), although this value is among the lowest of those for flavonoids [31]. By using Caco-2/TC7 cell lines it has been observed how the partitioning of Qu into simple bile salt micelles was low and, for mixed micelles, it was inhibited by increasing the bile salt concentration, confirming its relatively polar nature [32]. After oral administration, in plasma, Qu is present as methyl, glucuronide, and sulfate conjugates, with very little unconjugated Qu aglycone present [33]. The average peak plasma concentration of total Qu for 10 subjects was 463 ng/mL at 3.5 h (arithmetic mean) when dosing 500 mg Qu three times daily with meals [34], while the urinary recovery of Qu from its glycoside is rather low (0.07%–1.4% of the ingested dose) [35]. In the mouth, there are salivary proteins that can interact with Qu, forming soluble Qu-protein binary aggregates [20]. In the stomach, Qu is exposed to the strong acidic condition, and may be degraded to phenolic acids (e.g., protocatechuic acid) by bacterial ring fission, leading to the breakdown of the skeleton structure of Qu [36], though phenolic acids could also be absorbed in the stomach [37]. In the liver, Qu is obviously considered a xenobiotic, so its metabolism consists of three phases: modification (phase I), conjugation (phase II), and elimination (phase III) [38]. The phase I is not reported, but probably involves the cytochrome P450. The phase II reactions are glucuronidation, sulfatation, methylation that occur in the small intestine [39]. The involved enzymes are 5′-diphospho-glucuronosyltransferases (UGT), which give back Qu-monoglucuronide and Qu-diglucuronide; [40] sulfotransferase enzyme (SULT) yields the 3′-derivative [41]. Glycoside derivatives (Qu-glucosides and Qu-galactoside) can be deglycosylated to Qu in the small intestine, which is mediated by microbiota-derived β-glucosidase [42]. The most famous metabolites are carried out by catechol-*O*-methyltransferase (COMT), which give back 3′-*O*-methylquercetin (isorhamnetin) and 4′-*O*-methylquercetin (tamarixetin) [43]. The adsorption of Qu and its conjugates takes place in the large intestine, where colonic microorganisms can dissimilate those compounds. For example, *Clostridium orbiscindens* takes the fission of the **C** ring in Qu. The metabolites formed by the colonic microorganisms are absorbed and transported via the portal vein to the liver and undergo the conjugation reactions [44]. In vitro studies also demonstrated how other microorganisms such as *Cunninghamella elegans* ATCC 9245 yields three metabolites, Qu-3-*O*-β-D-glucopyranoside, kaempferol-3-*O*-β-D-glucopyranoside, and isorhamnetin-3-*O*-β-D-glucopyranoside, involving both dehydroxylation and *O*-methylation reactions [45]. Qu and Qu derivatives, in the liver, might also take part in further methylation reactions [46]. Phase III takes place in the small intestine by efflux of metabolites from the liver, mediated through breast cancer resistance protein 1 (BCRP1) and multidrug resistance associated-protein 2 (MRP2) [47]. Metabolites are now able to undergo conjugation reactions that may occur to favor excretion; in particular, glucuronides are important conjugated derivatives. In fact, although the C7 position may be not the most efficient conjugation site, most flavonoids with a 7-OH group are UGT1A3 and UGT1A9 substrates. In this context, Qu is better conjugated by UGT1A9 [48].

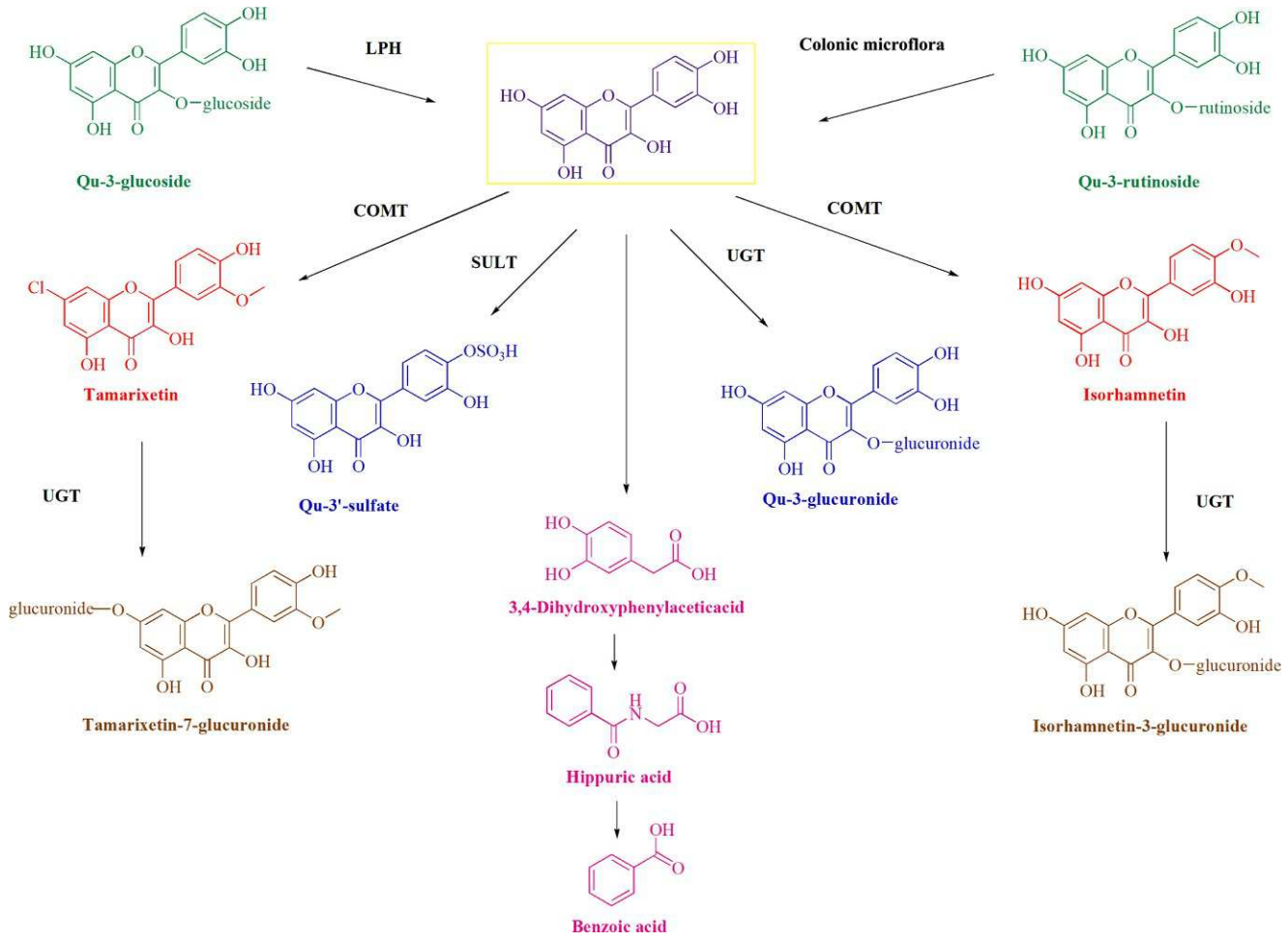

FIG. 26.3 The fate of dietary quercetin in the organism.

The adsorbed Qu is excreted in the urine [49] or excreted into the bile and eliminated in the excrement [50]. Principal metabolites of Qu are 3-hydroxyphenylacetic acid, benzoic acid, and hippuric acid, probably due to the fact that Qu is deconjugated before ring fission (which yields phenolic acids) [51]. Fig. 26.3 describes the fate of dietary Qu in the organism.

3 MECHANISTIC AND PRECLINICAL STUDIES OF QUERCETIN AND ITS METABOLITES

Increasing evidence suggests protective effects of various Qu-rich food products against chronic diseases, in particular cardiovascular and neurodegenerative conditions, and cancer [38]. It is claimed that the radical-scavenging activity of Qu is involved in these beneficial health effects [52]. Several in vitro mechanistic studies and in vivo interventions were performed to better understand how Qu and/or its metabolites interact with human physiological and pathological processes.

Qu might exhibit its antiinflammatory activity in vitro by significantly inhibiting the endogenous production of the multifunctional proinflammatory cytokine TNF-α. In addition to regulating the growth, proliferation, differentiation, and viability of activated leukocytes, TNF-α causes the nuclear accumulation of transcriptional factors, including NF-kB, induced by phosphorylation and degradation of Ikβ. NF-kB is considered a critical mediator of immune and inflammatory responses. In particular, Qu inhibits TNF-α production and gene expression in a dose-dependent manner, and this effect is mediated through modulation of NF-kB and Ikβ. TNF-α inhibition is a confirmed approach to treat several inflammatory diseases. These results indicate that Qu has potential application in antiinflammatory therapy [53]. Moreover, Qu reduces the TNF-α-induced expression of inflammatory genes, including IL-6, IL-1β, and IL-8 [54]. Qu significantly reduces nitrite production induced by the inflammatory cytokine IL-1β, iNOS protein and its mRNA expression levels [55]. The antiinflammatory activity of Qu is also accompanied by an increase in HO-1 protein levels, a downstream target of the

transcription factor Nrf2, known to antagonize chronic inflammation. Furthermore, Qu down-regulates the levels of mature microRNA-155 (miR-155), a component of the macrophage response to different types of inflammatory mediators, such as lipopolysaccharide (LPS) [56]. Finally, the antiinflammatory property of Qu was confirmed in studies involving both mice and patients. Qu supplementation attenuates the plasma levels of TNF-α and IL-8 as proinflammatory markers [56,57].

Qu exhibits in vitro antimicrobial activity, attributed at least in part to the inhibition of DNA gyrase [58].

It is known that ROS cause oxidative stress, which in turn plays a crucial role in the initiation and progression of pathological conditions leading to cardiovascular and neurodegenerative diseases, and cancer. Hence, the hypothesis that antioxidants can be used in disease prevention and therapy. Among antioxidants, Qu has attracted much attention due to its negligible adverse effects [59]. Qu exerts cardioprotective activity in vitro by digitalis-like increase of Ca^{2+} transients [60], and by a dose-dependent decrease in lipid content; [61] while Qu supplementation in vivo reduces blood glucose concentrations and blood pressure [61,62], and improves vascular health [63]. As demonstrated by both in vitro and in vivo studies, the antioxidant activity of Qu is the basis of its possible neuroprotective and anticancer effects [64], as well as its potential as alternative antiobesity therapy [65]. Finally, Qu might have multifaced therapeutic applications in pain management [66] and for allergy, arthritis, asthma, diabetes, infectious, and mood disorders [67].

As already mentioned, since dietary Qu is metabolized in the human body, its metabolites might influence not only the bioavailability but also the bioactivity of Qu in vivo [68]. Recently, conjugated Qu glucuronides have been found to act as bioactive metabolites and precursors of the aglycone form in vivo, contributing to the bioactivity of Qu [68]. It has also been found that Qu metabolites, such as Qu-3-*O*-glucuronide, Qu-3-*O*-glucoside, Q-3′,4′-di-*O*-glucoside, tamarixetin, isorhamnetin, isorhamnetin-3-*O*-glucoside, keep antioxidant properties both in vitro and in vivo [69,70], even though the activity was lower than Qu but greater than α-tocopherol [71]. In addition to the antioxidant activity, Qu metabolites also show antiinflammatory effects in vitro [72]. In particular, treatment of human macrophages with Qu-3-*O*-glucuronide reduces the transcription of genes involved in inflammation, including proinflammatory interleukins and enzymes implicated in oxidative stress responses [73]. Furthermore, Qu metabolites contribute to the chemopreventive effect of Qu by inhibition of COX-2 [74]. They also prevent metastasis through down-regulation of matrix metalloproteinases-2 (MMP-2) by up-regulating peroxisome proliferation-activated receptor- γ (PPAR-γ), which in turn participates, at least in part, in the inhibition of MMP-2 expression [75].

4 ESTERS OF QUERCETIN AND THEIR THERAPEUTIC APPLICATIONS

The various biological activities of Qu are often limited by its metabolism. The principal ploys to overcome metabolism are polymers in which Qu is trapped and exerts its activity [76]. In most cases, the bioactivity can be improved by modifying the structure with ester derivatives (Fig. 26.4). The ubiquitous presence of enzymes with esterase activity promises the rapid regeneration of the parent polyphenol in vivo [77]. This would improve bioavailability, increasing the levels of the aglycone in the circulatory stream and organs. In turn, this would allow more reliable assessments of the efficacy and innocuity of the compounds, mimicking circumstances of (patho)physiological relevance, and might eventually lead to the development of polyphenol-based molecules with preventive or curative applications [78]. In the first work reporting ester derivatives of Qu, the acetylated ester was chosen. These acetylated species were expected to be rapidly converted to Qu by esterases, so that the next result would be an increase in the fraction of Qu absorbed as such. Qu itself was extensively conjugated by tight monolayers of MDCK 1, MDCK 2 and Caco-2 cells. A few esters did not cross the monolayers, but others did, undergoing partial deacylation. No phase II conjugation was observed during transport of these compounds across MDCK or some Caco-2 clones [79]. The 3-OH is responsible for the autoxidation of Qu, so to prevent this behavior, three Qu-3-acyl derivatives (Qu-3-acetate, Qu-3-propionate, Qu-3-palmitate) have been proposed as suitable photoprotective agents, able to migrate through the aqueous environment and to interact with and cross phospholipid membranes, although the parent compound demonstrated a better antioxidant activity [80]. Other 3-*O*-acyl derivatives have been synthesized, such as Qu-3-ethyl, Qu-3-hexyl, Qu-3-propyl, Qu-3-butyl, Qu-3-lauryl and Qu-3-miristyl esters. Among these, propyl and butyl esters were more water-soluble than Qu, while ethyl, butyl, and lauryl derivatives were the most hydrolyzed by human plasma; propyl and butyl esters also penetrate human skin better than Qu from aqueous solutions [81]. Another synthesized derivative was the Qu-valerate ester, which showed a good lipophilicity but was less effective in inhibiting platelet aggregation compared with Qu and propyl- and butyl- acyl derivatives. These results indicated that the water solubility and the lipophilicity of the analogues must be improved in order to achieve higher antiplatelet activity, and an optimal acyl chain length is crucial for the synthesized Qu analogues to be more effective [82]. In addition to 3-OH, 7-OH is also considered a susceptible group, so in an interesting study, two pivaloxymethyl (POM) derivatives of Qu have been synthesized, 7-*O*-POM-Qu and 3-*O*-POM-Qu. They showed an increased

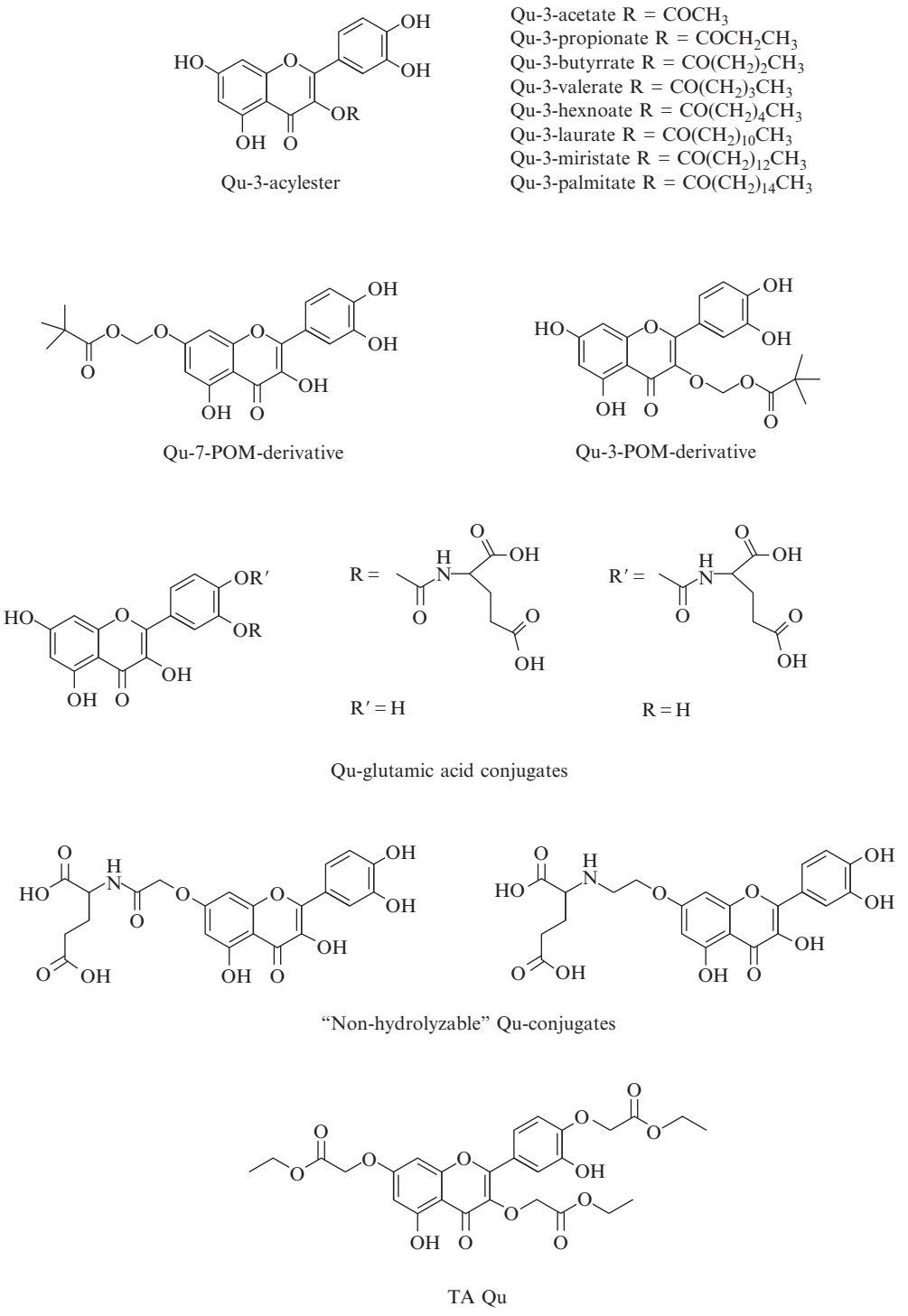

FIG. 26.4 Representation of some ester derivatives of quercetin.

stability ($t_{1/2}=4$ h for 7-*O*-POM-Qu and $t_{1/2}=52$ h for 3-*O*-POM-Qu) compared to the parent Qu. The C7 derivative also underwent efficient cellular uptake and intracellular levels of its hydrolyzed form (Qu) was maintained up to 12 h. The C3 derivative was more stable but failed to penetrate cell membranes. Both compounds were subjected to viability assays in various cancer cell lines, demonstrating a better apoptotic activity in the case of the 7-*O*-POM-Qu [83]. With the aim of increasing water solubility of Qu, amino-acid conjugates (3′ or 4′) have been synthesized. First, the glycine derivative QC12 (a carbamate prodrug water-soluble) was synthesized and assayed but it failed because after oral administration neither QC12 nor Qu were detected in the serum,

demonstrating the lowest bioavailability of the carbamate derivative [84]. Among other derivatives, all of them demonstrate good water solubility, especially aspartic and glutamic acid derivatives. In particular, glutamic derivatives showed a strong resistance against hydrolases and an increased intestinal permeability at MDCK cells [85]. In this context, the alanine, glutamic acid, phenylalanine, and leucine derivatives (on **B** ring) were designed to ameliorate the lipophilicity drawback of Qu, with the aim to synthesize new anticancer agents. The adopted molecular design evaluates the binding profile for Bcl-xL, an antiapoptotic protein, to enhance selectivity for cancer cell lines overexpressing peptide transporters. This was evidenced through docking calculations in the Bcl-xL protein, indicating that they bind to the BH3 binding site. The most active compounds were the Qu-glutamic acid derivatives with a K_d of 2.8 μM [86]. The ester derivatives of amino acids are often easily hydrolyzable, as in the case of Qu-glutamic acid ester, which is able to reverse multidrug resistance (MDR) of cancer cells through inhibition of P-glycoprotein (Pgp)-mediated drug efflux, but this activity has been attributed to the hydrolysate, that is Qu. To overcome this problem and evaluate if the glutamic chain is responsible for the biological activity, a nonhydrolyzable linker, such as —CH_2CO or —CH_2CH_2 substituents, was inserted in the core structure, by addition in the C7-position. The Pgp inhibition, Pgp ATPase, and MDR-reversal activity assays showed significantly higher activity compared to Qu, with EC_{50} values of 2.2 and 2.8 μM [87]. A particular linker (—$CH_2COOC_2H_5$) embedded in Qu has been identified as TAQU, a derivative that showed a better permeability coefficient than Qu; furthermore, TAQU was mostly adsorbed by passive diffusion, demonstrating how the lipophilic nature of TAQU contributed to the improved bioavailability [88].

Considering that Qu is present in natural sources together with hydroxycinnamic acids, a valuable strategy is to develop hybrids of Qu with these acids, in order to enhance the biological activities. In this sense, 3-*O*-galloylquercetin has been synthesized and assayed to evaluate the activation of Nrf2, that regulates the expression of many cytoprotective enzymes including HO-1. In RAW264.7 cells, the galloyl derivative had a better antioxidant profile but no effect on the expression of HO-1, unlike the parent Qu [89]. In a metabolic perspective, the galloyl ester was studied to understand its bioavailability. The identified metabolites were glucuronides and methyl derivatives, and ester bond cleavage was presumably performed by cellular esterases in a nonspecific manner [90]. Qu was also identified as an antiviral agent so, to fight influenza viruses, various compounds have been proposed, such as C-3-dihydroxybenzoate, C-3-aminohydroxybenzoates, C-3′-gallate, C-3′-aminopropyloxy, and C-5-propyloxy derivatives. The C-3′-gallate was the most active, showing antiviral activity against influenza virus porcine H1N1 with an ED_{50} value of 9.1 μM, which was higher than the parent Qu (ED_{50} = 48.2 μM) [91]. Other hydroxycinnamic-based esters have been synthesized and evaluated as potential remedies of chronic age-related metabolic or inflammatory diseases such as diabetes and neurodegeneration. In this field, monoacetylferuloyl-Qu (MAFQu) and chloronaphtoquinoneQu (CHNQu) are able to inhibit aldose reductase (IC_{50} values 2.1 μM for CHNQu and 18.66 μM for MAFQu) and α-glucosidase (IC_{50} values 49.3 μM for CHNQu and 74.8 μM for MAFQu), enzymes related to diabetes, or to prevent colon inflammation induced by acetic acid [92]. Very interesting modifications provided the use of fatty acids, not only in Qu as it is, but also in its glycosylated forms. In particular, eicosapentaenoic acid (EPA) ester of Qu-3-*O*-glucoside was synthesized as a potent antiinflammatory agent, being more effective in reducing the production of TNF-α, PGE2, COX-2 levels, and NF-kB expression compared to the parent compounds after LPS-induced inflammation in THP-1 derived macrophages. The study was also expanded to evaluate serum high-density lipoprotein (HDL)-cholesterol. The derivative was able to improve HDL-cholesterol and to reduce C-reactive protein (CRP), IL-6, and INF-γ [93]. The antiinflammatory activity of fatty acid acyl derivatives was also confirmed by α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) esters of Qu-3-*O*-glucoside, which also showed a protective effect against H_2O_2 insult in both human lung fibroblast (WI-38) and human primary hepatocytes upon exposure [94]. Not only glycosylated derivatives but also Qu derivatives have been proposed as antioxidant agents; in particular, various mono- and poly-oleoyl derivatives have been synthesized [95]. Oleic acid was also used to favor the interaction of Qu with the G-protein-coupled receptor 40, a new and prominent target to fight type 2 diabetes mellitus, demonstrating a potential agonist activity and a secretagogue effect, which causes the molecule to be considered a candidate for a promising antidiabetic agent [96]. Some ester derivatives of Qu with hydroxycinnamic and fatty acids are shown in Fig. 26.5.

5 CONCLUSION

The flavonol Qu has been proven to be an excellent antioxidant agent, with further antiinflammatory capacity in vitro; properties were confirmed in vivo as well. On the basis of these, Qu shows a spectrum of bioactivities that might promote human health through several pharmacological effects. As a result, Qu supplementation in the appropriate way, e.g., a pure supplement (usually in the aglycone form) or a diet intervention using Qu-rich

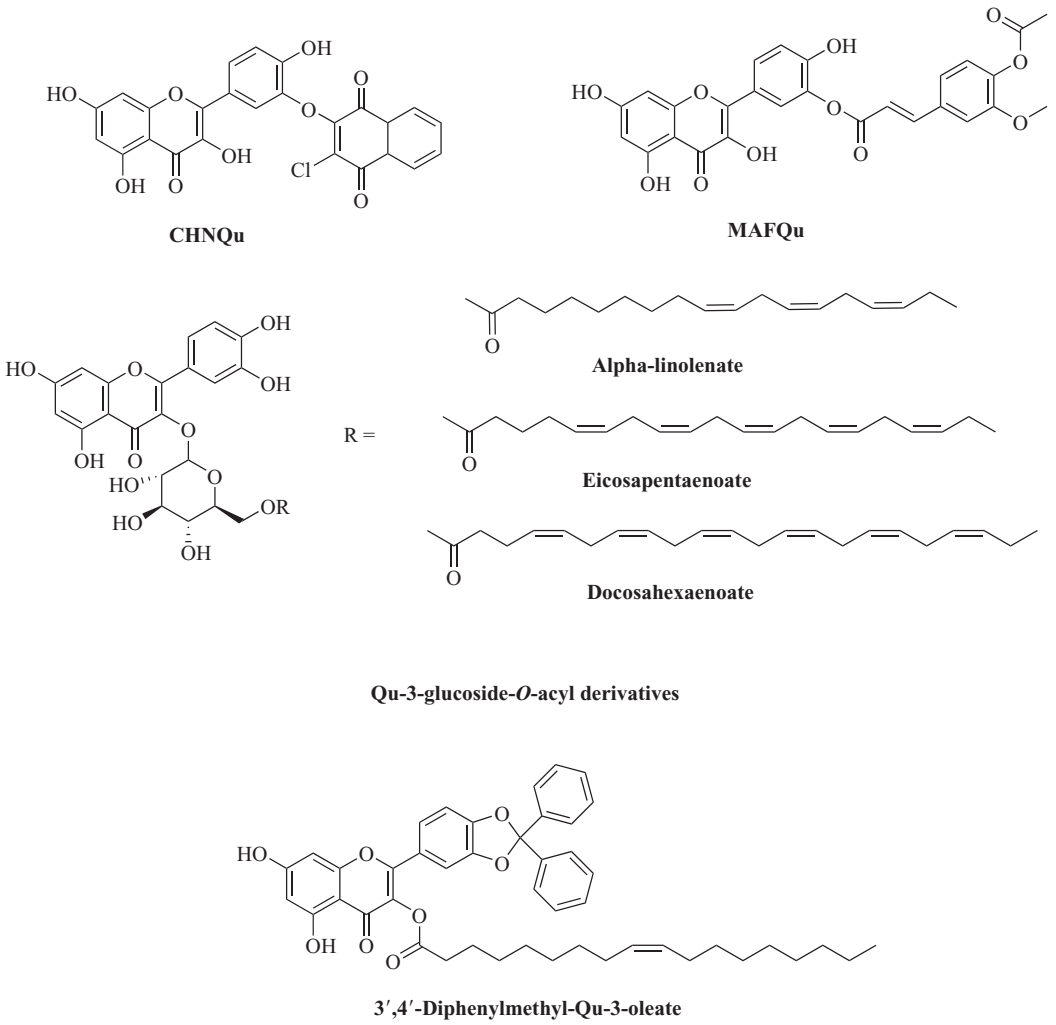

FIG. 26.5 Ester derivatives of quercetin with hydroxycinnamic and fatty acids.

food products (as glycosides), might be helpful for people suffering from a disease associated with both oxidative stress and inflammatory response.

Nonetheless it is not possible yet to establish dietary recommendations in terms of the type of supplementation or the amounts to be consumed. The huge heterogeneity of Qu metabolites related to the chemical structure and the distribution in the different districts in the organism needs further studies that aim to assess their bioactivities. In addition to its metabolism, Qu bioavailability is another important mediator of its health benefits. Hence, more research is necessary to completely understand the potential role of Qu in managing different diseases. Recently, several Qu esters have been designed and synthesized. They ameliorate its bioavailability as well as improve its bioactivities. In conclusion, based on these results, the development of new Qu-based nutraceutical and pharmaceutical agents for the treatment of various diseases becomes an attractive challenge.

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Further Reading

Terao J, Murota K, Kawai Y. Conjugated quercetin glucuronides as bioactive metabolites and precursors of aglycone *in vivo*. Food Funct 2011; 2(1):11–7.

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CHAPTER

27

Effects of Quercetin and Its Combinations on Health

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Acronyms and Abbreviations

$AUC_{0-\infty}$	area under pharmacokinetic curve "concentration-time" (from zero to infinity)
AUC_{0-t}	area under pharmacokinetic curve "concentration-time" (from zero to the latest blood sampling)
AMI	acute myocardial infarction
ANS	anylin naphthalene sulfonate
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CKD	chronic kidney disease
C_{max}	drug maximum concentration in blood plasma
CNS	central nervous system
CPK	creatine phosphokinase
CRF	chronic renal failure
ECG	electrocardiography
ED_{50}	median effective dose
F	absolute bioavailability
GAG	glucosaminoglycans
GFR	glomerular filtration rate
IC_{50}	half maximal inhibitory concentration
IL	interleukin
i.g.	intragastric
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
K_{el}	elimination constant
LD_{50}	median lethal dose
MCK	mass coefficient of kidneys
MRP	multidrug resistant proteins
NSAIDs	nonsteroidal antiinflammatory drugs
OA	osteoarthritis
PVP	polyvinylpyrrolidone
$T_{1/2}$	half-life period
TBARS	thiobarbituric acid reactive substances
T_{max}	time of achieving of maximum concentration
TNF-α	tumor necrosis factor alpha
TR	tubular reabsorption
UD_{50}	mean ulcerogenic dose

1 INTRODUCTION

Quercetin belongs to the class of flavones, which is abundant in plants; there are 175 known quercetin-containing substances to date.

Quercetin was isolated for the first time in 1854 from the flavonoid glycoside quercetrine. Later, in 1899, its chemical structure was determined via alkaline hydrolysis. This not only promoted study of the chemical structure of flavonoids, but also enabled their synthesis.

The first studies of these substances had very practical aims, since concentrated phenol substances from bark of plants was used for dyeing of woolen and silk fabrics, and in the East also in traditional medicine.

As a free substance, quercetin is rare, but the glycosylated form is very common in herbaceous and woody plants, pollen, flowers, leaves, and bark of tree and shrubs. Illustrating this point, here is the list of some plants containing quercetin or its derivatives: *Allium cepa, Allium fistulosum, Apium graveolens, Asparagus officinalis, Brassica oleracea, Calamus scipionum, Camellia sinensis, Capparis spinosa, Centella asiatica, Coriandrum sativum, Hypericum hircinum, Hypericum perforatum, Lactuca sativa, Malus domestica, Moringa oleifera, Morus alba, Nasturtium officinale, Prunus avium, Prunus domestica, Solanum lycopersicum, Vaccinium oxycoccos*.

All these plants have curative and prophylactic properties and are used in medicine or dietary interventions [1].

Generally, the polyphenol substance quercetin can be characterized as a powerful antioxidant, inhibitor of leukotriene synthesis, complex blocker of conduction and realization of signals in the calcium-mobilizing

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polyphosphoinositide system, and adenylate cyclase cascade activator. These mechanisms are definitive for most pharmacological effects, well known or being in the process of study, observed in quercetin administration.

This chapter gives a review of pharmacological properties of quercetin and outlines the results of development of quercetin-based drugs for heart, kidney, and joint pathologies.

2 BASIC PHARMACOLOGICAL PROPERTIES OF QUERCETIN

2.1 Antioxidant Effects of Quercetin and Quercetin Metabolites

Most flavonoid aglycones are known to have significant antioxidant properties. Quercetin in particular acts as a scavenger of superoxide radicals, singlet oxygen, and may inhibit the formation of lipoid hydroperoxide radicals [2].

The mechanism of antioxidant activity of quercetin or other similar flavonoids can be explained considering their chemical structure [1,3]. Thus, the presence of two phenol hydroxyls in the ortho position in the side phenyl fragment of the quercetin molecule enables this flavonoid to act as an antioxidant (Fig. 27.1).

In contact with active oxygen forms, this part of the flavonoid molecule undergoes mild oxygenation, leading to the formation of respective orthoquinone. Two hydroxyls at the C3 and C5 positions along with carbonyl oxygen at the C4 position of the quercetin molecule are the unique features necessary to create proper conditions for complexation with transition metal ions, primarily Fe^{3+} and Cu^{2+}. It should be also mentioned that these metals are often the ones present in the catalytic centers of many enzymes playing a role in redox processes [3]. In this way, yet another kind of antioxidant property of quercetin manifests itself.

Therefore quercetin in the cell may act mainly as an active oxygen radical scavenger. At the same time, quercetin induces the formation of complex chelate compounds with the metals of variable valencies, due to its ability to block chain reactions induced by different radicals. The antioxidant effect of quercetin, quercetin glycosides, and metabolites is one of the specific mechanisms of realization of quercetin pharmacologic properties.

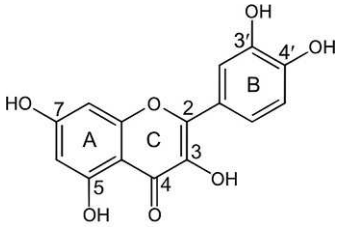

FIG. 27.1 Chemical structure of quercetin.

By neutralizing aggressive oxygen and NO-radicals and interfering with free radical reactions, quercetin may slow down many pathologic processes ensuring normal homeostasis of the cell and adequate functioning of the organism as a whole.

It is well known that the products of free-radical oxidation are the key agents of oxidative stress of the cell, leading to many pathologic processes and diseases. It is logical that the prevention of radical-containing compounds or their neutralization by flavonoids is fundamental for successive treatment.

The multiple variations of weak conjugation of quercetin with many cell proteins, including enzymes, are possible due to the unique structural features of this flavonoid. Quercetin interactions with the active centers of enzymes are realized mostly through the formation of intramolecular hydrogen bonds that require strict control over thermodynamic parameters to ensure synthesis-destruction equilibrium of these biologically active complex adducts [4]. Considering the low energy requirements for these biological compound formations, one can predict their disintegration and (or) transformation, reversibility of quercetin or quercetin metabolites' release as an aglycone, as well as release of any other enzyme or biological substrate.

Early studies on the pharmacologic effects of quercetin were concentrated primarily on its antioxidant properties and on its role in different enzymatic reactions and biochemical processes crucial for many pathologic conditions and processes leading to homeostatic disbalance [5].

Aside from the studying of the pharmacologic properties of quercetin, researchers' (chemists', pharmacologists', biochemists', etc.) attention has also been paid to the products of quercetin metabolism, since the biotransformation of quercetin leads to the formation of highly biologically active substances [6–8].

2.2 Interractions of Quercetin and Quercetin Metabolites With the Cell Enzymes

The biochemical and pharmacological effect of quercetin and most flavonoids are based on their selective inhibition of many vital cell enzymes, which makes these substances rank among the specific bioregulators of multiple enzymatic processes [4,9,10].

Many biochemical interactions of polyphenols, including quercetin, with enzymatic proteins are characterized by high selectivity and affinity to the active centers of the enzymes due to the specific structure of these molecules and a strong binding. Taking the aforesaid into account, it should also be mentioned that the stability of these

complex adducts may be characterized by distinct kinetic parameters. An example is the study on the interaction of quercetin with bovine serum albumin and insulin, accompanied by pronounced fluorescence of the complex [9,10].

Biochemical processes of inhibition of the enzymes with ATP-binding sites by quercetin are also worth mentioning [4]. These are mainly protein kinases, mitochondrial ATPases, myosin, Na^+/K^+ and Ca^{2+} serum ATPases, topoisomerase II, ATP-dependent transporting P-glycoproteins (P-gp high permeability carriers) [8–10] and P-glycoproteins of MRP1 and MRP2 (multidrug resistant proteins) subtypes [11]. Here the inhibitory action of quercetin is probably based on its selective binding to the active ATP-binding sites of these enzymes, integrated into the plasma membrane. Strong binding and subsequent inhibition are evidently due to the high affinity of this interaction.

At the same time, quercetin and, in particular, quercetin metabolites, may interact with ATP-dependent P-glycoproteins without inhibition of the latter, so this complex may play a role in intercellular transport [8–10,12,13].

In vitro studies have demonstrated the high-affinity ligand-receptor interactions of quercetin with adenosine [14], estrogen [15], and GABA-receptors [16]. Flavonoids, including quercetin, are also known to inhibit protein kinase, which is an important factor of cell division and proliferation regulation.

Structural and chemical features of flavonoid molecules, including quercetin, ensure their high-affinity interactions and inhibition of enzymes playing a role in oxidation reactions and processes. These enzymes are, among many others, lipoxygenases and cyclooxygenases, responsible for the biotransformation of arachidonic acid into its important biologically active derivatives, such as leukotrienes and prostaglandins, respectively [17].

Noteworthy, the inhibitory effect of quercetin on lipoxygenase is much more potent, than it is on cyclooxygenase. This phenomenon has been discovered during in vitro experiments with alternating quercetin concentrations [18].

Different quercetin-based drugs are characterized by unequally pronounced inhibition of different enzymes. The doses of quercetin that are effective in lipoxygenase inhibition ($IC_{50} = 4.2\,\mu M$) would not manifest any inhibitory effect on the mitochondrial respiratory enzyme succinate dehydrogenase ($IC_{50} < 3000\,\mu M$). Many others have confirmed the predominance of lipoxygenase inhibition over cyclooxygenase (the enzyme, inducing the prostacyclin synthesis) inhibition. This fact makes possible the wider range of dosage in clinical application, since there is no danger of inhibition of vital enzymes [18].

Xanthine oxidase is the enzyme responsible for catalysis of xanthine oxidative transformation into uric acid and simultaneous superoxide formation and is also susceptible to quercetin action [19]. In this case, quercetin inhibits the enzyme-inhibitor complex responsible for this kind of oxidative reactions in the cell.

There is also important evidence of quercetin interaction with hemoprotein-cytochrome P450-containing monooxygenases (such as isoforms CYP 1A1, 1F2, 1B1, 3A4, etc.). One of the quercetin pharmacological actions is also the highly selective inhibition of the cytochrome P450 isoform function, which is also based on the structural features of most flavonoids [20].

The studies in this field have demonstrated the ability of quercetin to regulate xenobiotic and drug transformations directing them at cytochrome P450 isoform pathway.

Another important property of quercetin is its ability to interact with calcium-mobilizing polyphosphoinositide system and other elements of this signal cascade. This modulates many intracellular reactions, including the formation and secretion of mediators, coagulation processes, smooth muscle contraction, some of the immune reactions, etc. [21].

The aforementioned reactions can also be inhibited by means of adenylate cyclase system activation, which may be stimulated by quercetin directly blocking phosphodiesterase and leading to cAMP and cGMP elevation in endotheliocytes [21,22].

The experimental studies on some other pharmacological effects of quercetin, such as its ability to prevent oxidative stress during ischemic reperfusion damage [23]; UV-radiation exposure [24]; and spontaneous hypertension [25] are also worth noting.

This analysis of literary data on in vitro and in vivo experiments demonstrates the powerful inhibitory effects of quercetin on multiple vital enzymes and even on entire enzymatic systems. Since most enzymes are responsible for their respective pathologic processes, one can evaluate the most important pharmacological properties of this flavonoid to prognosticate its possible applications in clinical practice.

2.3 Quercetin Interactions With Proteins

Publications [19,22] include the data on spectroscopic conformational studies on the quercetin and rutin (glycoside of quercetin) interactions with contractile proteins (myosin and actomyosin), and multiple possible conformational changes of contractile proteins resulting from their interaction with flavonoids. It was also discovered, that flavonoid compounds added to myosin solution produced characteristic differential spectra, with peaks at 258 and 375 nm for quercetin and 253 and 350 nm for rutin. These spectra helped the authors of publication [19] to confirm the fact of quercetin binding to the specific active centers of myosin molecules.

Using the fluorescent probe 1-anylyn naphthalene 8-sulfonate (ANS) to detect the possible changes of actomyosin and myosin molecules after quercetin and rutin binding, the researchers have found out that, aside from ultraviolet fluorescence, the spectrum of ANS fluorescence also involves a visible area. Adding quercetin to actomyosin-ANS solution caused the reduction of fluorescence of actomyosin and the shift of its spectrum to a longwave area. Altered spectral characteristics (spectrum shift and reduction) of actomyosin and ANS were probably caused by quercetin effect on the conformation of actomyosin [19,22].

Aside from the active centers for ATP hydrolysis, myosin heads contain also regulatory centers. Studies [19,22] have demonstrated that inhibition of myosin ATPase by quercetin is of a noncompetitive type, since quercetin binds the enzyme near its active center and realized its effect by allosteric mechanisms. Based on these studies and literary data analysis, the authors have suggested a conception of flavonoid attachment to the myosin head. Conformational changes, inducing the activation or inhibition of myosin ATPase, are caused by flavonoid binding the regulatory site near the active center of the myosin head.

Studies [19,22] have also discovered the two-phase effect of quercetin on ATPase. Low flavonoid concentrations (10–20 μM) activate ATPase, while high concentrations inhibit myosin ATPase along with actomyosin superprecipitation and myofibril contraction. Inhibitory effects of flavonoids are more pronounced on contractile structures of higher order, so it is most potent on myofibrils. Analysis of these studies and literary sources allowed us to conclude that the prominent effect of flavonoids on functional characteristics of contractile systems is based on conformational changes resulted from flavonoid binding the regulatory site near the active center of myosin head.

2.4 Antiinflammatory Activity of Quercetin and Its Mechanisms

The mechanism of antiinflammatory activity of quercetin is based mainly on 5-lipoxygenase inhibition—the key enzyme of arachidonic acid biotransformation. The lipoxygenase pathway of arachidonic acid metabolism ends with the formation of leukotrienes [17,18], associated with the development and progressing of inflammation. Therefore, the blocking of different stages of leukotriene synthesis by quercetin favors the inhibition of many pathologic processes.

The characteristic and important pharmacological properties of leukotrienes should be also taken into account, such as reduced blood flow in different organs, activation of lipid peroxidation, etc.

In vitro and in vivo experiments, as well as clinical trials of quercetin have demonstrated that inhibition of lipid peroxidation was an important manifestation of potent antiinflammatory [26], angioprotector [27], cardioprotector [28] and hepatoprotector [29] properties of this flavonoid.

It was also experimentally discovered that quercetin can directly inhibit the main mediators of inflammation, interfering with histamine secretion and functional activity alloantigenspecific cytotoxic T-lymphocytes, interleukin IL-8 and tumor necrosis factor (TNF-α) [30].

It was proved [31] that the antihistamine activity of quercetin is higher than that of sodium cromoglycate, which makes it potentially useful in treatment of allergic diseases.

2.5 Antiapoptotic Activity of Quercetin

Studies have proved that quercetin can prevent the programmed physiologic cell death of cardiomyocytes (apoptosis), induced by ischemia and reperfusion [32]. The mechanism of its cardioprotector activity is associated with the inhibition of proteasome-peptidase, controlling and regulating the key intracellular processes leading to apoptosis [32,33].

With antiapoptotic activity of flavonoids, including quercetin, is also associated their regulatory effects on expression of heat-shock genes [32].

Antiapoptotic activity of quercetin was also proven on the model of osteoarthritis in rats. The results showed a significant decrease in apoptosis of chondrocytes. This effect is one of the components of chondroprotective activity of quercetin [34].

3 PHARMACOKINETIC PROPERTIES OF QUERCETIN AND WAYS OF MODIFYING THEM

Quercetin has well-established pharmacokinetic properties both in laboratory animals and in humans. The studies demonstrated that, after oral administration of quercetin substance in the unchanged form, it has low bioavailability and consequently limited applicability in clinical practice [8,35–40]. According to the available data, oral bioavailability of quercetin in humans is about 1.0% [39] and in rats, 5.3% [8].

Quercetin is absorbed in the small intestine; after that, it is subjected to intense metabolism with the creation of at least 20 active metabolites, which are primarily excreted with urine [39,41,42]. Low bioavailability of the unchanged form of quercetin and high content of metabolites points to the active first-pass metabolism [8,35,40]. Quercetin and its metabolites are excreted as

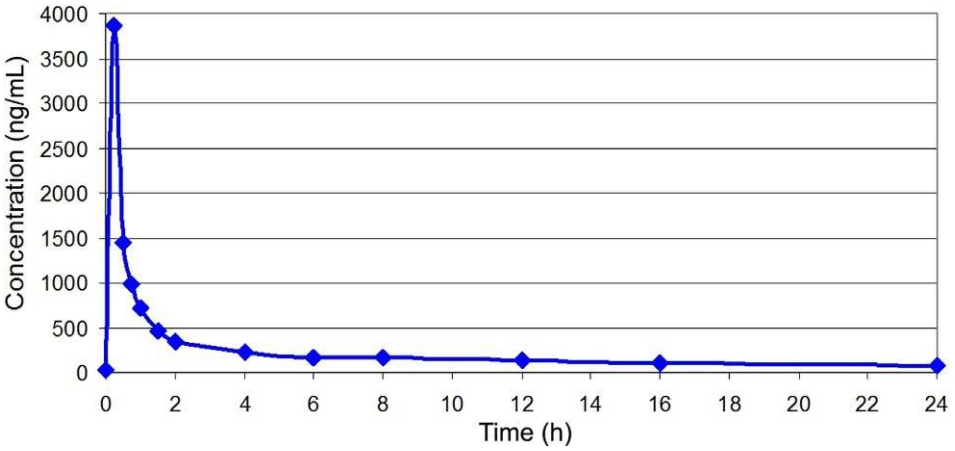

FIG. 27.2 Average concentrations of total quercetin and its metabolites in plasma of healthy volunteers after i.v. administration as a function of time.

conjugates (glucuronides, sulfates, methylated derivatives) over 48 h. Feces contain up to 53% of quercetin metabolites, which indicates its digestion by intestine microorganisms [39].

Half-life periods for different quercetin metabolites differ and are as follows: for unchanged quercetin form it is 20.7 min, for sulfonated quercetin forms 371.2 min, for quercetin glucuronides 560.7 min [36]. The degree of binding to proteins is very high and amounts to more than 98%. The volume of distribution is 0.34 g/kg. Following intravenous administration, 7.4% is excreted with urine in the form of conjugated metabolites and 0.65% in the unchanged form [8,39] while 1.5% is excreted with feces [8,43]. Other authors mention higher values of quercetin renal elimination: up to 18.4%–26.8 % in humans and 16.5% in rats [8,38].

For a long time the application of valuable pharmacological properties of quercetin in medicine was limited due to low bioavailability of its oral dosage forms. However, the research on the substances that can influence solubility of quercetin allowed drastically increasing the bioavailability and implementing quercetin in medical practice. Thus, application of polyvinylpyrrolidone (PVP), a low-molecular solubility modifier, resulted in the development of a soluble form of quercetin, which was introduced into medical practice as lyophilized powder for parenteral administration with the ratio of quercetin to PVP being 1:9 [18]. Now this drug is known as Corvitin (PJSC SIC "Borshchahivskiy CPP," Ukraine). Clinical trials confirmed the high efficiency of this form of quercetin in acute myocardial infarction [44].

The research team from the Department of Clinical Pharmacology and Clinical Pharmacy (National University of Pharmacy, Ukraine) conducted a series of clinical and experimental studies of pharmacokinetic properties of quercetin using different routes of administration.

TABLE 27.1 Mean Pharmacokinetic Parameters of Corvitin in Healthy Volunteers After i.v. Administration

Study group	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t} (h*ng/mL)	$AUC_{0-\infty}$ (h*ng/mL)	K_{el} (h^{-1})	$T_{1/2}$ (h)
Corvitin 500 mg v.v. (quercetin 50 mg)	3870.9	0.25	4136.8	4595.9	5.23	6.9

Pharmacokinetic properties of Corvitin at a dose of 500 mg i.v. (corresponds to 50 mg of quercetin), were studied in a clinical trial on healthy volunteers [45]. The obtained pharmacokinetic curve of quercetin is demonstrated in Fig. 27.2.

Pharmacokinetic parameters of Corvitin, determined by total quercetin and its metabolites, are presented in Table 27.1.

The obtained results showed that the main metabolites of quercetin after i.v. administration are its methoxy derivatives (isorhamnetin and tamarixetin) and their glucurone and sulfatide conjugates. Metabolic transformations occur in approximately 20% of the administered doses of quercetin and take 20 min. Free quercetin is conjugated by 33% and isorhamnetin by 70% in 20–30 min after i.v. administration. Corvitin is excreted with urine in the form of quercetin and isorhamnetin conjugates, with maximum elimination rate on average being 1.39 mg/h at 1.63 h after the administration of the drug.

Subsequent experimental research in rabbits further scrutinized pharmacokinetic modifications achieved by combining quercetin with different solubility modifiers following oral and parenteral administration [46,47].

The obtained results attest to intensification of quercetin gastrointestinal absorption in combination with

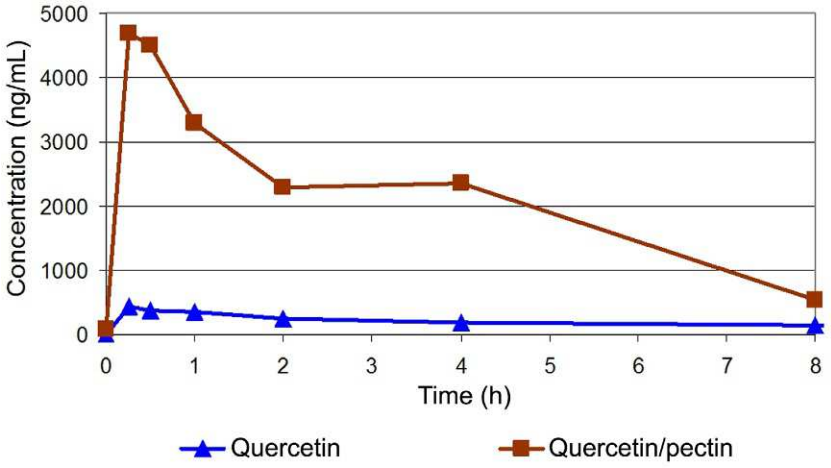

FIG. 27.3 Average total concentration of quercetin and its metabolites in plasma of rabbits as a function of time.

pectin, as was evident from the increase of maximum concentration by 11.0 times and bioavailability by 10.0 times, compared to pure substance (Fig. 27.3) [46]. This allows substantial improvement of quercetin solubility properties and correction of its pharmacokinetic parameters for therapeutic use.

These results became the basis for development and introduction into medical practice of a quercetin/pectin combination drug—Quertin (PJSC SIC "Borshchahivskiy CPP," Ukraine), in the form of chewable tablets [18].

Clinical studies on healthy volunteers scrutinized pharmacokinetics of oral Quertin at the doses of 40 and 240 mg [47].

Pharmacokinetic parameters of orally administered quercetin were calculated by total quercetin and its metabolites and are presented in Table 27.2. They show that C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of Quertin at doses 40 and 240 mg change in direct ratio. T_{max} decreases lightly with the 240-mg dose, probably as a result of high content of excipients. $T_{1/2}$ changes significantly depending on the dose and equals 9.03 h for the 40-mg dose and 12.8 h for the 240-mg dose of Quertin.

Experimental study of the pharmacokinetics of quercetin combination with amino sugars (glucosamine hydrochloride and *N*-acetylglucosamine) after i.g. (intragastric) administration showed an increase of quercetin maximum concentration (C_{max}) by 2.0 times, and absolute bioavailability (F) by 2.5 times, as well as an increase of the half-life period ($T_{1/2}$) from 4.8 to 6.4 h (Table 27.3).

TABLE 27.2 Mean Pharmacokinetic Parameters of Quertin in Healthy Volunteers After Oral Administration

Study group	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t} (h*ng/mL)	$AUC_{0-\infty}$ (h*ng/mL)	K_{el} (h^{-1})	$T_{1/2}$ (h)
Quertin 40 mg p.o.	201.0	2.96	1456.7	1631.1	0.11	9.0
Quertin 240 mg p.o.	924.2	2.17	7223.2	7703.5	0.06	12.8

The pharmacokinetic curve analysis testifies that the curve after quercetin administration has a flat form with a maximum at 15 min and without apparent peaks while, after complex administration with amino sugars, it repeats the profile of the curve of single quercetin administration but with twice as high a level of concentrations and presence of an apparent "plateau" at 500 ng/mL. [48].

At a further stage of the research, the physicochemical interactions of quercetin with amino sugars was excluded by the results of computer modeling in ACD/pK_aDB software [49].

TABLE 27.3 Mean Pharmacokinetic Parameters of Quercetin in Rabbits, Achieved by Different Routes of Administration With Solubility Modifiers

Study group	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t} (h*ng/mL)	$AUC_{0-\infty}$ (h*ng/mL)	K_{el} (h^{-1})	$T_{1/2}$ (h)	F (%)
Quercetin i.g.	441.7	0.25	1731.8	2694.4	0.14	4.8	27.5
Quercetin/amino sugars i.g.	920.6	0.25	4394.0	9686.0	0.11	6.4	69.8
Corvitin i.m.	1882.9	1.00	7948.9	8938.4	0.29	2.4	126.3
Corvitin i.v.	8013.2	0.25	6294.3	6785.0	0.42	1.7	100.0

These results became the basis for development and introduction into medical practice of a quercetin/glucosamine derivative (glucosamine hydrochloride and *N*-acetylglucosamine) combination drug—Gluquamine (PJSC SIC "Borshchahivskiy CPP," Ukraine), in the form of capsules for oral administration. This combination is characterized both by the improved pharmacokinetic properties of quercetin and broadening of its pharmacodynamic spectrum.

Data in Table 27.3 attest that after i.m. (intramuscular) administration of quercetin as the Corvitin drug, its bioavailability (F) increases by 1.3 times, and its half-life period ($T_{1/2}$) increases by 1.4 times as compared to i.v. (intravenous) administration. Increase in quercetin bioavailability following its i.m. administration is of scientific interest and is explained by its greater release rate from the complex with solubility modifier PVP as compared to i.v. administration. Thus, parenteral administration is an alternative way of improvement of quercetin pharmacokinetics, i.e., i.v. administration allows a C_{max} to be achieved fast while i.m. administration grants prolongation of pharmacokinetic parameters and increase of bioavailability of the flavonoid.

Thus, the obtained results allow concluding that application of quercetin solubility modifiers in combined oral and parenteral dosage forms substantially increases pharmacokinetic parameters of this flavonoid and improves its applicability in medicine.

4 GASTROPROTECTIVE EFFECTS OF QUERCETIN

Along with its potent antiinflammatory activity, quercetin, unlike the synthetic nonsteroid antiinflammatory drugs, does not possess any ulcerogenic properties and may even act as a gastroprotector [18,50,51].

The gastroprotective effect of quercetin is based on its antihistamine activity, associated with its ability to inhibit the functioning of H^+-K^+-ATPase (decrease of hydrochloric acid secretion), to stimulate the synthesis of cytoprotective prostaglandin E_2 by the cells of gastric mucosa, and to decrease the containment of the active form of oxygen in the gastric wall [51,52].

It is also curious that quercetin is able to inhibit the growth of *Helicobacter pylori*, which is probably one of the mechanisms of its antiulcerogenic properties [18,52].

The antileukotriene properties of quercetin may also prevent the damage of vascular endothelium of ischemic tissues, including the gastric wall, and to reduce the reperfusion disorders of microcirculation [49]. These pathologic conditions always involve leukotriene activity. Therefore, quercetin may protect the vascular endothelium from multiple pathogenic agents resulting from the initiation of peroxide oxidation and leukotriene induction.

5 ANGIOPROTECTIVE ACTIVITY OF QUERCETIN

The important pharmacological effect of quercetin is its ability to prevent the peroxidation of low-density lipoproteins, mitigating their atherogenic effects. This is realized through the interfering with endothelial retraction and reducing the thickening of intima, which ultimately leads to the expansion of the intraendothelial space.

Quercetin was also proved to affect the adhesive properties of endothelium, interfering with lipopolysaccharide-induced molecular synthesis [53]. In these interconnected processes quercetin may inhibit the key enzymes responsible for the induction of endothelial cell adhesiveness. In the first place, quercetin inhibits the activity of protein kinase C, the key enzyme of polyphosphoinositide system, responsible for tumor necrosis factor activation [54].

The antiatherogenic activity of quercetin is directly related to the reduction of free radical oxidation of lipids, which mitigates the cytotoxicity of low-density lipoproteins [55].

The aforementioned processes may alter the hypolipidemic processes and atherosclerosis course. Significant hypolipidemic and antiatherogenic effects of quercetin on high-cholesterol diet was experimentally proven.

6 EFFECTS OF QUERCETIN ON HEMOSTASIS

It is well known that micro- and macrorheological disturbances of blood are associated with the intensification of free radical processes. Thus, activation of these reactions decreases the fluidity and elasticity of erythrocyte membranes and affects erythrocyte morphology. It alters the ability of erythrocytes to aggregate and change their shape.

Antiradical properties of quercetin and its inhibitory effects on lipid peroxidation are playing an important role in the regulation of blood rheology, including viscosity of blood and plasma, fibrinogen content and erythrocyte aggregation [56].

Many examples of thrombocyte hyperaggregation are described, as this condition accompanies many acute pathologic conditions, such as myocardial infarction, strokes, and arterial hypertension. That's why the hemorrheological and antithrombocytal properties of quercetin may be important for prevention of ischemic diseases of heart and brain. Protector effects of this flavonoid and its metabolites on hemostasis result from suppression of catalytic activity of thrombin and stabilization of erythrocyte membranes [56].

Moreover, hyperaggregation of thrombocytes is a pathologic basis of myocardial infarction and strokes.

Hyperaggregation is caused by arachidonic acid transformation into thromboxane A_2 and aggregation threshold of thrombocytes resulting from their increased sensitivity to thromboxane A_2, which acts as an aggregation inductor [57].

Many experimental studies on quercetin and its effects on hemostasis have demonstrated that quercetin and other flavonoids may act as coronary artery vasodilators, increasing the level of cAMP in endothelial cells and inhibiting thrombocyte aggregation [58–60]. The authors of these studies also recommended using quercetin for ischemic heart disease complication prophylaxis.

Disaggregation effects of quercetin are based on the mechanisms of leukotriene synthesis inhibition, interfering with calcium transport into thrombocytes and inhibition of phospholipase D. Disaggregation of thrombocytes here is caused by the decreased activity of different stages of thrombocyte glycoprotein VI transformation and the inhibition of many intracellular enzymes, playing a role in proteinase phosphorylation and calcium mobilization [60].

Quercetin inhibits the activity of protein kinase C and phosphatidylinositol-3-kinase, which is an important factor in the reverse control of thrombocyte aggregation, induced by low concentration of aggregation agonists [18]. As mentioned earlier, the antiplatelet effects of quercetin may be realized through the inhibition of thromboxane A_2 synthesis. Decreased synthesis of thromboxane A_2 here is probably caused by the cyclooxygenase inhibition [17].

It is also worth mentioning that anticoagulation activity of quercetin is realized directly through the inhibition of catalytic activity of thrombin in the reactions of fibrinogen binding. Anticoagulation activity in this case results from the phosphodiesterase inhibition leading to the increase in cAMP [61].

Moreover, quercetin is also proven to have not only anticoagulant and disaggregation effects, but also an ability to potentiate the effects of endogenous anticoagulants and antiaggregants [61].

7 CARDIOPROTECTIVE PROPERTIES OF QUERCETIN

7.1 Quercetin Effects on Clinical Course of Acute Myocardial Infarction

The variety of the previously mentioned pharmacological properties of quercetin, such as antioxidant, antiischemic, antiatherogenic, angioprotective, and antiplatelet, constitutes the pronounced cardioprotective effect of this flavonoid, ensuring its positive effect on the course of cardiological pathology and providing prophylaxis of possible complications.

The results of the studies prove the intravenous form of quercetin—Corvitin—to be the most relevant for the treatment of various clinical forms of ischemic heart disease, including myocardial infarction [18].

Early i.v. administration of Corvitin in the complex therapy of acute myocardial infarction (AMI) improved the clinical course of the disease, by reducing the severity of arrhythmic syndrome, acute left-sided heart failure, the incidence of chest pain, and need for narcotic analgesic injections. The antiarrhythmic action of Corvitin may be the result of its membrane-stabilizing action, as well as of the improvement of intracardiac hemodynamics due to reduction of myocardial stress. At the same time, the positive effects of the water-soluble form of quercetin flavonoid are associated with its ability to affect the biologically active regulators (nitric oxide, leukotrienes) and to reduce prooxidative stress.

Quercetin action results in faster stabilization of the necrotic zone and the decreased mass of the necrotized myocardium. The time of reaching the peak activity of creatine phosphokinase (CPK) in the group of patients receiving Corvitin was 13.5 ± 0.6, and in the control group was 17.1 ± 0.8 h; the difference was highly reliable ($P < 0.001$). Time of reaching the peak activity of CPK-MB was, respectively, 9.9 ± 0.4 and 13.9 ± 0.6 h ($P < 0.001$). CPK activity returned to normal on the average 8.1 h earlier, and CPK-MB activity 9.4 h earlier during the Corvitin therapy in comparison with the control group ($P < 0.001$). The early peak of cardiac specific enzymes in the blood of the patients treated with Corvitin is the evidence of the faster formation of the necrotic zone, and the shorter time of elimination of CPK and CPK-MB from the necrotic zone demonstrates the ability of Corvitin to prevent further damage of viable cardiomyocytes. The mass of necrotic damage, calculated based on the dynamic of serum CPK-MB activity in patients receiving Corvitin, was 26.4% less than that of the control group (45.4 ± 2.1 and $61.6 \pm 2.9\,\mathrm{g\,eq^{-1}}$ respectively, $P < 0.001$). The most significant reduction of the necrotic zone was observed in the patients undergoing thrombolytic therapy and percutaneous transluminal coronary angioplasty [44,62].

7.2 Cardioprotective Action of Quercetin Combinations

The combination of quercetin and glucosamine derivatives, implemented into the clinical practice as the drug Gluquamine, is a promising cardioprotective agent. Such a combination allows broadening and increasing cardioprotective pharmacological properties of quercetin. Glucosamine enters the cell membranes of cardiomyocytes, leading to their stabilization, boosting resistance against ischemia and hypoxia, neutralizing free radicals, and causing an antienzymatic effect.

Pharmacological screening for cardioprotective properties of quercetin/glucosamine (glucosamine hydrochloride and *N*-acetylglucosamine mixture) combinations with different ratios of the active substances on the model of isadrine myocarditis showed varying degrees of cardioprotective activity for different test objects. The most pronounced effect was observed for a 1:3 quercetin/glucosamine ratio (which corresponds to the drug Gluquamine) in a dose of 81.9 mg/kg, which served as a rationale for researching exactly this test object [63].

The subsequent study under the conditions of neurogenic myocardial damage in rats revealed that Gluquamine reliably decreases the levels of enzymemia (by 1.5–2.0 times) and products of lipid peroxidation (by 21% in serum and 35% in the heart tissue), normalizes endogenous *N*-acetylglucosamine metabolism, functional state of the myocardium and, as a consequence, causes anticytolitic, antioxidant, and membrane-stabilizing effects, which determine the expressed cardioprotective effect [64].

The experiment on the model of heart failure in rats proved that Gluquamine possesses composite cardioprotective activity, which manifested in animal lethality decrease by 4.0 times, improvement of physical condition of the animals, reduced degree of free-radical and inflammatory processes in the myocardium, as well as a healthier electrophysiological state of the latter. Among the findings of this study is also the decrease of aspartate aminotranspherase by 1.5 times, lactate dehydrogenase by 1.3 times, and diene conjugates by 1.6 times in the serum; at the same time in the myocardium, there was 1.8× lower concentration of thiobarbituric acid reactive substances (TBARS) and 2.0× higher concentration of *N*-acetylglucosamine.

Histomorphological study of Gluquamine under the conditions of proliferative myocardial damage showed that the studied combination facilitated preservation of the morphological structure of the heart muscle, i.e., there was normalization of cytoarchitecture, less venous congestion, and lower intensity of proliferative, degenerative, and dystrophic processes in cardiomyocytes [65].

Gluquamine has had a positive effect on furazolidone-isadrine myocarditis as demonstrated by normalization of the functional state of the myocardium and biochemical parameters, i.e., serum level of enzymemia decreased by 1.5 times, both serum *N*-acetylglucosamine and TBARS by 1.7 times, while *N*-acetylglucosamine in myocardium increased by 11.0 times.

In alterative myocardial damage in rats, Gluquamine preserved morphological structures of the cardiac tissues, lowered the number of foci of necrosis, myocytolysis, and cell infiltration, and prevented destructive changes in cardiomyocytes. Administration of the test drug facilitated restoring of cardiomyocytes' ultrastructure with less destruction of myofibrils, less degenerative changes in mitochondria, and improved mitochondria orientation. Only mild features of myocardial dystrophy were observed [66].

The totality of the obtained results suggests that Gluquamine is a potent cardioprotector with a composite mechanism of action affecting most links of pathogenesis of various cardiomyopathies. The degree of cardioprotective activity of the studied combination significantly exceeds that of quercetin or glucosamine alone. Cardioprotective activity of the test object is realized by means of synergistic interaction of its components, i.e., antioxidant and antiproliferative effects of quercetin accompanied by membrane-stabilizing and antialterative properties of glucosamine.

Being a cardioprotector, quercetin may have application in treatment of inflammatory cardiac diseases such as myocarditis. While possessing its own antiinflammatory activity via lipoxygenase inhibition, quercetin, however, cannot be deemed a powerful antiinflammatory drug and consequently for such indication may benefit from synergistic interaction with a drug from the group of NSAIDs, which are known to block cyclooxygenase, thus complementing quercetin in inhibiting products of arachidonic acid metabolism. This idea is realized in a pharmaceutical composition of quercetin and diclofenac. Another rationale for such a combination is that quercetin may prevent the known problem of cardiotoxicity of diclofenac.

The results of the study on the model of furasolidone-isadrine myocarditis in rats showed that administration of quercetin/diclofenac combination significantly lowered enzymemia and decreased TBARS and diene conjugates by 1.3 times compared to the untreated animals. ECG demonstrated normalization of RR, PQ, QT intervals, QRS complex, and P, T and R waves indicating protective action of the drug on the function of the myocardium. Moreover, tachycardia became milder—472 vs. 555 bpm in untreated animals [67].

The subsequent morphological study demonstrated that the quercetin/diclofenac combination decreased intensity of alterative processes and prevented development of degenerative-dystrophic damage of the myocardium in rats with furasolidone-isadrine myocarditis. The level of activity of the combination was at the same level as that of quercetin and significantly exceeded diclofenac [68].

The study of ultrastructure of the myocardium of rats showed that administration of the quercetin/diclofenac combination restored the ultrastructural organization of the myocardium with only mild manifestations of myocardiodystrophy. This positive effect is due to antialterative properties of the combination and correlates to the results of the morphological study. The level of activity of the combination was again at the same level as that of quercetin and significantly exceeded diclofenac.

Another experimental model utilized to study cardioprotective properties of the quercetin/diclofenac combination was doxorubicin cardiomyopathy in rats, which allowed focusing on antiproliferative activity of the test drug. The lethality of the animals treated with the quercetin/diclofenac combination decreased by 2.7 times compared to the untreated animals while the mass coefficient of heart reached 0.38% with significant difference from the untreated animals. Moreover, biochemical tests showed decreased levels of the lipid peroxidation products and enzymemia. Furthermore, the studied combination caused significant normalizing effect on the level of endogenous *N*-acetylglucosamine with reliable difference compared to diclofenac administration.

ECG findings show reliable superiority of the quercetin/diclofenac combination over diclofenac in normalizing depolarization and repolarization processes in the myocardium of rats, which can be explained by enhancing of oxidative metabolism and consequent compensation of dystrophic and ischemic changes.

The subsequent morphological study demonstrated that, under the conditions of doxorubicin cardiomyopathy, the combination shows pronounced cardioprotective activity decreasing the intensity of proliferative and destructive processes of cardiomyocytes of rats. Moreover, the level of activity of the studied combination was the same as that of quercetin and significantly exceeded diclofenac.

In conclusion, pronounced cardioprotective properties of a new quercetin/diclofenac combination were proven on the models of experimental myocarditis, giving prospects for applying this drug for inflammatory diseases of the myocardium.

8 APPLICATION OF QUERCETIN-BASED DRUGS IN CHRONIC KIDNEY DISEASE

8.1 Intravenous Form of Quercetin in Kidney Failure

Optimization of chronic kidney disease (CKD) treatment is a serious problem of medical practice today. This is not only due to the high risk of adverse drug effects of polypharmacy and low compliance of the patients but is also on account of inevitable development of renal failure, necessity of kidney transplantation, disability, reduction of quality of life, and decreased patients' social significance.

Quercetin is a potentially effective medication for correction of chronic renal failure (CRF) and, consequently, can be used for treatment of CKD. The pharmacological complex of quercetin, namely, its antioxidant, angioprotective, hypotensive, antiplatelet, and diuretic effects, allows suggesting nephroprotective activity. Furthermore, owing to the higher bioavailability, the parenteral form of quercetin—the drug Corvitin—has the best prospects.

Experimentally, the nephroprotective action of Corvitin was evaluated on the different models of renal failure, selected by the severity of its clinical manifestation. Corvitin's role in the successful therapy of CRF was studied in the Department of Clinical Pharmacology and Clinical Pharmacy (National University of Pharmacy, Ukraine). The following issues have been studied:

(1) toxicological characteristics of Corvitin after i.p. (intraperitoneal) administration [69];
(2) establishment of the median effective dose (ED_{50}) of Corvitin for rats with chronic renal failure [69];
(3) Corvitin effects on the course of acute renal failure in laboratory animals [70];
(4) pharmacological properties of Corvitin in experimentally induced chronic renal failure [71];
(5) pharmacological properties of Corvitin in renal failure resulting from the experimentally induced autoimmune glomerulonephritis [72–74];
(6) Corvitin effects on morphology and ultrastructure of renal parenchyma in experimentally induced glomerulonephritis and renal failure.

During the pharmacological investigations an acute toxicity of Corvitin at i.p. administration was studied. Therefore the median lethal dose (LD_{50}) was 5000 mg/kg, being the highest dose which did not lead to death of the test animals [69].

A median effective dose of Corvitin has been chosen based on the model of renal failure, induced by subcutaneous injection of potassium chromate [69]. The rats that successfully developed the pathologic condition were receiving Corvitin at the doses of 50.0–6.3 mg/kg, which equals to 1/100–1/800 LD_{50}. On the 10th day of the experiment, daily diuresis, creatinine levels in blood and urine, glomerular filtration rate (GFR) were evaluated. In consideration of renal failure, relationship between the drug activity and the dose administered, should also be accounted for, hence the ED_{50} of Corvitin was calculated using the method of probit analysis.

The basis for evaluation of nephroprotective action of Corvitin was its ability to increase glomerular filtration, which was estimated by means of measuring the GFR in the group of animals and comparing it with that of the control pathology.

During the experiment, the elevation of GFR, diuresis and serum creatinine normalization after Corvitin administration were discovered. Corvitin affected glomerular filtration to the greatest extent when administered in the 25–50 mg/kg dose range. Hence, the calculated median effective dose of Corvitin for rats with CRF was 33.8 mg/kg and this dose was used in the next investigations. Adjusted for humans by using the

coefficient of species sensitivity the median therapeutic dose is 8 mg/kg, for a human with a body weight of 70 kg the daily dose of Corvitin is 560 mg.

In further experimental studies, as a reference drug, Lespenephryl (a diuretic with hypoammoniemic action) was chosen. In these experiments, the ED_{50} dose of Corvitin (34 mg/kg) was injected i.p. and 1.2 mL/kg of Lespenephryl was administered i.g., which equals the median therapeutic dose for humans (adjusted for rats by using the coefficient of species sensitivity).

Corvitin effects on acute renal failure disease course were studied on the glycerin nephropathy model in rats [70]. Renal failure was modeled by means of i.m. injections of 80% glycerin solution. On the 7th day of the experiment lethality in the intact pathology group was 75%, while in both the Corvitin and Lespenephryl groups lethality was 50%.

In animals with acute renal failure the significant impairment of tubular reabsorption (TR) was observed. In the experiment, this parameter was improving much faster following the administration of Corvitin as compared to Lespenephryl, which probably may be explained by the more potent diuretic action of Corvitin. The same tendency was also observed for the ammonia metabolism parameters. Corvitin was demonstrated to lead to elevation of serum creatinine—it was almost 5 times higher than that of the intact animals.

Pharmacological effects of Corvitin on CRF development were also studied on the model of CRF, induced by subcutaneous administrations of mercuric chloride solution [71]. In 2–3 weeks this pathology assumes a chronic character with high lethality. Thus, in the group of animals with CRF, lethality on the 14th day was 50% and 63% on the 21st day. In the group with Lespenephryl administration, lethality on the 14th and 21st days was 25%, while in the group with Corvitin administration it was 13%.

Analysis of these experimental data with the respective biochemical parameters shows that nephroprotective action of Corvitin results from a significant oxidative stress reduction. This resulted in the normalization of creatinine excretion, potentiation of glomerular filtration, reduction of proteinuria and water reabsorption and normalization of electrolyte and acid-base balance regulation by kidneys.

Nephroprotective properties of Corvitin were also studied at glomerulonephritis with renal failure on the model of active Heiman nephritis in rats [72–74]. The disease progress was monitored on the 30th, 60th and 90th day of the experiment by measuring diuresis, proteinuria level, and urinary sediment examination.

The important characteristic of glomerulonephritis in its late stages is relative diuresis. After administration of Corvitin relative diuresis was significantly higher in comparison with that in the intact pathology group of animals and was equal to that in the group receiving a reference drug. The next important index of kidney filtration is proteinuria. In the group of animals with renal pathology and massive proteinuria (up to 144 mg/day), Corvitin reduced this parameter by 2.3 times—to 63 mg/day—which was lower than that in the group of Lespenephryl.

In this experiment, Corvitin has demonstrated its significant protective action, ensuring normal physiological weight gain and keeping the mass coefficient of kidneys (MCK) in this group of test animals at the level of healthy animals. These parameters give Corvitin an advantage over Lespenephryl, since the latter does not affect the MCK; this parameter, after Lespenephryl treatment, significantly differs from that of the tested substance and does not differ from that observed in untreated animals.

According to the results of histomorphological examination, Corvitin was able to detain morphologic and ultrastructural alterations in animals with experimentally induced glomerulonephritis and renal failure. The data, obtained on the positive effects of Corvitin on renal function in pathologic conditions may be an experimental basis of nephrotective activity of the test drug.

Thus it was determined that Corvitin reduced nephrotic and hydropic syndromes, normalized parameters of kidney function state, electrolyte metabolism, blood biochemical parameters, intensity of free radical oxidization and destruction processes in renal tissue on various models of renal failure, which stipulates general positive influence of the drug on the course of CKD.

8.2 Nephroprotective Effects of Quercetin and Amino Sugars

Data analysis of promising components among antioxidant and membrane-stabilizing substances for a potential medication with nephroprotective action suggests using quercetin and amino sugar glucosamine (or its derivatives) for effective treatment of various clinical forms of CKD.

Quercetin is a well-known antioxidant that has an antiischemic effect and stabilizes cell membranes via lipoxygenase blockade. Glucosamine is a natural human metabolite of connective tissue components such as glycosaminoglycans (GAG), glycoproteins, proteoglycans, etc. Forming the kidney basal and cell membranes and intermediate structural molecules, it realizes nephroprotective action. Thus, the application of the proposed combination for the treatment of CKD is promising since it allows influencing various links of pathogenesis, which may help to synergize nephroprotective effects of the active substances. This proves relevancy of the experimental study of quercetin/glucosamine combination nephroprotective properties and effects of the combination on the development of CKD.

In connection with the foregoing, our research team conducted a comparative study of nephroprotective properties of quercetin/glucosamine combination under the conditions of membranous nephropathy development in rats [75]. In this case, in an experiment on rats with nephropathy, a total index of nephroprotective activity was determined for several quercetin/glucosamine combinations with different ratios between the active substances.

The highest level of the total index of nephroprotective activity—89.9%—was shown by a combination containing quercetin, glucosamine hydrochloride, and *N*-acetylglucosamine in a ratio 2:3:3, which was introduced into the clinical practice in the form of the drug Gluquamine. Thus, it was expedient to conduct further experimental studies to evaluate the prospects of Gluquamine application in kidney diseases.

First, it was necessary to carry out preliminary comparative studies of Gluquamine efficiency on the models of membranous nephropathy and renal failure [76,77].

Gluquamine at a dose of 82 mg/kg, which corresponds to ED_{50} for antiinflammatory activity [78], showed significant nephroprotective action on the model of membranous nephropathy in rats caused by i.p. administration of doxorubicin [76]. In this case, the test drug was administered i.g. for 3 weeks.

The experimental results (Table 27.4) show that on the 21st day of the research Gluquamine caused a significant decrease of proteinuria by 3.5 times, MCK by 3.5 times and an increase of GFR by 2.7 times, which indicates the normalization of the functional state of the kidneys. At the same time, Gluquamine was superior to the comparative drugs (quercetin and amino sugars-glucosamine derivatives) by the degree of effect on some parameters. Thus, the studied drug exerts a pronounced nephroprotective effect on the membranous nephropathy in rats.

A preliminary experimental study of the efficiency of Gluquamine in renal failure was carried out on the model of mercuric chloride nephropathy in rats [77]. The study design was the same as in the previous research.

The data in Table 27.5 show that Gluquamine causes a significant increase of GFR by 5.0 times relative to untreated animals, which indicates the normalization of the renal function. There was also a positive effect on nitrogen metabolism, as evidenced by a significant decrease of the blood urea level by 3.0 times and an increase of the urea clearance rate by 3.3 times. In this case, the test drug was significantly more effective than the comparative drugs (quercetin and glucosamine derivatives). To sum up, Gluquamine exerts a pronounced hypoazotemic and nephroprotective effect in conditions of development of mercuric chloride nephropathy in rats and is a promising drug for correction of renal failure.

For an in-depth study of the nephroprotective and hypoazotemic properties of Gluquamine, as well as its efficiency on the models of CKD, it was necessary to determine its effective doses for this type of activity. The experimental study was performed on a model of renal failure in rats caused by subcutaneous administration of potassium chromate [79]. The test drug was administered i.g. every day for 15 days at doses of 25, 50, and 100 mg/kg, followed by determination of GFR in rats and calculation of the activity parameters and ED_{50} for Gluquamine.

The obtained data (Table 27.6) showed that under the influence of the Gluquamine at 25 mg/kg, the diuresis and GFR increased by 1.2 times and the blood creatinine decreased by 1.4 times. When the test drug was used at a dose of 50 mg/kg, the diuresis increased by 1.3 times, GFR by 1.5 times, and the blood creatinine decreased by 1.8 times. The greatest effect on the renal functions was caused at a dose of 100 mg/kg, which was

TABLE 27.4 Kidney Function Parameters of Rats With Doxorubicin Nephropathy Under the Influence of Gluquamine

Study group	MCK (%)	Proteinuria (mg/day)	GFR (mL/day)
Intact control	0.30±0.01	1.1±0.1	397.8±17.9
Control pathology	0.40±0.02	59.0±6.8	152.9±10.8
Gluquamine 82 mg/kg	0.31±0.01[a]	17.0±2.1[a]	414.7±39.6[a]
Amino sugars 61 mg/kg	0.33±0.01[a]	25.3±1.8[a,b]	368.7±22.3[a]
Quercetin 21 mg/kg	0.37±0.01[b]	35.4±2.2[a,b]	281.3±13.3[a,b]

[a] *Significant difference vs. group of control pathology* ($P<0.05$).
[b] *Significant difference vs. group of animals treated with Gluquamine* ($P<0.05$).

TABLE 27.5 The Effect of Gluquamine on the Parameters in Rats With Kidney Failure

Study group	GFR (mL/day)	Blood urea (mmol/L)	Urea clearance (mL/day)
Intact control	396.4±4.7	4.2±0.2	166.4±8.8
Control pathology	60.9±4.4	22.5±1.3	40.9±1.9
Gluquamine 82 mg/kg	312.8±12.1[a]	7.3±0.7[a]	134.3±5.7[a]
Amino sugars 61 mg/kg	234.8±13.2[a,b]	11.2±0.7[a,b]	118.3±4.9[a,b]
Quercetin 21 mg/kg	182.8±11.9[a,b]	16.3±0.7[a,b]	78.3±5.5[a,b]

[a] *Significant difference vs. group of control pathology* ($P<0.05$).
[b] *Significant difference vs. group of animals treated with Gluquamine* ($P<0.05$).

TABLE 27.6 Nephroprotective Parameters of Gluquamine at Different Doses in Rats With Renal Failure

Study group	Diuresis (mL/day)	Blood creatinine (μmol/L)	GFR (mL/day)	Activity (%)
Intact control	6.9±0.2	57.4±2.7	391.6±12.1	–
Control pathology	3.9±0.1	212.8±16.8	168.7±8.3	–
Gluquamine 25 mg/kg	4.6±0.2[a,b]	154.9±14.1[a,b]	209.5±5.3[a-c]	18.3±2.4[b,c]
Gluquamine 50 mg/kg	5.0±0.1[a,b]	120.7±9.6[a,b]	258.7±8.9[a,b]	40.4±4.0[b]
Gluquamine 100 mg/kg	6.1±0.2[a,c]	71.2±4.8[a,c]	300.7±11.1[a,c]	59.2±5.0[c]

[a] *Significant difference vs. group of control pathology* ($P<0.05$).
[b] *Significant difference vs. group of animals treated with Gluquamine at 100 mg/kg* ($P<0.05$).
[c] *Significant difference vs. group of animals treated with Gluquamine at 50 mg/kg* ($P<0.05$).

manifested in an increase of the diuresis by 1.6 times, GFR by 1.8 times, and a reduction of blood creatinine by 3.0 times.

Under the influence of Gluquamine, there was a significant, dose-dependent increase of glomerular filtration relatively to the untreated animals. Thus, when the test drug was used at a dose of 25 mg/kg, the activity was 18.3%, 50 mg/kg was 40.4%, and 100 mg/kg was 59.2% (Table 27.6). These results allowed use of the probit analysis method to calculate ED_{50} of Gluquamine for the treatment of renal failure, which equaled 79.7 mg/kg. This dose is useful for further experimental study of Gluquamine as a drug for treatment of CKD.

Glomerulonephritis has great medical and social importance, since it is one of the main causes of CKD. Currently, there are no effective drugs for glomerulonephritis treatment.

Nephroprotective properties of Gluquamine were studied on the model of glomerulonephritis with renal failure [80]. The study was carried out on the late stages of active Heiman nephritis in rats. The test drugs were administered i.g. for 2 months starting 2 months after the start of the experiment.

The results of the experiment on the 120th day (Table 27.7) showed that Gluquamine caused a significant decrease of proteinuria by 2.3 times (to 52 mg/day) relative to the untreated animals. In addition, there was an increase of relative diuresis by 23% and GFR by 1.8 times (up to 255 mL/day), which indicates the normalization of the functional state of the kidneys. Also, there was an increase of the blood total protein by 1.7 times and a decrease of creatinine by 1.8 times and urea by 1.6 times, which indicates an improvement of protein and nitrogen metabolism and a reduction of the renal failure manifestations. At the same time, the efficiency of the reference drugs (quercetin and Lespenephryl) was significantly lower than that of Gluquamine. In conclusion, the test drug has a pronounced positive effect on the development of glomerulonephritis with renal failure in rats and is a promising drug for CKD therapy.

The main cause of the development of the terminal stage of CKD is diabetic nephropathy. In connection with this, the efficiency of Gluquamine has also been confirmed in experimental studies on the model of diabetic nephropathy in rats, which was caused by i.p. administration of alloxan. Gluquamine was administered i.g. everyday for 3 months.

The data given in Table 27.8 indicate that, on the 90th day of the experiment under the influence of Gluquamine, the mortality of animals with pathology significantly decreased by 2.3 times. Also, the drug caused a significant decrease of MCK (to 0.45%) and proteinuria

TABLE 27.7 The Effect of Gluquamine on the Course of Glomerulonephritis With Renal Failure in Rats

Study group	Relative diuresis (%)	Proteinuria (mg/day)	GFR (mL/day)	Blood total protein (g/L)	Blood creatinine (μmol/L)	Blood urea (mmol/L)
Intact control	50.8±0.6	1.4±0.1	422.5±13.0	72.3±2.1	52.0±2.5	4.3±0.3
Control pathology	37.9±0.7	118.8±9.7	138.6±6.6	33.2±1.5	139.4±9.6	17.6±0.6
Gluquamine 80 mg/kg	46.7±0.5[a]	51.7±3.5[a]	255.0±8.5[a]	55.3±1.6[a]	79.3±3.8[a]	11.2±0.6[a]
Quercetin 20 mg/kg	43.3±0.7[a,b]	65.3±4.6[a]	213.7±8.0[a,b]	47.9±2.5[a,b]	110.7±8.8[a,b]	14.4±0.9[a,b]
Lespenephryl. 1.2 mL/kg	53.4±0.7[a,b]	83.0±5.6[a]	235.8±7.7[a]	39.4±1.1[a,b]	94.1±4.4[a,b]	13.5±0.7[a,b]

[a] *Significant difference vs. group of control pathology* ($P<0.05$).
[b] *Significant difference vs. group of animals treated with Gluquamine* ($P<0.05$).

TABLE 27.8 The Effect of Gluquamine on the Course of Diabetic Nephropathy in Rats

Study group	Mortality (%)	MCK (%)	Proteinuria (mg/day)	GFR (mL/day)	TR (%)	Blood urea (mmol/L)
Intact control	–	0.31±0.01	1.3±0.1	446.3±13.6	98.2±0.1	4.4±0.3
Control pathology	58.3	0.55±0.02	35.6±4.1	203.2±8.8	93.3±0.3	12.0±1.1
Gluquamine 80 mg/kg	25.0[a]	0.45±0.01[a]	17.9±1.3[a]	309.1±12.0[a]	96.7±0.1[a]	8.5±0.3[a]
Quercetin 20 mg/kg	33.3	0.50±0.01[a,b]	25.3±2.0[a,b]	258.7±8.9[a,b]	95.4±0.2[a,b]	10.5±0.7[b]
Lespenephryl 1.2 mL/kg	41.7	0.52±0.02[b]	30.0±2.7[b]	268.4±6.8[a,b]	94.3±0.2[a,b]	10.0±0.4[b]

[a] *Significant difference vs. group of control pathology* ($P<0.05$).
[b] *Significant difference vs. group of animals treated with Gluquamine* ($P<0.05$).

by 2.0 times, which suggests a decrease of renal damage and inflammatory-destructive processes in kidneys. The total improvement of the functional state of the kidneys is indicated by a significant increase of GFR by 1.5 times (up to 309 mL/day) and TR (up to 96.7%). Also, there was an increase of blood urea level by 1.4 times, which indicates an improvement of nitrogen metabolism. Importantly, Gluquamine was superior to the comparative drugs (quercetin and Lespenephryl) by the degree of pharmacological effect. Thus, under the conditions of diabetes mellitus type I nephropathy in rats, Gluquamine exerts a pronounced nephroprotective and hypoazotemic action.

In conclusion, the results of the conducted studies prove that the combination of quercetin with the glucosamine derivatives (glucosamine hydrochloride and *N*-acetylglucosamine) makes it possible to expand the pharmacodynamic spectrum of this flavonoid and enhance its nephroprotective and hypoazotemic effects. Thus, the original complex drug Gluquamine, containing quercetin and glucosamine derivatives, is a promising medicament for the treatment of CKD.

9 QUERCETIN-BASED DRUGS IN JOINT DISEASES

9.1 Chondroprotective Properties of Quercetin

Osteoarthritis (OA) is the most common rheumatological disease, affecting, according to different sources, up to 14% of the adult population. This fact determines relevancy of the constant research for new effective chondroprotective drugs.

In such conditions, quercetin is of scientific interest, since its broad pharmacodynamics spectrum allows expecting the presence of chondroprotective activity. Antiinflammatory properties, caused by 5-lipoxygenase inhibition, as well as antioxidant, antihypoxic, and antiapoptotic effects are especially noteworthy, concerning the ability of quercetin to increase the resistance of chondrocytes against adverse conditions of a pathologically changed joint.

Chondroprotective properties of quercetin were confirmed in experimental studies, conducted by our research team. Quercetin was studied in a pectin-modified form—the drug "Quertin." Such modification provided expressed pharmacokinetic advantages, i.e., higher C_{max}, $AUC_{0-\infty}$, bioavailability, and decreased elimination rate and $T_{1/2}$ [46]. ED_{50} was calculated to be 10 mg/kg and LD_{50} exceeded 5000 mg/kg after i.g. administration, reinforcing quercetin in the status of a low-toxicity drug [78]. The new, modified form of quercetin demonstrated its superiority on the models of exudative, proliferative, and alterative inflammation [81,82].

The study on the model of systemic steroid OA showed antiinflammatory, chondroprotective, and antiapoptotic activity of quercetin as levels of connective tissue markers decreased with significant differences with untreated animals and without significant differences with healthy animals [83].

Morphological study confirmed a more pronounced reparative and antiinflammatory activity of quercetin in comparison with unmodified quercetin and diclofenac. The study of ultrastructure if the joints demonstrated substantial intensification of metabolic processes in chondrocytes and reduced manifestations of degenerative and dystrophic processes. The test object possesses an antiapoptotic effect, which can be interpreted as one of the mechanisms of realization of its chondroprotective activity [34].

On the model of collagen-induced arthritis, quercetin exhibits pronounced positive action on the joint and bone tissue metabolites as shown by the results of clinical, biochemical, and immunoenzymatic studies. The blood concentration of eicosanoids is a highly informative index of experimental arthritis treatment efficacy. It was shown, that quercetin reliably decreases the blood concentration of leukotriene B_4 in comparison with untreated animals. Histomorphological findings confirm positive changes after administration of quercetin [84].

One of the advantages of quercetin is that it can be administered to patients with rheumatoid arthritis with secondary osteoarthritis as opposed to traditional chondroprotectors.

Subsequent clinical trials were dedicated to the study of pharmacokinetics and tolerability of quercetin. It was demonstrated, that the overall tolerability of a single 40 and 240 mg dose of a modified form of quercetin was assessed as "good" in 50% of healthy volunteers and "acceptable" in another 50% of cases [47,85]. The received results allow regarding Quertin as a virtually safe medication for wide application in clinical practice.

9.2 Application of Quercetin and Amino Sugars for Osteoarthritis

A quercetin/glucosamine combination is a promising medication for treatment of OA. This approach allows significantly enhancing chondroprotective properties of quercetin, since glucosamine is a well-known symptom-modifying remedy for treatment of OA.

As a result of our research, the optimal correlation of active ingredients was established, that is, the mixture of amino sugars and quercetin, which was (3:1) in a 81.9-mg/kg dose [78]. It was determined that combined application of glucosamine derivatives with quercetin results in the origin of a pharmacodynamic synergism effect in the form of potentiation of action, which was confirmed on the models of exudative, alterative, and proliferative inflammation [86,87]. The conducted experiments proved that the composition has a complex mechanism with predominant influence on the lipooxygenase path of arachidonic acid metabolism. It was verified that the investigated composition belongs to the relatively harmless substances.

On the models of different variants of OA (posttraumatic, nonspecific, and systemic steroidal OA) the composition containing the mixture of amino sugars and quercetin showed the higher antiinflammatory and chondroprotective activity in comparison with its monocomponents, diclofenac and Teraflex [88–90]. Such composition's properties stipulate the possibility of its subsequent clinical study in patients with different variants of OA. Posttraumatic OA is characterized by local changes in joints with prior destructive elements; nonspecific OA is local changes in joints with prior inflammatory changes; steroidal OA shows systemic generalized dystrophic degenerative character. These experimental models allow encompassing all etiopathogenetic factors of OA.

The application of the composition promotes the normalization of biochemical parameters in rat blood serum [88]. Histological and morphologic researches of animals' joint cartilage after treatment with the composition confirm the decreasing of inflammatory and intensification of reparative processes in joints [90].

The investigated composition has a proapoptotic effect, promotes the prevention and deceleration of chondrocytes death in joint cartilage, which can be interpreted as one of the possible mechanisms of chondroprotective activity realization [91]. The composition decreases the number of apoptotic chondrocytes to 7%, amino sugars complex to 9.1%; quercetin to 16.3%, and diclofenac to 38.5%.

The medical and preventive effect of the composition is connected with the increase of chondrocytes' protective properties against the influence of apoptotic factors, enhancing the penetrating ability of chondrocytes' plasmatic membranes for amino sugars and ability to store in more higher concentrations, which will help to decrease the level of proteoglycan deficiency of matrix and to offset the processes of catabolism and anabolism of cell chondrocyte homeostasis.

The results of experimental research make it possible to recommend the clinical application of Gluquamine in patients with diseases of the musculoskeletal system. The recommended dose and regime of capsule application is the following: the daily dose is 14.0 mg/kg or 980 mg/day (according to the amount of active ingredients); 1 capsule 3 times a day during 6 weeks.

9.3 Quercetin and Diclofenac Combination in Joint Diseases

Nonsteroidal antiinflammatory drugs (NSAIDs) are recommended by the leading rheumatologic societies (EULAR, ESCEO, OARSI, ACR) as first-line symptomatic treatment of both inflammatory joint diseases, like rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthropathy, and primarily degenerative diseases, such as osteoarthritis.

Furthermore, in spite of recent concerns about the cardiovascular safety of diclofenac, this particular NSAID continues to hold leadership within its pharmacological group in many countries [92].

The fact that treatment with NSAIDs, on the one hand, is undoubtedly effective and necessary and, on the other hand, poses a substantial problem with possible development of gastro-, cardio-, nephro-, hepato-, and chondrotoxic adverse drug effects, necessitates ongoing scientific research aimed at creating better drugs of this group. One of the ways to fulfil such a goal consists of combining NSAIDs with other pharmacological substances that provide positive effects.

Following, we provide the results of our pharmacological research, which uses such an approach and is dedicated to the study of a pharmaceutical composition of quercetin and diclofenac. The rationale for such a combination is the antiinflammatory, antioxidative, and antiapoptotic activity of quercetin. The scope of the research involves the study of acute toxicity, median effective dose, ulcerogenic activity, analgesic activity, and

antiinflammatory activity on stages of alteration, exudation, and proliferation activity on the model of collagen-induced osteoarthritis and steroid arthrosis.

It has been proved that the quercetin/diclofenac combination may improve safety and effectiveness of antiinflammatory therapy and is the potentially resultative drug for treatment of degenerative-destructive and degenerative-dystrophic joint diseases.

The quercetin/diclofenac combination belongs to the class of low-toxic drugs, since its LD_{50} after i.g. administration is 952.5 ± 45.2 mg/kg for rats. Gastric toxicity of diclofenac as a component of this combination reduces by 1.3 times, since the mean ulcerogenic dose (UD_{50}) equaled 164.5 ± 10.3 mg/kg (63.3 ± 4.0 mg/kg for diclofenac), while the UD_{50} of diclofenac alone is 48.0 mg/kg. The tolerance index of the test drug is higher than that of diclofenac by 1.5 times (9.2 against 6.0), and the therapeutic index is higher by 4.5 times (53.5 against 11.9), which proves the higher level of safety of the test combination.

The mean effective dose of the quercetin/diclofenac combination for its antiinflammatory activity is 17.8 ± 1.9 mg/kg for the sum of the active substances, which corresponds to 6.8 ± 0.8 mg/kg for diclofenac. The drug is recommended for clinical trials in the mean effective daily dose of 4.0 mg/kg or 280 mg.

The studied drug demonstrates prominent antiexudative effects, since it realizes a complex effect on the inflammatory reaction, induced by different proinflammatory agents, and induces positive therapeutic and prophylactic effects on toxic pulmonary edema (Fig. 27.4).

On all of the six models the quercetin/diclofenac combination demonstrated more potent inhibition of inflammation in comparison with both quercetin and diclofenac. Yet this activity was unequally pronounced on the different models and demonstrated different advantages above the reference drugs. This can be explained by the different roles of individual proinflammatory mediators in the particular inflammation model and by the different activity of diclofenac and quercetin towards these proinflammatory mediators [93].

We can summarize the results of these studies in the following way:

- the quercetin/diclofenac combination has a complex mechanism of action, inhibiting the exudative inflammation induced by different proinflammatory agents (carrageenan, histamine, serotonin, zymosan and prostaglandins);
- quercetin and diclofenac, combined in single dosage form mutually increase antiinflammatory effects of each other, realizing an additive action;
- prominent antiexudative effect of this combination drug, demonstrated on the model of carrageenan- and prostaglandin-induced inflammation, is based primarily on anticyclooxygenase effect of diclofenac;
- effects of the combination on zymosan-induced inflammation are realized through anti-leukotriene properties of quercetin;
- effects of the studied drug on the models of histamine- and serotonin-induced edema in the first hours of inflammation are based on anti-leukotriene and antihistamine action of quercetin, while in the next hours they are realized through anti-cyclooxygenase action of diclofenac;
- on the model of toxic pulmonary edema, activity of the quercetin/diclofenac combination was 3.0 times higher than that of quercetin and 1.2 times higher than that of diclofenac;

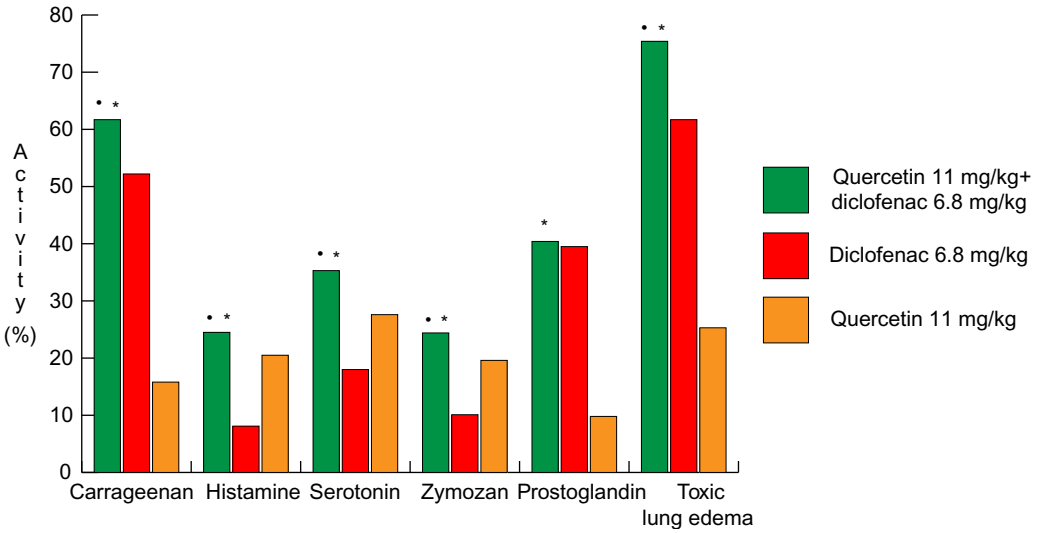

FIG. 27.4 Antiexudative activity on various models of exudative inflammation in rats: •—significant difference vs. group of animals treated with diclofenac ($P < 0.05$); *—significant difference vs. group of animals treated with quercetin ($P < 0.05$).

- due to inhibition of the inflammatory reactions induced by the practically full range of the known proinflammatory mediators, the quercetin/diclofenac combination in the dose of 17.8 mg/kg may be recommended as an effective antiinflammatory agent, characterized by its complex action on the course of inflammatory and destructive processes of different etiology.

The discussed drug also positively affects the alteration phase of inflammation on the model of scarified wounds in rats (Fig. 27.5), revealing higher antialterative activity in comparison with quercetin (by 1.2 times) and diclofenac (by 1.2–1.4 times during different periods of observation). Complete epithelization was observed on the 21th day in the control group; on the 15th day in the quercetin/diclofenac group; on the 19th day in the quercetin group; on the 21st day in the diclofenac group [93].

Anti-proliferative activity of the quercetin/diclofenac combination on the model of cotton granuloma was moderate, demonstrating antiproliferative activity, which was 1.3 times higher than that of quercetin and 1.8 times higher than that of diclofenac [93].

Therefore, studying the effects of the test drug on different phases of inflammation, we detected the highest activity on the models of exudative and alterative inflammation. The studied combination also demonstrated a moderate activity toward the proliferation phase of inflammation. This pattern corresponds to the negative correlation between antialterative and antiproliferative activity of NSAIDs, which can be explained by the ability of these drugs to inhibit the synthesis of collagen. This property is positive in the treatment of rheumatoid arthritis and negative in the treatment of osteoarthritis.

But in our case, the modification of the antiinflammatory potential of diclofenac by quercetin increases the antialterative activity, which can improve the efficiency of the therapy of destructive diseases, while not decreasing the efficiency for proliferative ones.

During the evaluation of analgesic effects on the model of inflammatory hyperalgesia, we established the ED_{50} of the quercetin/diclofenac combination for this kind of activity, which is 12.0 ± 1.9 mg/kg (4.6 ± 0.7 mg/kg for diclofenac) [94]. In the animals with spontaneous pain reaction due to acute gonarthritis, the test drug in the dose of 12.0 mg/kg after single and repeated administrations induced a potent analgesic effect, which was reliably higher than that of diclofenac on the 5th day. Based on these figures, the studied drug is considered effective in the therapy of chronic joint pain in rheumatologic patients and has certain advantages above diclofenac [95].

The quercetin/diclofenac combination positively affects the course of autoimmune collagen-induced osteoarthritis, which was evident from the normalization of functional condition of joints, clinical and biochemical parameters, serum dynamics of eicosanoids, and from the condition of morphological structure of articular tissues of rats. This effect was practically equal to that of diclofenac, being even higher for several parameters [96].

Especially representative in this regard are the results of the immunoenzymatic analysis, which perfectly reflect the mechanism of antiinflammatory action of the active substances of the test and reference drugs. Following the administration of the test combination, prostaglandin E_2 level decreased by 1.4 times; prostaglandin I_2 decreased by 1.1 times; thromboxane B_2 decreased by 1.9 times; leukotriene B_4 decreased by 1.2 times. Thus, the quercetin/diclofenac combination revealed a more potent effect than quercetin on the level of prostaglandin E_2 and thromboxane B_2 due to the anticyclooxygenase activity of diclofenac. On the other hand, the studied drug

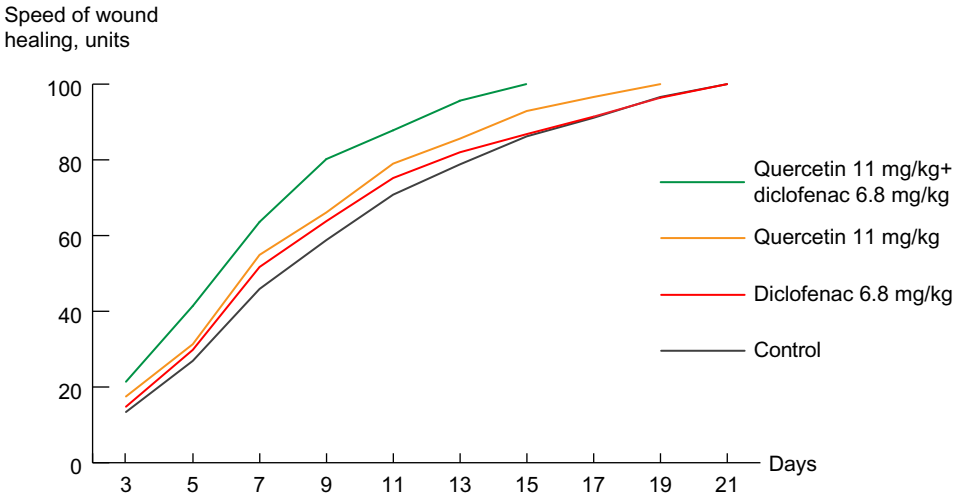

FIG. 27.5 Scarified wound healing in rats. $P < 0.05$ for the quercetin/diclofenac combination vs. both reference drugs at all stages of observation.

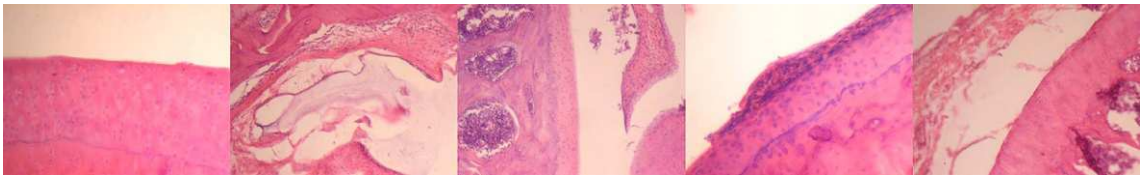

FIG. 27.6 Histomorphology of joint tissues of rats with collagen-induced arthritis on day 28 of the experiment, from left to right: intact control (healthy), control pathology (untreated), quercetin/diclofenac combination treatment, quercetin treatment, diclofenac treatment.

was more potent than diclofenac in terms of its effects on the level of leukotriene B_4 due to the ability of quercetin to inhibit lipoxygenase. Therefore, out of those three drugs, the quercetin/diclofenac combination covers the widest range of proinflammatory eicosanoids and has the complex mechanism of action on the cyclooxygenase and lipoxygenase metabolic pathways of arachidonic acid [96].

The histological examination confirmed that the test combination, just like diclofenac, was able to normalize the morphologic structure of the joints in rats with collagen-induced arthritis, nullifying cartilage destruction and reducing synovial hyperplasia (Fig. 27.6). After the treatment with the test drug, there were only residual changes in synovial membranes, while after the diclofenac therapy there were residual changes in periarticular tissue; quercetin therapy was less effective in terms of the reduction of articular cartilage destruction and synovial and periarticular inflammation; the hyperplastic processes in synovia and pannus formation were still ongoing [97].

Therefore, just like in the previous phases of the study, the experiment on the collagen-induced osteoarthritis model confirmed the high antiinflammatory activity of the quercetin/diclofenac combination.

On the model of systemic steroid arthritis in rats, the combination revealed moderate chondroprotective effects, improving the course of the disease due to normalization of clinical and biochemical parameters and positively affecting morphology and ultrastructure of articular tissues. The mechanism of chondroprotective effect of the quercetin/diclofenac combination is based on the prevention of apoptosis of chondrocytes, which was evident from the reduction in TUNEL-positive apoptotic cell number. The chondroprotective action of this drug is practically equal to that of quercetin and is reliably higher than that of diclofenac [98].

The quercetin/diclofenac combination induced a mild decrease in the serum level of all the markers of connective tissue disorganization, mainly chondroitin sulfates and sialic acids, which showed reliable deviations in comparison to the control pathology group. Thus, the level of chondroitin sulfates and glycoproteins decreased by 1.2 times, and the level of sialic acids decreased by 1.3 times. These results can be explained by the high antialterative activity of quercetin when used as the component of this combination, and as a monocomponent drug, which is known to prevent destruction of animal cartilage and therefore chondroitin sulfate leak into the bloodstream [98].

Another important biochemical marker of the activity of the pathologic changes in articular tissues of the animals with systemic steroid arthritis is the total level of GAG and GAG fractions in the rat blood. After administration of the test drug to the animals, its pharmacological activity manifested in the reliable decrease in total GAGs in comparison with the control group, with the decrease not only in chondroitin-4-sulfates, but also in chondroitin-6-sulfate level, which reached the level observed in the intact animals. Moreover, the ratio of these fractions was practically stable, and the previously mentioned changes were considered reliable in comparison to the control group only for the dynamics of chondroitin-4-sulfates. On the contrary, diclofenac did not demonstrate any positive effects on GAG levels. The best effect on total GAGs and their fractions in the blood was detected after the administration of quercetin [98].

Moreover, the treatment with the quercetin/diclofenac combination has led to a reliable increase in levels of free *N*-acetylglucosamine, when compared to that during the treatment with diclofenac. We know that the low concentration of this fraction is associated with decreased regenerative abilities of damaged cartilage. Therefore, we can assume that connective tissue has a higher regenerative potential during experimental therapy [98].

The results of morphologic examination demonstrate a moderate therapeutic effect of the studied drug—the articular surface of cartilage becomes smoother, with no signs of exfoliation; cytoarchitecture normalizes; the matrix is stained more evenly, with minimal signs of disorganization and dystrophic changes of chondrocytes (Fig. 27.7).

It was also discovered, that the quercetin/diclofenac combination is able to decrease the number of chondrocytes dying by way of apoptosis during systemic steroid arthritis. Thus, the percentage of TUNEL-positive cells in the quercetin/diclofenac group was 11.8%, in the diclofenac group 40%, in the quercetin group 16.3%, in the control pathology group 55%–70% and in the intact group 1% [99]. The modifying effects of quercetin are probably based on its antioxidant and antileukotriene activity and by the inhibition of proteasomes on a molecular level [33].

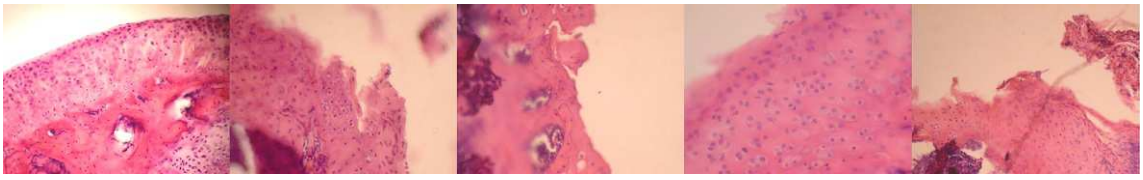

FIG. 27.7 Histomorphology of joint tissues of rats with systemic steroid arthritis on day 56 of the experiment, from left to right: intact control (healthy), control pathology (untreated), quercetin/diclofenac combination treatment, quercetin treatment, diclofenac treatment.

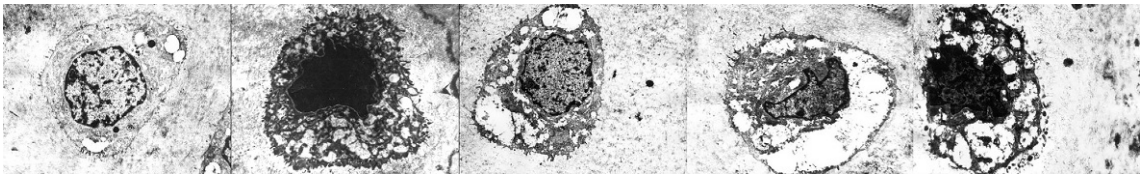

FIG. 27.8 Ultrastructure of joint tissues of rats with systemic steroid arthritis on day 56 of the experiment, from left to right: intact control (healthy), control pathology (untreated), quercetin/diclofenac combination treatment, quercetin treatment, diclofenac treatment.

During the examination of chondrocyte ultrastructure, we also detected positive changes, such as the stimulation of biosynthetic processes of cells, induced by the administration of the test drug to the animals (Fig. 27.8). This is evident from the growth of the membrane structures, visible as long profiles of granular endoplasmic reticulum and tubular Golgi complexes, and from the absence of chondrocytes in different stages of cell death in the examined microslides [100].

The quercetin/diclofenac combination underwent phase I of clinical trials in healthy volunteers. The trial scrutinized pharmacokinetics after single oral administration in comparison with quercetin and diclofenac.

The results conclude that quercetin and diclofenac do not have negative interactions and go together well in one dosage form. The pharmacokinetic profile of diclofenac as part of the quercetin/diclofenac combination was similar to both the profile of individual diclofenac administration and the profile when diclofenac was administered together with quercetin but in separate dosage forms. However, for combined diclofenac, there was a higher rate of absorption, bioavailability (by 5%–8%) and blood retention time, implying longer duration of the pharmacological effect of the drug.

The pharmacokinetic profile of quercetin as part of the quercetin/diclofenac combination had major advantages over individual quercetin (Quertin) administration with bioavailability of the flavonoid increasing by more than 2 times. Thus, pharmacokinetic properties of quercetin as part of the quercetin/diclofenac combination significantly improved compared to Quertin, leading to undoubted increase of therapeutic effect.

The study of this combination further revealed good parameters of single dose tolerability, that didn't differ from those of quercetin and diclofenac.

The study of tolerability of the course of increasing doses also yielded positive results. The first group of volunteers was given 120 mg of quercetin/75 mg of diclofenac daily while the second group was administered 240 mg of quercetin/150 mg diclofenac daily for 5 days. During the whole period of the study, there were no adverse drug reactions in the first group and the tolerability was assessed as good. In the second group, in 25% of the volunteers, there were mild adverse drug reactions which didn't necessitate the withdrawal of the studied drug.

To sum up, the quercetin/diclofenac combination is a promising drug for implementation into clinical practice with the aim of treatment of rheumatologic patients.

10 CONCLUSION

Quercetin is a polyphenol substance with multidirectional activity on the human body, i.e., antioxidant, modulation of multiple enzymes, interaction with contractile proteins, antiplatelet, apoptosis decrease, antiinflammatory activity, nephro- and gastroprotective effects, antiarrhythmic, myocardial necrosis decrease in myocardial infarction, and chondroprotection.

Such a wide variety of favorable properties makes quercetin an extremely interesting substance for both maintaining and restoring of health. This manifests in development of quercetin-based drugs, such as combinations of quercetin with glucosamine and quercetin with diclofenac.

Combining quercetin with glucosamine derivatives enhances nephro-, cardio-, and chondroprotective effects of the flavonoid as well as increases its oral bioavailability. High effectiveness of this combination as compared to ordinary quercetin and other reference drugs is proven in experimental studies on the models of glomerulonephritis, kidney failure, membranous, and diabetic nephropathy. Besides, the combination has also showed marked effectiveness in experimental myocarditides and osteoarthritides.

Currently, the quercetin/glucosamine combination has passed clinical approbation and has been implemented into clinical practice. It is successfully used as a nutriceutic not only for kidney, heart, and joint pathologies but also for prophylaxis of complications of these conditions.

Taking into account the results of preclinical studies, the potential indications for treatment with the combination drug containing quercetin and diclofenac are inflammatory-destructive diseases (rheumatoid arthritis, ankylosing spondylitis, rheumatic fever), degenerative-dystrophic diseases (osteoarthritis, osteoporosis) and pain syndrome in gout, lumbago, tunnel syndrome, traumatic concussions, sprains, gynecologic diseases, and myalgia. This is due to higher antiinflammatory and analgesic activity of this combination compared to diclofenac owing to the antioxidant, antihypoxic, and antiapoptotic properties of quercetin.

Moreover, the quercetin/diclofenac combination demonstrated high effectiveness on the models of inflammatory myocardial damage, suggesting expediency of its application in rheumatic myocarditis.

Positive results of preclinical studies were confirmed on the clinical stage. The quercetin/diclofenac combination passed stage I of clinical trials, having demonstrated good tolerability compared to diclofenac and improved pharmacokinetic parameters compared to quercetin. All the foregoing implies expediency of implementation of this combination into clinical practice for treatment of inflammatory and destructive diseases of the joints as well as myocarditis.

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SECTION V

POLYPHENOLS IN DISEASE

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CHAPTER

28

Green Tea Polyphenols in the Amelioration of Osteoarthritis: Memoir on the Preclinical Observations

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1 INTRODUCTION

Osteoarthritis (OA), an age-related condition, caused in part by injury, loss of cartilage structure and function, and an imbalance in inflammatory and antiinflammatory pathways, is an ailment as old as mankind [1]. It is not life threatening and primarily affects the articular cartilage and subchondral bone of synovial joints and results in joint failure, leading to pain upon weight bearing, including walking and standing [1]. The ailment progresses in a gradual manner and is associated with painful, inflammatory conditions that affect the mobility. Global reports indicate that around 20–40 million suffer from the disability [2]. In the recent past, the prevalence of the disease has increased and has reached high proportions in countries where there is considerable proportion of elderly people [2,3]. OA is an important public health challenge due to its increasing incidence in both the developed and developing world due to the high socioeconomic impact [3].

From a histological perspective, osteoarthritis is associated with destruction of articular cartilage and the exposure of the subchondral bone, causing pain during movement [4]. Some of the most important symptoms associated with osteoarthritis of the knee include joint stiffness, deformity, pain on movement of the knee, joint swelling, and limited range of movement. The pain and disability caused affects the individual's social life [4]. Studies have indicated that the pathogenesis of OA is influenced by a myriad of factors and the interplay of genetics, gender, age, obesity, and joint trauma are known to be the chief contributory factors [4]. Additionally, mechanical aspects, such as joint instability and repetitive microtrauma, have also been implicated in perpetuating/aggravating the pathogenic process [4].

Currently there is no cure to OA as there is no treatment that can reverse or restore the changes in the damaged cartilage [1]. Under these circumstances the goals of treatment are to relieve pain, maintain or improve joint mobility, increase the strength of the joints, and minimize the disabling affects of the disease [1,5]. From a pharmacological perspective, analgesics that are effective in mitigating pain are the mainstay and may be prescribed along with the chondroprotective agents [4,6,7]. However, long-term consumption of these agents is associated with untoward effects and negates the beneficial effects [2,3]. In advanced stages, when cartilage is eroded or absent, the analgesic treatment is of no benefit and surgical intervention, where replacement of the articular surfaces of the afflicted part is performed, becomes useful [3]. However, the fear of surgery is a major deterrent for some patients.

The chronic nature of the disease, severe morbidity, and suboptimal response to conventional drugs, and the fear of surgery persuades the patient to use unconventional treatments, with a hope that it will decrease the symptoms of the disease and increase the quality of life

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[2,3]. Unsurprisingly patients are looking to alternative and complementary medicine and plant-based medicines and dietary agents are widely marketed and used by people afflicted by OA [8]. Recent studies have shown that dietary agents like turmeric, green tea, soy protein, citrus fruits, and pomegranate are useful in delaying the pathological process and mitigating the OA-associated pain [1]. This chapter collates the reports on usefulness of green tea and its constituents in OA by focusing on the molecular antiosteoarthritic mechanisms responsible for the beneficial effects.

2 GREEN TEA IN ARTHRITIS

Green tea is one of the highly invested plants and preclinical studies have now shown it to be beneficial in delaying the process of osteoarthritis and in mitigating the pain [9–13]. Green tea is a widely consumed beverage and has been cultivated and consumed by humans for thousands of years [12,13]. Historical evidence suggests that the tea plant was a native of China, Burma, Thailand, Laos, and Vietnam, but today is also cultivated in Sri Lanka, India, and Japan [12,13].

Depending on the way the leaves are harvested and processed, they are categorized as green, black, and oolong tea powder [12,13]. Traditionally tea was consumed in the green tea form in China and is still consumed in this form in most Asian countries. However, over time and especially in the last century, black tea has gained popularity over green tea in the United States and Europe [12,13]. From a scientific perspective, of the three tea types, green tea has been highly investigated and reported to be useful in a myriad of ailments and diseases afflicting humans [12,13]. This has consequentially increased public awareness and popularized its consumption [12,13].

3 PHYTOCHEMISTRY OF GREEN TEA

Green tea is one of the highly investigated plants and detailed information on the phytochemical constituents is available. The active compounds of green tea are the

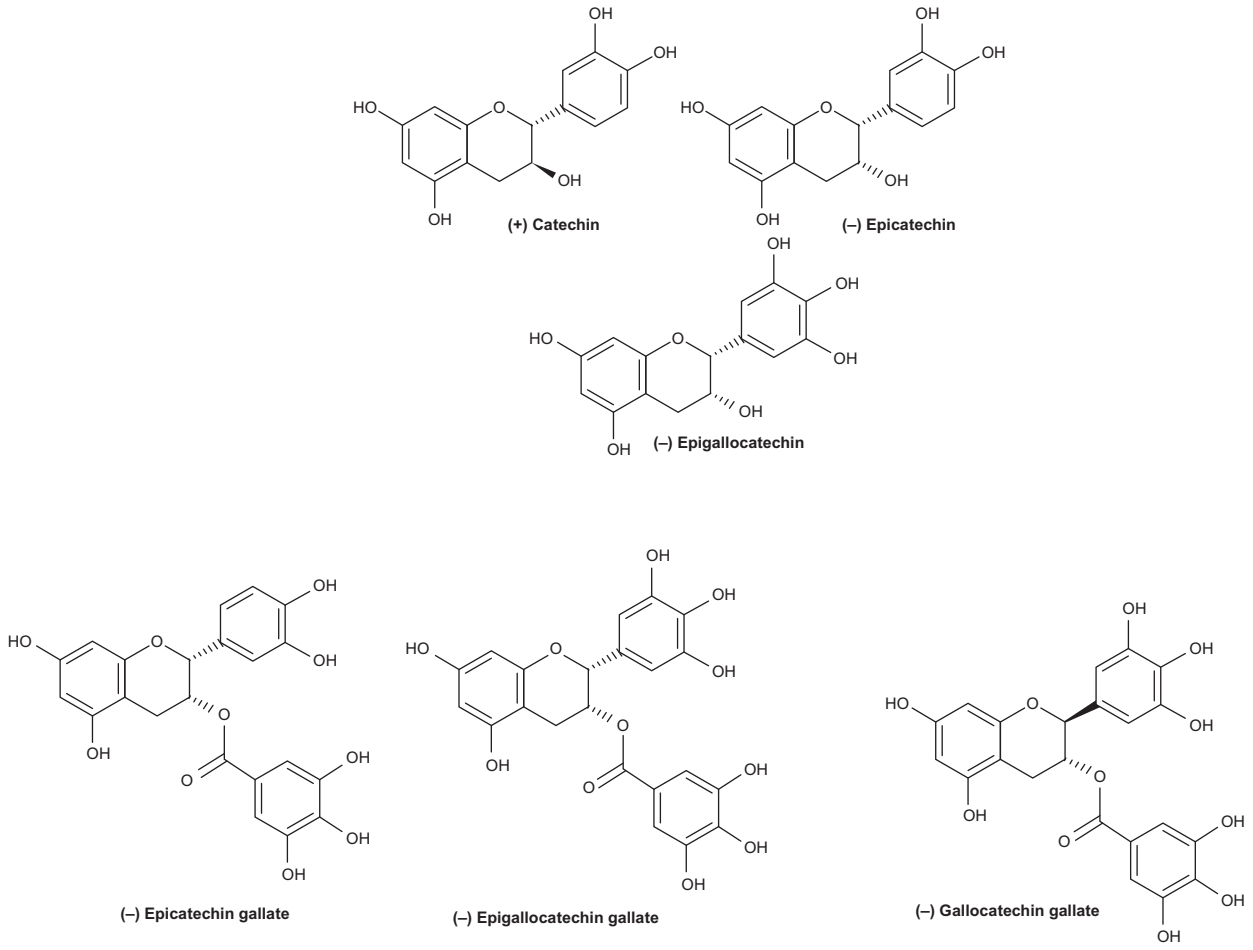

FIG. 28.1 Polyphenols present in green tea.

catechins epicatechin, epigallocatechin, epicatechin-3-gallate, epigallocatechin-3-gallate, proanthocyanidins, flavonols (kaempferol, quercetin, and myricitin in the form of glycosides), and gallic acids [9–13]. The structures of important polyphenols are represented in Fig. 28.1. Green tea also contains proteins, carbohydrates, vitamins, minerals, and trace elements like Ca, Mg, Mn, Fe, Cu, and Zn, constituents known to be useful in maintenance of health [12,13].

4 TRADITIONAL AND VALIDATED USES

In traditional systems of medicine, green tea is reported to be useful as a stimulant, a diuretic, an astringent, wound healing agent, and to improve the function of the heart [9–11]. Other traditional uses of green tea include treating gas, regulating body temperature, regulating blood glucose, improving digestion, improving mental processes, controlling body weight and cardiovascular disease, helping bone mineral density, improving cognitive function, treating neurodegenerative disease, and preventing cancers [9–11,13]. The most important aspect that makes green tea an important dietary agent with medicinal benefit is its potent antiinflammatory, antibacterial, antiviral, antimutagenic and antiaging properties [9–11,13]. In the following section the mechanistic observations are addressed in detail.

5 FREE RADICAL SCAVENGING AND ANTIOXIDANT PROPERTIES

Free radicals have an important role in the pathogenesis of knee osteoarthritis and studies have shown that reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause oxidative and nitrosative stress. They can damage proteins, lipids, nucleic acids, and matrix components, which are known to play a important role in OA [14,15]. Among ROS and RNS, the superoxide anion radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide (NO), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2) are the most cytotoxic and damage the cell [14].

Generation of ROS and RNS by abnormal chondrocyte metabolism exceeding the physiological buffering capacity triggers cell damage and triggers an important intracellular signaling that amplifies the inflammatory response [15,16]. Scientific studies have confirmed that green tea extracts and their polyphenols possess free radical scavenging properties in numerous cell free systems assays [17–26]. Additionally, studies with cultured mammalian cells have also shown that treatment with green tea and its polyphenols was effective in decreasing intracellular generation of free radicals and thereby reducing the stress, mutagenesis, and cell death [27–36].

6 GREEN TEA INCREASES ANTIOXIDANT ENZYMES AND REDUCES LIPID PEROXIDATION

The antioxidant enzymes SOD, GPx, and catalase cooperate or work in a synergistic way to protect cells against oxidative stress [14]. The SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide, which then is acted upon by the GPx and catalase to give water [14]. By this process the toxic free radicals are converted into the nontoxic water molecule. When an appropriate balance exists between these three enzymes, the oxidative stress is reduced and the cells are protected from the cytotoxic and mutagenic effects of the ROS [14].

Seminal studies by Meki and coworkers [37] have shown that green tea was effective in reducing inflammation in collagen II-induced inflammatory rheumatoid arthritis in rats. The investigators observed that feeding green tea extracts to the rheumatoid arthritic rats increased the levels of SOD, GSH and concomitantly decreased the concentration of lipid peroxides, NO and PGE(2) [37]. A similar mechanism may be operating in osteoarthritis and needs to be validated in relevant study models.

7 ANTIINFLAMMATORY EFFECTS

Inflammation plays a pivotal role in osteoarthritis and numerous studies have been aimed at deciphering the biochemical, cellular, and metabolic changes through the time line in cells. It is known to play a cardinal role in the process of OA, having validated its role in pathogenesis [38–40]. Of all the molecular players involved in the inflammatory reactions that cause distress in OA, the cytokines IL-1b, TNF-α, and prostaglandin E have been shown to play a cardinal role [12]. Both TNF-α and IL-1β mediate the pathogenic process by down-regulating the synthesis of major ECM components as well as inhibiting the anabolic activity of chondrocytes [40–43]. IL-1β is also one of the major chemoattractant factors and contributes towards synovitis [12].

Green tea and its polyphenols are shown to be effective in reducing pain by reducing chondrocyte inflammation by directly/indirectly interacting with the joint-associated tissues (articular cartilage and synovium) [1]. The principal polyphenol EGCG is also reported to be effective in reducing the cytokines IL-1β and TNFα [12,44]; to mitigate pain by reducing the levels of IL1 beta in the dorsal root ganglion of mice [44]; to reduce the up-regulation of cyclooxygenase-2 (COX-2), production of prostaglandin E_2, and activation of nuclear factor-kappaB (NF-κB), mitogen-activated protein kinases

(MAPKs) [45] and activator protein-1 (AP-1) in the IL-1β stimulated human OA chondrocytes. EGCG also modulates the levels of epithelial neutrophil activating peptide, granulocyte macrophage colony stimulating factor, macrophage inflammatory protein-1beta (MIP-1β), granulocyte chemotactic protein-2 (GCP-2), MIP-3alpha, interferon-gamma-inducible protein-10 (IP-10), nucleosome assembly protein-2 (NAP-2), and leukemia inhibitory factor (LIF) [45]; together all these numerous processes lead towards the beneficial antiinflammatory and analgesic effects.

8 GREEN TEA POLYPHENOLS INHIBIT THE ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASES

Mitogen-activated protein kinases (MAPKs), consisting of growth factor-regulated extracellular signal-related kinases (ERKs), the stress-activated MAPKs, c-jun NH2-terminal kinases (JNKs), and p38 MAPKs, play key regulatory roles in the production of proinflammatory cytokines and downstream signaling in synoviocytes and chondrocytes is now being considered important in OA [46].The activation of p38, ERK, and JNK signal pathways induces the production of matrix metalloproteinases that accelerate the degradation of articular cartilage, and concomitantly also initiates cartilage cell proliferation, apoptosis, and differentiation [47].

Experiments have indicated that green tea and its polyphenols inhibit enzyme activities and molecules vital in signal transduction pathways important for inflammation and joint destruction [13]. EGCG has been reported to reduce the TNFalpha induced expression and formation of advanced glycation end products and to mediated this by suppressing the activation of p38-MAPK and c-Jun N-terminal kinases (JNK) [42]. in vitro studies have also shown that EGCG inhibits phosphorylating activity of IK-beta kinase and the AGE-mediated activation and DNA binding activity of NF-kappaB by suppressing the degradation of its inhibitory protein I-kappa & B-alpha in the cytoplasm [42].

9 GREEN TEA AND ITS POLYPHENOLS DECREASE ACTIVATION OF NF-κB

NF-κB is an important transcription factor and mediates an array of signaling pathways in the cell [48]. It is stimulated by proinflammatory cytokines, chemokines, stress-related factors, and extracellular matrix (ECM) degradation products [48]. When stimulated, the activated NF-κB molecules activate the expression of a myriad of genes that induce destruction of the articular joint, leading to osteoarthritis (OA) onset and progression [48,49]. In the milieu of these observations, NF-κB signaling is considered to be a novel potential therapeutic option for OA treatment [48]. Treatment with EGCG decreased the expressions of chemokine receptors and the production of catabolic mediators NO and PGE(2) in human chondrocytes, by regulating the expression and catalytic activity of their respective enzymes and activation of NF-kB [44].

10 EGCG AND ITS INFLUENCE ON MATRIX METALLOPROTEINASES

Matrix metalloproteinases are important for the degeneration of matrix associated with cartilage and bone. Osteoclast activity is essential for the role of regulation of bone matrix regeneration [50,51]. EGCG has been reported to reduce the activity of MMP-2 and MMP-9. These molecules when inhibited also suppress the differentiation of osteoclasts. EGCG has been reported to inhibit IL 1 Beta induced production and release of MMPs [50]. Among the various MMPs, MMP-13 is of particular importance, because it is elevated in joint disorders and can cleave type II collagen, the major component of the cartilage matrix, more efficiently [50,51].

11 CHONDROPROTECTIVE EFFECTS OF EGCG

Chondrocytes made up of adult hyaline cartilage are terminally differentiated cells and are pivotal in the maintenance of the matrix of cartilage [52]. From a cell turnover perspective, their turnover is low and reports suggest that biochemical and mechanical stress hastens the process of OA lesion formation by disrupting chondrocyte-matrix associations and altering metabolic responses [52].

Cell culture studies with primary human osteoarthritic chondrocytes have shown that EGCG was effective in reducing the generation of IL-1β induced nitric oxide and in mediating this by suppressing the induction and expression of NF-κB [53]. EGCG was also effective in decreasing the AGE-stimulated gene expression and production of TNF-α and MMP-13 in human chondrocytes [54]. Subsequent studies with laboratory mice have shown that EGCG offers chondroprotective properties and delays and mitigates the development of OA and associated pain [55].

Mechanistic studies indicated that, when compared to the placebo-treated cohorts, administering EGCG decreased the expression of the chemokine receptor (CCR2) and proinflammatory cytokines IL-1β and TNF-α in the dorsal root ganglia (DRG) [55]. Together, both

of these observations indicated the usefulness of EGCG as a chondroprotective agent.

12 EGCG INHIBITS OSTEOCLAST ACTIVATION AND DIFFERENTIATION

Osteoclasts, which are involved in subchondral bone remodeling, are important in maintaining the bone homeostasis, and their increase leads to development of osteoporosis, inflammatory arthritis, and rheumatoid arthritis [56]. The activated osteoclasts attach to the bone, trigger the release of H+ ions to demineralize the matrix and produce cathepsin K, which initiates the breakdown of type I collagen [56]. Cumulatively, these concomitant steps contribute towards development of calcified cartilage, microcracks, and focal subchondral bone loss, and the pharmacological agent bisphosphonate is shown to reduce this [56].

Seminal studies by Oka and coworkers [57] have shown that green tea polyphenols inhibit osteoclast differentiation. Cell culture studies indicated that treatment of rat osteoclast precursor cells with EGCG decreased the formation of multinucleated osteoclasts and actin rings. Additionally, the levels of MM-2 and MM-9 were decreased, indicating its usefulness [57]. Subsequent studies have also shown that feeding of green tea polyphenols to laboratory mice was effective in mitigating the levels of neural endopeptidase activity and reduced the total IgG and type II collagen-specific IgG in both serum and arthritic joints [45].

13 CONCLUSION

Green tea polyphenols possess antiinflammatory properties that are well established in preclinical models of study. Randomized control studies are required to know the efficacy and safety in treating osteoarthritis. Due to its easy availability and low cost, green tea has a tremendous potential to be used as an agent against osteoarthritis. In addition to its antiarthritic effects, green tea polyphenols are observed to possess free radical scavenging, antioxidant, chemopreventive, and antiinflammatory effects, and to reduce muscular discomfort. All these beneficial effects will also be of help in improving the general health of the individual, without imparting any toxic effects.

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CHAPTER

29

Polyphenolics Evoke Healing Responses: Clinical Evidence and Role of Predictive Biomarkers

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A dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans. ***Kevers et al. [1].***

Polyphenols give color and protection to plants and, in adequate amounts, promote animal health as synergistic antioxidants. Flavanoids and flavanols are the two major categories of polyphenols (polyphenolics). Tens of thousands of polyphenols have been identified, confirming them as abundant and beneficial antioxidants in nature. This review examines the virtues of various polyphenols and documents that the literature recognizes quercetin dihydrate flavanoid and soluble oligomeric/ortho-proanthocyanidin (OPC) flavanol, with or without ellagic acid, as the "dream team" that supports safer, more effective protection from free radical excess. Free radical excess induces repair deficits known as inflammation when inadequate antioxidants are available at the times needed. Examples are provided from the research literature indicating the adaptogenic and efficacious nature of specific polyphenolics.

Assessing a person's level of health in terms of oxidative stress and inflammatory repair resilience provides individual biomarker benchmarks of need for high ORAC-value diet and supplementation. ORAC is an acronym for *oxygen radical absorbance capacity*. Given the ubiquitous free radical and toxic exposures intrinsic to industrial society today (allostatic load), the question of supplementation has high relevance both in the clinic and in the community. Absent of biodynamic and organic super-sources, it is likely that a sensible diet no longer provides enough of the needed essentials to maintain health. The use of global predictive biomarkers referenced to "least risk" or "best outcome" enables more accurate determination of the level of supplementation sufficient to meet individual needs. As discussed in following sections, laboratory tests are reframed from having statistical meaning to having individually predictive meaning.

Polyphenolics are classified first structurally into subgroups and then discussed functionally with emphasis on the evidence supporting safer, more effective flavanoid and flavonol supplementation to meet 21st century oxidative total burden.

1 POLYPHENOLIC CONSUMPTION

The average person consumes several grams of polyphenols daily. This is much more than for all other classes of phytonutrients and known dietary protectants. Amounts ingested are typically approximately 10 times higher than the usual dietary intake of vitamin C and 100 times higher than the intake of vitamin E and carotenoids. The primary dietary sources are fruits, vegetables, and plant-derived beverages such as dark chocolate, fruit spritzers, teas, coffees, beers, and wines. Vegetables, cereals, root vegetables, and dry legumes also contribute to polyphenolic intake [2].

Several hundred polyphenolics are found in commonly consumed food. These molecules are secondary

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metabolites of plants and are generally involved in protection from ultraviolet radiation or defense against aggression by pathogens (both examples of oxidative stress that the polyphenolics are designed to resist or repel).

2 FLAVANOIDS AND FLAVONOLS

Flavanoids and flavonols are the major chemical groups of polyphenols, with multiple subgroups. Most polyphenolics have poor bioavailability or toxicity risks that make them inappropriate for health management and promotion. This chapter focuses on the safer exceptions and their effective use. Related reviews are included in the discussion. Nontoxic forms of polyphenolics with good bioavailability indicated in the following list include quercetin dihydrate and orthoproanthocyanidins (OPC), with or without ellagic acid (see Table 29.1).

- Flavanoids (higher bioavailability; lower risks): quercetin dihydrate, ellagic acid
  - Flavanones (lower bioavailability; higher risks): hesperetin, naringenin, eriodictyol
- Flavonols: kaempferol, myricetin, isorhamnetin
  - Flavones (lower bioavailability): luteolin, apigenin
  - Isoflavones (lower bioavailability): diedzin
  - Anthocyanidins (higher bioavailability): orthoproanthocyanidins (OPC), cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin
  - Flavan-3-ols (lower bioavailability): catechin, epicatechin, aflavin, arubigin, quercitrin

3 MEASUREMENTS OF ANTIOXIDANT CAPACITY

Oxidative stress from free radical damage is increasingly associated with the development and progression of chronic, degenerative, and autoimmune diseases. **Oxygen radical absorbance capacity (ORAC)** and total oxyradical scavenging capacity (TOSC) assays have been developed to measure antioxidant capacity in foods. A high ORAC value indicates increased activity against free radicals and subsequent reduction in reactive oxygen species (ROS) due to high antioxidant content. Both assays are useful in identifying phytochemicals with high antioxidant activity [3].

The ORAC assay measures the degree of inhibition of peroxy-radical-induced oxidation by the compounds of interest in a chemical milieu. It measures the value as Trolox equivalents and includes both inhibition time and the extent of inhibition of oxidation. In addition to the ORAC assay, other common measures of antioxidant capacity

TABLE 29.1 Polyphenols: Food Sources and Bioavailability

		Polyphenol content	
Compound	**Source (serving size)**	**By wt or vol mg/kg fresh wt (or mg/L)**	**By serving mg/serving**
Hydroxybenzoic acids	Blackberry (100 g)	80–270	8–27
Protocatechuic acid	Raspberry (100 g)	60–100	6–10
Gallic acid	Black currant (100 g)	40–130	4–13
p-Hydroxybenzoic acid	Strawberry (200 g)	20–90	4–18
Hydroxycinnamic acids	Blueberry (100 g)	2000–2200	200–220
Caffeic acid	Kiwi (100 g)	600–1000	60–100
Chlorogenic acid	Cherry (200 g)	180–1150	36–230
Coumaric acid	Plum (200 g)	140–1150	28–230
Ferulic acid	Aubergine (200 g)	600–660	120–132
Sinapic acid	Apple (200 g)	50–600	10–120
	Pear (200 g)	15–600	3–120
	Chicory (200 g)	200–500	40–100
	Artichoke (100 g)	450	45
	Potato (200 g)	100–190	20–38
	Corn flour (75 g)	310	23
	Flour: wheat, rice, oat (75 g)	70–90	5–7
	Cider (200 mL)	10–500	2–100
	Coffee (200 mL)	350–1750	70–350
Anthocyanins	Aubergine (200 g)	7500	1500
Cyanidin	Blackberry (100 g)	1000–4000	100–400
Pelargonidin	Black currant (100 g)	1300–4000	130–400
Peonidin	Blueberry (100 g)	250–5000	25–500
Delphinidin	Black grape (200 g)	300–7500	60–1500
Malvidin	Cherry (200 g)	350–4500	70–900
	Rhubarb (100 g)	2000	200
	Strawberry (200 g)	150–750	30–150
	Red wine (100 mL)	200–350	20–35
	Plum (200 g)	20–250	4–50

TABLE 29.1 Polyphenols: Food Sources and Bioavailability—cont'd

Compound	Source (serving size)	Polyphenol content: By wt or vol mg/kg fresh wt (or mg/L)	Polyphenol content: By serving mg/serving
	Red cabbage (200 g)	250	50
Flavonols	Yellow onion (100 g)	350–1200	35–120
Quercetin	Curly kale (200 g)	300–600	60–120
Kaempferol	Leek (200 g)	30–225	6–45
Myricetin	Cherry tomato (200 g)	15–200	3–40
	Broccoli (200 g)	40–100	8–20
	Blueberry (100 g)	30–160	3–16
	Black currant (100 g)	30–70	3–7
	Apricot (200 g)	25–50	5–10
	Apple (200 g)	20–40	4–8
	Beans, green, or white (200 g)	10–50	2–10
	Black grape (200 g)	15–40	3–8
	Tomato (200 g)	2–15	0.4–3.0
	Black tea infusion (200 mL)	30–45	6–9
	Green tea infusion (200 mL)	20–35	4–7
	Red wine (100 mL)	2–30	0.2–3
Flavones	Parsley (5 g)	240–1850	1.2–9.2
Apigenin	Celery (200 g)	20–140	4–28
Luteolin	Capsicum pepper (100 g)	5–10	0.5–1
Flavanones	Orange juice (200 mL)	215–685	40–140
Hesperetin	Grapefruit juice (200 mL)	100–650	20–130
Naringenin	Lemon juice (200 mL)	50–300	10–60
Eriodictyol			
Isoflavones	Soy flour (75 g)	800–1800	60–135
Daidzein	Soybeans, boiled (200 g)	200–900	40–180
Genistein	Miso (100 g)	250–900	25–90
Glycitein	Tofu (100 g)	80–700	8–70
	Tempeh (100 g)	430–530	43–53
	Soy milk (200 mL)	30–175	6–35
Monomeric flavanols	Chocolate (50 g)	460–610	23–30
Catechin	Beans (200 g)	350–550	70–110
Epicatechin	Apricot (200 g)	100–250	20–50
	Cherry (200 g)	50–220	10–44
	Grape (200 g)	30–175	6–35
	Peach (200 g)	50–140	10–28
	Blackberry (100 g)	130	13
	Apple (200 g)	20–120	4–24
	Green tea (200 mL)	100–800	20–160
	Black tea (200 mL)	60–500	12–100
	Red wine (100 mL)	80–300	8–30
	Cider (200 mL)	40	8

Source: Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79(5):727–747.

(AC) include ferric ion reducing antioxidant power (FRAP) and Trolox equivalence antioxidant capacity (TEAC) assays [4,5]. These assays are based on discrete underlying mechanisms that use different radical or oxidant sources and therefore generate distinct values and cannot be compared directly. The ORAC assay is therefore considered to be a preferable method because of its biological relevance to the in vivo antioxidant efficacy.

The U.S. Department of Agriculture published an initial list of ORAC values for more than 100 common foods in 2004, expanded to 277 foods, and most recently in 2010, this list has grown to 326 foods. The items that contain the highest antioxidant value per serving on the ORAC list are beans (pinto, red kidney, and small red beans) and various types of berries (blueberries, black currants, raspberries, and cranberries) [6].

ORAC reigns as the vitamin-industry standard and as one of the easiest ways to compare the antioxidant power of foods and supplements [7]. The ORAC value of a food

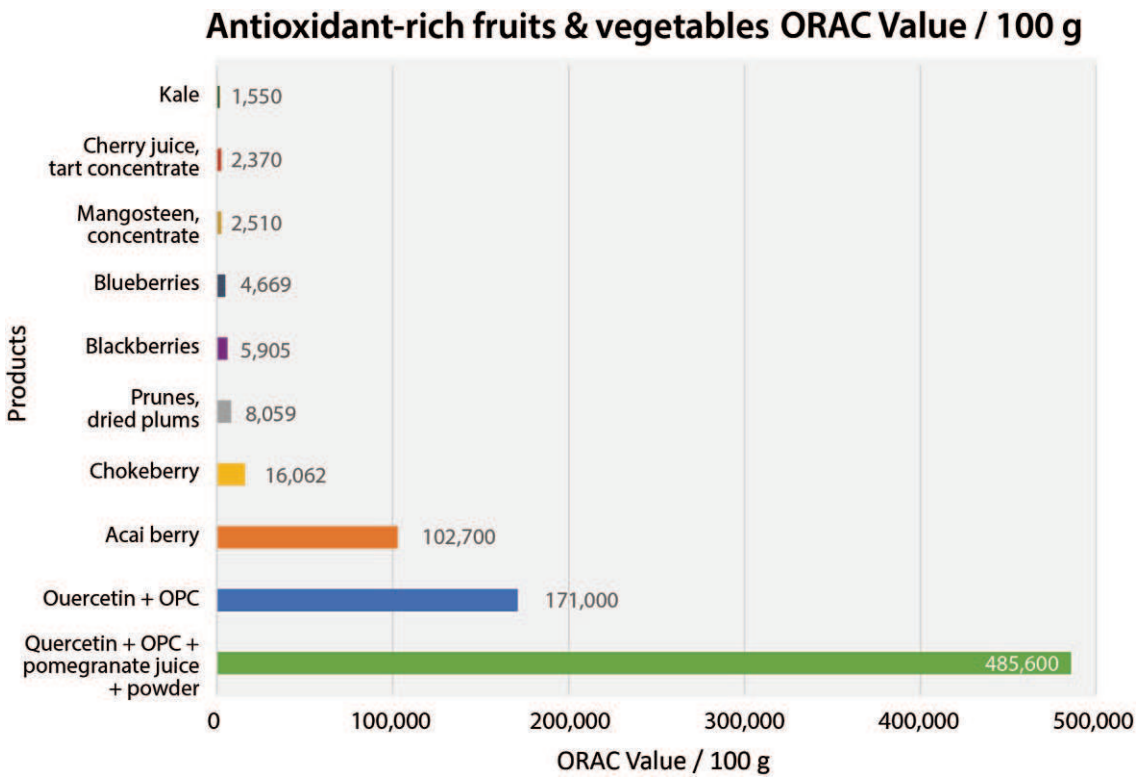

FIG. 29.1 ORAC values of antioxidant-rich foods. *sOPC*, soluble orthoproanthocyanidin; pomegranate juice powder (freeze-dried).

varies significantly, based on whether dry weight or wet weight of the substance is being measured.

Fig. 29.1 shows a comparison of ORAC values among common high-antioxidant fruits and vegetables compared to the preferred polyphenolics recommended in this chapter. Basic research and direct clinical experience confirm that high-activity antioxidants are needed to balance the increased oxidative stress and free radical activity that exist commonly today.

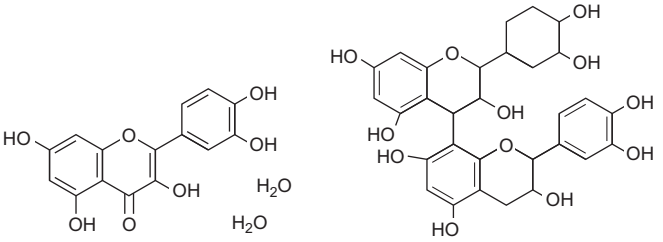

FIG. 29.2 Structure of quercetin dihydrate and oligomeric/orthoproanthocyanidins (OPC).

4 QUERCETINS

Quercetins are naturally occurring flavonoids that function as active dietary antioxidants. These flavonoids are ubiquitous in foods, including vegetables such as onions, garlic, and ginger; fruit such as apples; and in tea and wine. All quercetins however are *not* equal. Certain forms of quercetin such as *quercetin rutinoside* (rutin) are poorly absorbed by the body and are more likely to be irritating or allergenic [8]. Another example is *quercetin chalcone*, a special hesperidin, which has an exceptionally short half-life and is therefore not effective unless it is taken every hour or so.

While quercetin dihydrate is insoluble in water, in physiologic or biological salt solutions it is easily available, especially to first-responder phagocytic and dendritic cells. Quercetin *dihydrate* therefore seems to have the apparent best bioavailability followed by *glycosides*, *aglycone*, and finally *rutinoside* [9]. The chemical description of quercetin dihydrate is *3,3′4′, 5,7-pentahydroxy flavone* (Fig. 29.2).

5 SYNERGISTIC POLYPHENOLS: QUERCETIN DIHYDRATE AND SOLUBLE ORTHOPROANTHOCYANIDIN

Among the various polyphenols, quercetin dihydrate and soluble orthoproanthocyanidin (OPC) are most notable for their safety and functional bioavailability.

Quercetin dihydrate and soluble OPC combine to achieve an ORAC value of 171,000 units per 100 g (Fig. 29.1). Given this highly protective antioxidant

power, it is evident that this flavonoid-flavanol combination has a role to play in mitigating inflammation, by reducing oxidative stress and promoting repair processes. These same polyphenolics also more effectively decrease the need for gene induction of proinflammatory, repair-stimulating cytokines.

Flavanoids such as quercetin dihydrate and flavanols like OPC can benefit connective tissue by promoting repair of injured tissue, improving local circulation, and promoting and maintaining strong collagen, elastin, and basement membrane infrastructure for cells [10].

Quercetin dihydrate *reduces* IL-12 signaling and Th1 differentiation, indicating its potential as therapy for multiple sclerosis and other Th1 cell-mediated autoimmune diseases. We achieve similar results on a twice-daily regimen of two to four 500 mg quercetin dihydrate capsules taken with 5 mg OPC.

When warranted, higher doses are used in conjunction with fully buffered, fully reduced L-ascorbates whose need is determined by a vitamin C cleanse protocol (systematic use of buffered vitamin C as the next generation beyond bowel tolerance to determine need from time to time) [11].

Quercetin intake has been shown to provide some protection against osteoporosis, pulmonary and cardiovascular diseases, and chronic degenerative diseases, including cancers. Quercetin scavenges highly reactive oxygen species such as peroxynitrite and hydroxyl radicals, accounting for the beneficial health effects observed [12].

5.1 Antiinflammatory Effects

Oral intake of quercetin dihydrate (160 mg/kg given five times a day) decreases pain, confirming quercetin's role as a potent antiinflammatory agent [13]. Its antiarthritic properties correlate with a corresponding decrease in proinflammatory mediators produced by peritoneal macrophages, further solidifying the use of quercetin dihydrate as a potential antiinflammatory agent. This effect is significant since chronic inflammation can cause connective tissue degradation due to blocked repair processes. Typically, nonsteroidal antiinflammatory drugs (NSAIDs) and/or corticosteroids are used to control inflammation. However, long-term use of NSAIDs and other antiinflammatory medications is associated with adverse effects on the liver, kidney, and gut [14].

While there are some who suggest avoidance of all items that influence CYP family/cytochrome function, our experience over the last 30 years is that specifically quercetin dihydrate has been safer and helpful. This means that people find they need less of OTC antihistamines or antiinflammatory products. Indeed, many find they can discontinue the repair dampening effects of OTC products when they enhance the innate immune defense and repair system abilities. Quercetin dihydrate, fully reduced and buffered L-ascorbate, and enhanced uptake magnesium are among the team that overcomes oxidative distress by enhancing repair cell energetics and capacities.

5.2 Decreased Oxidative Stress

Quercetin dihydrate reduces oxidative stress and has been found to inhibit NF-kappa B activation in an experimental model of portal hypertensive gastropathy [15].

5.3 Normalized Cholesterol and Fatty Acid Levels

Quercetin dihydrate has a significant cholesterol-lowering action and decreases fatty acid synthesis in the liver better than other polyphenolics. It reduces the activity and mRNA levels of various enzymes involved in hepatic fatty acid synthesis, helping to explain its role in lowering blood fats [16].

5.4 Improved Diabetic Function

Quercetin dihydrate taken at 10 mg/kg dosage improves vascular function in diabetes, reduces blood glucose levels, and shows antiatherogenic effects [17].

5.5 Reduces Obesity

Oxidative stress suppresses the endocrine functions of adipose tissue by disrupting the secretion of adipokines such as adiponectin. Preliminary studies have shown that quercetin can reduce body weight (almost 40%) and suppress expression of adipogenic, lipogenic, and inflammation-related cytokines. The potential to inhibit lipid accumulation and obesity-induced inflammation in the cell is achieved by quercetin's ability to down-regulate MAPK signaling [18].

5.6 Reduced Stroke Risk

At higher doses (30 mg/kg, i.p.), quercetin also protects against cerebral ischemic damage with value in stroke risk reduction and recovery [19]. Quercetin dihydrate has also been shown to reduce lung inflammation, goblet cell metaplasia, and benefit those with chronic obstructive pulmonary disease (COPD).

5.7 Antihistamine Activity

Quercetin dihydrate prevents recruitment of mast cells while stabilizing their membranes and blocking

subsequent degranulation [20]. Compounds such as histamine, serotonin, and proteases are prevented from release and the cascade of symptomatic allergic effects is avoided. Quercetin dihydrate also plays a role in the early stages of an allergic reaction, avoiding the activation of the mast cells and by down-regulating the imbalance between Th2 and Th1 lymphocytes. Th2 response involves allergic response whereas Th1 is a specific immune defense reaction. By decreasing the Th2 response, Ig-E production is inhibited and consequently mast cell, monocyte, and macrophage degranulation are reduced, and symptoms abate when white cells are rebalanced [21].

5.8 Anticancer Effects

Quercetin possesses anticancer properties in part by enhanced degradation of NF-kappa B consistent with a down-regulation of the NF-kappa B binding activity. This activates the AP-1/JNK pathway, important in apoptosis. Quercetin significantly suppresses head and neck cancer-derived tumor initiating cells (HNC-TICs). It also down-regulates ALDH1 activity of head and neck cancer cells in a dose-dependent manner and reduces cell production and "stemness signatures expression" in head and neck cancer-derived sphere cells [22].

The risk of colorectal cancer (CRC) is high in patients with chronic inflammatory disease. Quercetin induces helpful cell cycle arrest and apoptosis, inhibiting excess cell proliferation and stimulating antimetastatic and anti-angiogenic responses when antioxidant activity and repair ability are available [23].

Quercetin synergizes with epigallocathechin gallate (EGCG), found in green tea, in inhibiting the self-renewal properties of prostate cancer stem cells (CSCs), inducing apoptosis, and blocking CSC's migration and invasion. This complementary action may explain the natural prostate cancer prevention and treatment benefits [24].

6 ORTHOPROANTHOCYANIDINS (SOLUBLE OPCs)

Soluble proanthocyanidins refer to a larger class of polyphenols, termed *flavan-3-ols.* Oligomeric orthoproanthocyanidins (OPCs) are thus classed and are among the safer and more bioavailable of the flavanols. OPCs are powerful antioxidants, in a class of polyphenolic bioflavonoids found commonly in fruits and vegetables, and highly concentrated in the seeds of grapes and the bark of maritime pine trees. Low-molecular-weight soluble oligomeric proanthocyanidins (LMW OPCs) were first identified by Jack Masquelier, who developed and applied techniques for their extraction [25]. The active fractions of these substances are antioxidant, antiinflammatory, antidiabetic, and cancer chemopreventive, as well as antimicrobial.

OPCs are made up of proanthocyanidin subunits termed "monomers." "Oligomeric" simply means more than one. Thus, oligomeric proanthocyanidins consist of two or more monomers chemically linked together. Strong binding to proteins appears to form the basis of many of their biological actions [26]. Flavanols are distinguished chemically by the hydroxyl group as opposed to the ketone near the same position on the pyran ring.

6.1 Adaptogenic and Cytotoxic Effects

OPCs have been reported to possess a broad spectrum of pharmacological and medicinal properties active against oxidative stress and have even more free radical scavenging ability than vitamins C, E, or beta-carotene. In addition, OPC has demonstrated significant cytotoxicity towards adenocarcinoma cells affecting the human breast, lung, and stomach, while concurrently enhancing the growth and viability of normal cells. OPCs have an ability to block antideath signaling mediated through the proapoptotic transcription factors and genes such as JNK-1 and c-JUN.

6.2 Cardiovascular Benefits of OPC

Free radicals and oxidative stress play a crucial role in the pathophysiology of a broad spectrum of cardiovascular diseases, including congestive heart failure, vascular heart disease, cardiomyopathy, hypertrophy, atherosclerosis, and ischemic heart disease. Cardio-protective properties and mode of action of OPCs are varied.

Reduction in foam cells, a biomarker of early stage atherosclerosis, has been observed following supplementation of 50 mg and 100 mg OPC/kg body weight (with reductions of approximately 49% and 63%, respectively). At 50 mg/kg this means OPC intake of 4 g for an 80-kg or 176-pound person and at 100 mg/kg this translates to OPC intake of 8 g for an 80-kg or 176-pound person. OPC supplementation has shown significant reduction in oxidized LDL, another important biomarker of cardiovascular diseases. OPCs have also been found to inhibit endothelial CD36 expression, a cardio-regulatory molecule.

Grape seed extract is one of the most potent sources of OPC and has demonstrated excellent protection against myocardial ischemia-reperfusion injury and myocardial infarction [27]. We suggest this as useful for all patients undergoing angioplasty or heart surgery. In addition, adequate grape seed extract (OPC) supplementation given to people consuming a high-fat diet has been shown to help normalize body weight, support epididymal

tissue, normalize lipid concentrations, and improve carnitine levels by improving lipid metabolism.

6.3 Antiaging and Neuroprotection Functions

OPCs enhance cerebral connectivity by increasing the densities of axons, dendrites, and synapses. In addition, OPCs increase the phosphorylation of vascular endothelial growth factor receptor (VEGFR-2), suggesting a protective role against memory deficit. It also extends the lifespan of the senescence-accelerated prone mouse (SAMP8) and elevates sirtuin 1 c (SIRT1) expression, a recognized essential factor for lifespan extension in the brain [28].

6.4 Nephropathy and Soluble OPC

Studies have shown that OPCs reduce oxidative damage associated with nephropathy and improve renal pathology [29]. Activation of reactive oxygen species and inflammation are implicated in renal ischemia/reperfusion (I/R) injuries. OPCs reduce renal dysfunction and injury caused by renal I/R. Adequate OPC intake significantly reduces blood urea, creatinine, and cystatin C levels, and kidney superoxide dismutase, which are up-regulated when additional repairs are needed. In addition, glutathione peroxidase levels increase and OPCs reduce malondialdehyde levels, indicating more efficient recycling of B vitamins [30].

6.5 Osteoarthritis and OPC

Given the antiinflammatory role of OPCs, it seems fitting that they benefit osteoarthritis. OPCs have been shown to reduce the loss of chondrocytes and proteoglycan and reduce the number of subchondral bone fractures, thus promoting bone
health [31].

6.6 Photo-Protection and OPC

Topical application of OPCs has shown significant skin protection from ultraviolet radiation, resulting in fewer sunburn cells and promising to evolve into an effective, natural preventative photo-protection agent that works synergistically with natural vitamins E and selenomethionine [32].

7 ELLAGIC ACID CONTENT: POMEGRANATE JUICE

Pomegranate (*Punica granatum* L., Punicaceae) is a fruit cultivated in many countries and widely consumed. The edible flesh of pomegranate is rich in anthocyanins and polyphenolic compounds, including quercetins that possess antioxidant, antiperoxidative, antiinflammatory, and prorepair activities. The most abundant polyphenols in pomegranate juice are the hydrolyzable tannins called ellagitannins, formed when ellagic acid binds with a carbohydrate.

Pomegranate's antioxidant capacity is three times that of the popular antioxidant-containing beverages such as red wine and green tea, presumably due to the presence of larger amounts of anthocyanins, quercetin dihydrate, and ellagic acid derivatives [33]. As a result, the activities of catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase are enhanced in the liver. Consuming pomegranates exerts repair-promoting, antiinflammatory effects that include:

- Down-regulation of COX-2 activity (an enzyme induced when enhanced repair is needed);
- PGE2 levels are reduced (associated with prostaglandin end-products derived from arachidonic acid and considered proinflammatory);
- Nitric oxide (NO), a potent activator in cell functions, is reduced. Mast cells and basophils are known to play a central role in inflammatory and immune events, inducing edema, destroying connective tissue, and supporting lymphocyte chemotaxis, key in the development of an inflammatory condition like rheumatoid arthritis (RA). Research shows that pomegranate juice inhibits the inflammatory activity of activated human mast cells, suggesting its benefit in RA and other proinflammatory conditions [34].

We find the combination of freeze-dried pomegranate juice, quercetin dihydrate, and OPCs a best-practice solution for a host of repair-deficient inflammatory conditions, promoting remissions in arthritis, cardiovascular diseases, insulin resistance, and diabetes. Chronic fatigue and fibromyalgia abate with the restoration of healthful homeostasis, digestive and detoxification competence, neurohormonal balance, and immune tolerance [35]. In addition, this combination is clinically well suited to resolve local inflammation, reflected in symptoms from headaches and repair deficits to muscular injuries, sprains, contusions, and bruises.

8 CLINICAL CONSIDERATIONS: WHOLE FRUIT AND FRUIT JUICE

To provide the body with comprehensive nutritional support, vegetables, nuts, seeds, and herbs are promoted as good sources of complex carbohydrates and nutrients. Although fruits provide some the highest levels of antioxidants found in nature, their sweetness increases the risk of "sugar overload." Regardless of

the fruit and the method used for juicing, the most diverse and intact collection of nutrients comes to us through the whole fruit. Focusing upon two components of fruit—the skin and the pulp fiber—helps to clarify why there is such a significant difference between whole fruit and fruit juice.

8.1 Nutritional Constituents of Fruit Skins

The edible skins of most fruits are sites of important biological activity in the life of the fruit. The skin is one of the components of the plant where the fruit interacts with sunlight and forms a variety of colored pigments that absorb different wavelengths of light. These pigments include healthy carotenoids and polyphenolics. The skins of whole fruits such as grapes have been studied for their ability to help provide protection from ultraviolet light (a well-recognized source of free radicals) and to help lower risk of cancer. Unfortunately, when fruits are juiced, the fruit's skin may be removed, or their nutritional benefits are lost due to atmospheric oxidation or heating in processing. As a result, the full antioxidant benefits of the whole fruit do not make their way into the juice.

8.2 Pectin Fiber in Fruit Pulp

Orange juice is a good example of the health difference between the fruit and its juice. The white pulpy portion of the orange is a primary source of its flavonoids. The juicy orange-colored flesh of the fruit contains most of its vitamin C. In addition to the skin, the pulp of the fruit is a source of fiber and other nutrients. In the human body, flavonoids and vitamin C often work together, supporting health through their synergistic actions.

When the pulpy white portion of the orange is removed in the processing of orange juice, the flavonoids in the orange are lost. The words "pulp added" on commercial juice product labels indicate an attempt to correct the situation; however, the added pulp may not even be the original pulp found in the whole fruit, and may not adequately restore flavonoid levels.

Another important benefit of fruit fiber is blood sugar regulation. Pectin content in the fiber slows the uptake of sugar into the blood stream, while also nourishing the digestive microbiome.

8.3 Nutritional Issues With Fruit Juices

Fruit juice that has been robbed of its fiber and nutrients is basically just a concentrated source of sugar that lacks most of the supportive nutrients necessary to aid in digestion and metabolism. Fruit juice elevates blood sugar more rapidly than whole fruit, and the level of sugar that can be obtained from fruit juice is substantially higher than the level found in whole fruit. For example, 120 calories' worth of whole apples contains approximately 24 g of sugar, while 120 calories' worth of apple juice contains about 30% more or 30 g of sugar. In terms of glycemic effects, a cup of apple juice has a glycemic load of 6, which is twice that of a cup of diced apple with a glycemic load of 3 due to the fiber that slows sugar uptake.

While whole fruit is always a better choice than fruit juice, if the juice is replacing a can of soda then it may be the best option under the circumstances. Making water the beverage of choice is highly recommended. We also encourage the use of herbal beverages, fruit spritzers, diluted fruit juice, and other beverages with minimal sugars and low glycemic effects.

It is important to note that most fruit juices sold in supermarkets contain only a small percentage of actual fruit juice, and usually contain added sweeteners. As a result, it is easy to consume a large amount of calories without getting any actual nutrition. "Overfed and undernourished" are trends that are all too common in the developed world.

Practical tip: Preparing juice at home can allow almost full retention of pulp and skin. The Hurom, Norwalk, and Vitamix juicers are particularly recommended as they extract more of the fiber and polyphenolics. Fresh fruits and vegetables juiced together fresh can increase total skin and pulp intake, while minimizing the content of natural sugars.

9 PREDICTIVE BIOMARKERS REFERENCED TO GOAL VALUES: PERSONALIZED CARE

We transition into an overview of eight key predictive biomarkers (PBs) that were developed on the premise that epigenetics influence 92% and genetics influence the remaining 8% of health. Biochemical individuality supports supplement use in physiologically meaningful amounts for each individual, monitored through predictive biomarker lab tests to determine individual need.

The polyphenols elucidated earlier in this chapter prove to be extremely effective for systemic repair, especially in response to oxidative stress and repair deficit commonly known as inflammation. The issue many clinicians face is understanding what the true benchmarks of these health states are and the complexity of individual needs. The primary predictive biomarkers described here are different from most laboratory tests physicians would generally order. They are an interdependent suite of tests referenced to best outcome goal values rather than usual or normal statistical ranges. PBs can be used as valuable tools in individual clinical assessment of risk and resilience, of need and of sufficiency.

Biochemical individuality has been recognized since Roger Williams's pioneering work in the 1950s [36]. We find the following predictive lab testing to be helpful, particularly when referenced to a goal value that reflects the least risk or most gain for a given individual. These PBs were developed on the premise that epigenetics influences 92% and genetics influences the remaining 8% of health [37] and selected on the basis that they have a variance of 5% or less, which is desirable. The less the variance, the more predictive is the observed value.

Additional basis for the emphasis on these particular evaluations is discussed in more detail elsewhere [38–41].

1. Hgb A1c with a goal value of <5%
2. hsCRP with a goal value of <0.5 mg/L
3. Homocysteine with a goal value of <6 μmol
4. LRA by ELISA/ACT functional immune memory tests with goal values of no delayed allergies
5. First urine after 6 h rest with a goal value pH of 6.5–7.5
6. Vitamin D (25 OH-D) with a goal value of 50–80 ng/mL
7. Omega-3 index with a goal value of >8%
8. 8-oxo-guanine (8 OHdG) with goal values of <5 g/ng creatinine

10 CONCLUSIONS

Polyphenolics are, collectively, nature's most versatile antioxidant botanical family and essential synergists to ascorbate in mammalian cells.

This overview highlights safer, more effective choices among polyphenols: the flavonoid quercetin dihydrate and the flavanol soluble OPC, with or without freeze-dried pomegranate juice (ellagic acid and other polyphenolic-rich nutrients). In our experience over the last 30 years, use of flavonoids both alone, and in synergistic combination, promote safer, more effective repair and immune competence. Multiple physiologic benefits are noted when the full team of antioxidants are also present in physiologic balance that includes nature's preferred antiinflammatory prorepair molecules. Among the polyphenols, flavanoids are the largest group of compounds, with flavonols next in importance in this broad class of colorful plant-derived antioxidants.

It is predictable that the amount of antioxidants required to sustain health has increased, compared to the needs of a generation ago. Many patients experience massive exposures to oxidative free radicals in their food sources, their workplace, and environment associated with high-tech urban living. Today, diet alone is rarely enough to provide sufficient protective antioxidants. Polyphenols such as quercetin dihydrate and soluble OPC's, as well as ascorbates, tocopherols, carotenoids, B complex, and other vitamins, and buffering minerals may be required in larger amounts to balance and maintain well-being at a time when most challenges to health have increased substantially. While a healthy diet is essential, supplementation is increasingly needed to meet essential nutritional requirements due to dietary deficits, individual learned responses, and toxin exposures. With the increase in oxidative stresses and xenobiotic free-radical generating toxins to which people are routinely exposed, it is likely that increased amounts of antioxidants are being utilized by the body and depleted. Supplementation is warranted in proportion to the total allostatic and homeostatic oxidative load.

We suggest *not* using polyphenols in combination with antiinflammatory medications such as cyclooxygenase inhibitors because the mechanisms of action compete and do not cooperate. In our experience, supporting repair with a comprehensive, integrative, physiology first approach provides better clinical outcomes at lower net costs. Since we are taught today not to combine different classes of antiinflammatory medications, the use of nutrients in place of elective medications reduces potential risks and enables people to amplify healing responses without increased toxicity or adverse effects.

Standardized natural, professional products are now available and recommended—the polyphenols described here are notable for their safety, efficacy, and lack of toxicity when present in their native, forms.

We find better outcomes when biochemically individual needs are assessed through the use of predictive biomarker tests, and interpreted or referenced to best outcome goal values. With the predictive biomarkers discussed in this chapter we provide a timely solution to the increasing number of health care consumers who are interested in predictive, proactive, personalized primary prevention practices. Each biomarker has an epigenetic meaning and elucidates metabolic pathways. Scientists evaluate biomarkers to evaluate a suspected disturbance in particular pathways. Four strongly validated predictive biomarkers are HgbA1c, homocysteine, hs-CRP and Lymphocyte Response Assay (Table 29.2). These biomarkers provide the practitioner with a window into individual glucose, inflammation, repair methylation, and immune status. Evidence-based clinicians can utilize this valuable set of tools to provide a higher level of health.

Life for most people contains increasing external distress and allostatic, oxidative, and acid-rich metabolic dysfunctions. This report builds on the evidence that personalized, proactive, predictive, primary prevention practices saves lives and can reduce total cost of care more than 20% while improving quality of care and quality of life. We can choose life. In the 21st century this requires more personalized, proactive, predictive primary prevention practices and products.

TABLE 29.2 Predictive Biomarkers, Healthier Goal Values, Personalized Care

Predictive Biomarker test (PB)	Best outcome goal value	Measures	Significance
Hgb A1c, HbA1c (Hemoglobin A1c)	<5%	Blood sugar, diabetic risk and insulin resistance	Highly predictive of certain aspects of physiology having to do with sugar metabolism and insulin functions, which in turn are linked to cell energy status, metabolism, weight and related chronic health conditions such as metabolic syndrome, diabetes, cardiovascular disease, and bone and collagen related conditions
hsCRP (high sensitivity C-Reactive Protein)	<0.5 mg/L	Repair and inflammation status	Highly predictive of repair deficits
Homocysteine (high sensitivity homocysteine)	<6 μmol/L	Methylation, detoxification, cardiovascular risk	Helps to measure adequate methylation, sulfur metabolism, detoxification, and epigenetic modulation
hsLRA by ELISA/ACT	No delayed allergies	Immune tolerance to foods and chemicals	*LRA* measures immune defense and repair tolerance and intolerance across all delayed allergy pathways
First AM Urine pH	6.5–7.5	Mineral need assessment and cellular acid/alkaline balance	Measurement after 6 h rest reflects net acid excess and metabolic acidosis. Consequences of metabolic acidosis include hormonal changes, insulin resistance, loss of bone and muscle protein degradation
Vitamin D (25-OH cholecalciferol)	50–80 ng/mL	Cellular equilibrium and communication	Vitamin D pleiotropic functions dictate cellular function, physiology and proliferation—crucial for immune health, bone metabolism, neurological and cognitive function
Omega-3 index	>8%	Omega-3 level of oxidative stress	Reflects the relative amount of omega-3 fatty acids within red blood cell membranes and expressed as percentage of total fatty acids, useful in predicting oxidative stress
DNA oxidative stress (8-OHdG)	<5 ng/mg creatinine	Oxidative stress and nuclear antioxidant status	DNA oxidative stress marker that can be a predictor of repair deficit and risk of conditions like diabetes and atherosclerosis

These biomarkers provide a revealing snapshot of functional, biochemical health status at a given moment in time. Knowing the client's level of health in terms of oxidative stress and inflammatory processes such as the hsCRP and 8-OHdG among others can alert us to the need for intervention with high ORAC-value supplementation.

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CHAPTER

30

Hepatoprotective Effects of Green Tea and Its Polyphenols: A Revisit

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1 INTRODUCTION

The liver, the chemical factory of the body, plays a cardinal role in many metabolic processes that include digestion (bile synthesis) and excretion of waste metabolites [1]. A healthy liver has a remarkable ability to regenerate and maintain function, while a pathological one with suboptimal functioning will affect the health and even the life of the individual [2–5]. Diet has a substantial influence on liver functions and health, and numerous studies have shown that *Camellia sinensis* (L) Kuntze, commonly known as tea, possesses beneficial effects as a hepatoprotective agent against various hepatotoxins [1].

Globally, tea is the second most widely consumed beverage and has been cultivated and consumed by humans for thousands of years [6]. Historical evidence suggests that the tea plant was originally a native of China, Burma, Thailand, Laos, and Vietnam but today is also cultivated in Sri Lanka, India, and Japan [6]. Depending on the way the leaves are harvested and processed, they are categorized as green, black, and oolong tea powder [6,7].

From a scientific perspective, of the three tea types, green tea has been the most highly investigated and has been reported to be useful in myriad ailments and diseases afflicting humans [8,9]. It is reported to be useful as a stimulant, a diuretic, an astringent, a wound-healing agent, and to improve the function of heart [10–12]. Green tea is considered to be useful in regulation of blood glucose, to improve digestion, controlling body weight, bone mineral density and cognitive function; and to prevent various cancers [9–12].

The various pharmacological effects of green tea are attributed to the presence of epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG), proanthocyanidins, flavonols such as kaempferol, quercetin, and myricitin in the form of glycosides [8,9]. The structures of important polyphenols are represented in Fig. 30.1. Green tea also contains proteins, carbohydrates, vitamins, minerals, and trace elements like Ca, Mg, Mn, Fe, Cu, Zn, constituents known to be useful in maintenance of health [8,9].

Mechanistically, the most important aspect that makes green tea an important dietary agent with medicinal benefit is its potent antiinflammatory, antibacterial, antiviral, antimutagenic, immunomodulatory, and antiaging properties [9–12]. This has consequentially increased public awareness and popularized its consumption [8,9]. In the following section, the hepatoprotective observations are addressed in detail.

2 TEA PROTECTS AGAINST ALCOHOL-INDUCED HEPATOTOXICITY

Globally, chronic alcohol-induced liver cirrhosis and cancer represent a major health problem [1]. Alcoholic liver disease is a spectrum of disease states, ranging from simple steatosis (fat accumulation), to inflammation, and eventually to fibrosis and cirrhosis if untreated [13]. Scientific studies have shown that epigallocatechin-3-gallate (EGCG), the most abundant catechin polyphenol in green tea, protected against alcohol-induced, cytochrome P 450-dependent liver damage and formation of fatty liver [14]. Dietary supplementation with EGCG (3 g/L with liquid diet, 7 weeks) prevented an increase in serum ALT and AST, and ameliorated the reduced hepatic

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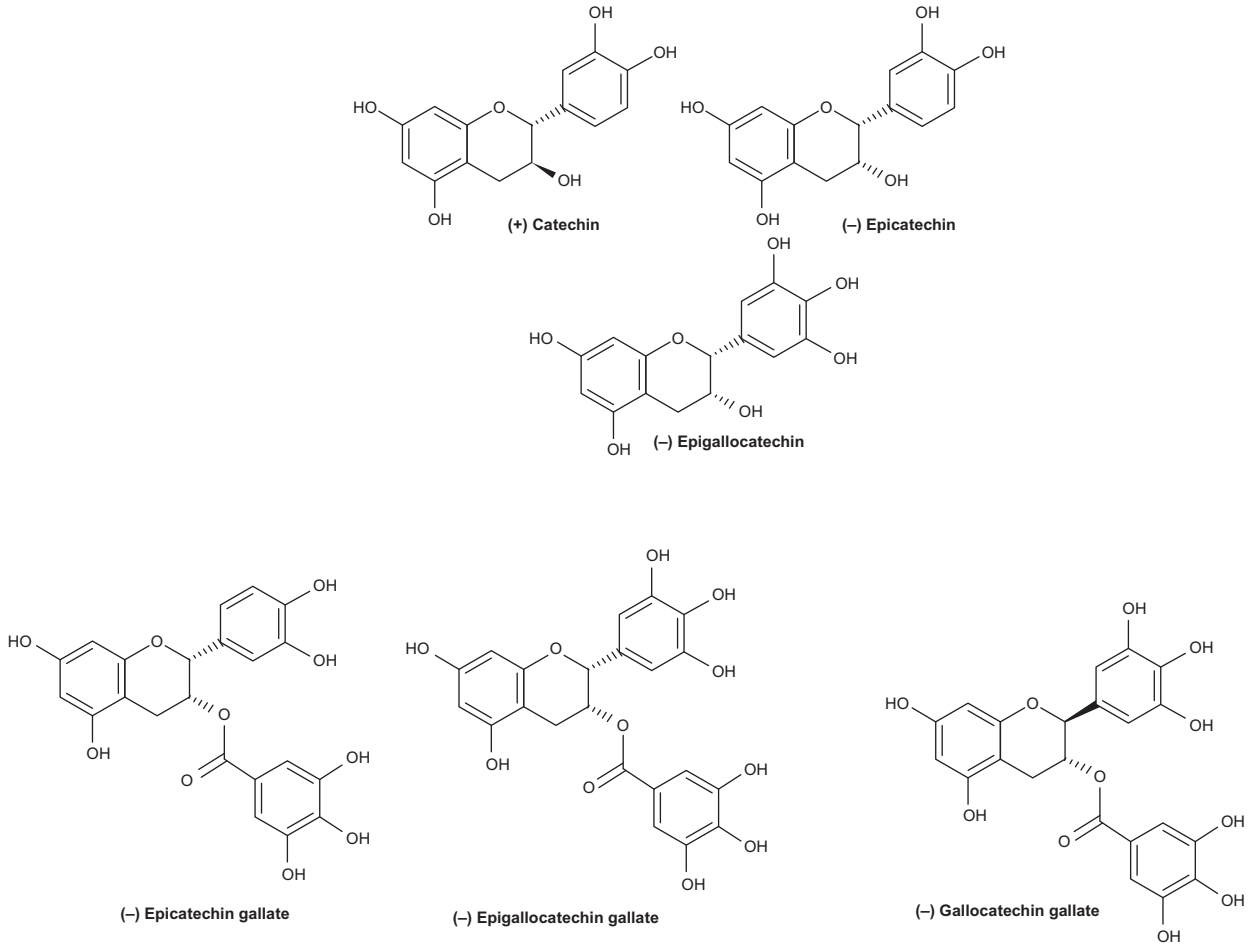

FIG. 30.1 Polyphenols present in green tea.

phospho-acetyl CoA carboxylase (p-ACC) and carnitine palmitoyl-transferase 1 (CPT-1) levels [14].

3 TEA PROTECTS AGAINST CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY

Carbon tetrachloride (CCl_4) is arguably the most important xenobiotic compound used to study underlying mechanisms on how the liver functions on exposure to a toxin [15,16]. Studies with laboratory rats have shown that administering green tea extract was effective in decreasing the CCL_4-induced increase in ALT, AST in serum [15,16], concomitantly reducing the depletion of GSH and increase in lipid peroxide levels in the liver [15], and increasing plasma total antioxidant capacity [16]. Additionally, studies with laboratory mice have also shown that the polyphenol-enriched extract from Huangshan Maofeng green tea was effective in mitigating the CCl_4-induced hepatic damage in mice and in mediating these effects, at least in part by the free radical scavenging and antioxidant activities [17].

4 EFFECT OF TEA ON N-ACETAMINOPHEN-INDUCED HEPATOTOXICITY

Acetaminophen (paracetamol or *N*-acetyl-p-aminophenol) is an important pharmacological agent and is very useful as a antipyretic or analgesic drug. Chronic intake of high doses of acetaminophen causes hepatotoxicity by triggering generation of free radicals, glutathione depletion, oxidative stress, and mitochondrial dysfunction [18]. Seminal studies by Salminen and coworkers have shown that administration of green tea extract prior to acetaminophen was beneficial in preventing/mitigating acetaminophen-induced hepatotoxicity [19]. However, supplementation of green tea extract after acetaminophen was observed to increase the acetaminophen-induced hepatoxicity, indicating that the scheduling of

administration of green tea extract prior to acetaminophen was important [19].

5 TEA IS EFFECTIVE IN VIRAL HEPATITIS

Globally, hepatitis caused by hepatotropic viruses is the most common cause for various liver disease and cancer [20,21]. Of the various viruses, hepatitis B and C are responsible for most of the liver diseases and hepatitis C virus (HCV) specifically is a major cause of liver cirrhosis and hepatocellular carcinoma [20,21]. Studies have shown that green tea polyphenols were useful and exhibited antiviral and antioncogenic properties [20]. Cell culture studies with hepatoma cell lines and primary human hepatocytes have shown that EGCG treatment was effective in inhibiting HCV infection [20]. Additionally, studies with Beijing ducklings have also shown that green tea polyphenols were effective against viral hepatitis and reduced the levels of DHBsAg and DHBV-DNA and histopathological changes in the liver [21].

6 EFFECT OF TEA ON ISCHEMIA REPERFUSION INJURY

In liver transplantation, ischemia-reperfusion injury caused by free radicals is a life-threatening complication [22]. A study conducted with fasting Sprague-Dawley rats have shown that a single dose of green tea extract was highly effective in reducing the ischemia reperfusion injury, and it mediated these effects by decreasing cellular activation and improving the sinusoidal circulation [22]. Subsequent experiments in mice have validated these observations and mechanistic studies have indicated that tea polyphenols mediated these effects by decreasing the mRNA and protein expression of cytokine-inducible nitric oxide synthase and inhibiting oxidative stress and apoptosis [23].

7 EFFECT OF GREEN TEA PHYTOCHEMICALS ON HEPATOTOXICITY OF LEAD

Globally, lead toxicity caused by occupational and environmental sources is a major concern [24]. Mechanistic studies principally with laboratory animals indicate that exposure to lead causes alterations in hepatic xenobiotic metabolism, cholesterol metabolism, liver cell proliferation, and lead-induced hepatic hyperplasia due to increased DNA synthesis [24]. Studies with Sprague-Dawley rats have shown that administering green tea extract was effective in mitigating lead-induced hepatotoxicity [24]. The cohorts administered the green tea extract had a significant reduction in the levels of ALT, AST, and ALP, thus proving its protective effects on the hepatic cells [24].

8 EFFECT OF TEA PHYTOCHEMICALS ON HEPATOTOXICITY OF AZATHIOPRINE

Azathioprine (AZA), a purine analogue with immunosuppressive effects, is an important drug in organ transplantation and autoimmune diseases [25]. Principally, AZA causes apoptosis of activated T lymphocytes and curbs the overactive immune defense mechanisms [25]. AZA-induced hepatotoxicity is a rare adverse event and is manifested as nodular regenerative hyperplasia, veno-occlusive disease, peliosis hepatis, fibrosis, and sinusoidal dilatation [25]. Studies with laboratory rats have shown that administering the green tea polyphenols mitigates the AZA-induced hepatotoxicity [25]. Green tea prevented the elevation of enzymes like ALT, AST, and ALP and increased the levels of GSH, GPx, CAT, and GSSG, contributing to its antiinflammatory activity. It also decreased the levels of tumor necrosis factor alpha (TNF α) and caspase-3, thus reducing apoptosis [25].

9 EFFECT OF TEA PHYTOCHEMICALS ON GALACTOSAMINE-INDUCED LIVER DAMAGE

D-galactosamine (GalN) is an important experimental hepatotoxin and the pathogenesis it causes is akin to that in acute hepatitis [26]. GalN causes insufficiency of UDP-glucose and UDP-galactose and alters the intracellular calcium homeostasis, consequently affecting the cell membranes, cell organelles, energy metabolism, and the synthesis of proteins and nucleic acids [26]. Experimental studies with laboratory rats have shown hepatoprotective effects and attenuation of the levels of plasma ALT and AST [26].

10 EFFECT OF TEA PHYTOCHEMICALS ON LIPOPOLYSACCHARIDE-INDUCED LIVER DAMAGE

Lipopolysaccharide (LPS) causes hepatoxicity by induction of oxidative stress and consequent oxidative damage to biomolecules [27]. Studies with laboratory rats have shown that pretreatment with tea polyphenols attenuated LPS-induced liver injury and mitigated the levels of TNF-alpha and lipid peroxidation and expressions of TNF-alpha, iNOS in the liver [28].

11 EFFECT OF TEA PHYTOCHEMICALS ON FUMONISIN B1-INDUCED LIVER DAMAGE

Fumonisin B1, a mycotoxin produced by several species of fusarium molds, is an important toxicant and a hepatocarcinogen [29]. Treatment with green tea polyphenols was effective in people who were exposed to fumonisin B1 through cereals, and at high risk with hepatocellular carcinoma. Supplementation with green tea polyphenols (1000 mg, 3 months) resulted in decreased urinary excretion of fumonisin B1, sphingosine, sphinganine, and the ratio of sphinganine to sphingosine. This study indicates a beneficial anticarcinogenic effect of green tea polyphenols [29].

12 EFFECT OF TEA ON HEPATOTOXICITY OF AFLATOXINS

Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* and are potent inducers of hepatotoxicity. An aflatoxin treatment of 30 days caused significant impairment in liver structure and function. Oxidized tea polyphenols have been shown to form a complex with AFB1 (aflatoxin), thus inhibiting the absorption of AFB1 in intestine, promoting the elimination of AFB1 in feces, and inhibiting the liver injury caused by AFB1 in rats [30].

13 EFFECT OF TEA PHYTOCHEMICALS ON PHENOBARBITOL-INDUCED LIVER DAMAGE

Hepatotoxicity is an infrequent but fatal adverse effect of phenobarbitol toxicity. Phenobarbitol has been shown to induce cytochrome P450 (CYP), oxidative stress, and consequently induce liver damage [31]. An animal study conducted on diethylnitrosamine-initiated male Wistar rats showed that administration of epicatechin complex extracted from green tea along with the administration of phenobarbitol during hepatocarcinogenesis produced significant inhibition to the promotive effects of phenobarbital [32]. Further studies with green tea extract must be conducted to develop it as a potential agent against hepatocarcinogenesis promoted by phenobarbitol.

14 EFFECT OF TEA ON HEPATOCARCINOGENESIS

Liver cancer, a malignancy with extremely aggressive features and poor survival rate is globally the fifth commonest cancer [33]. It remains an important public health issue and causes a substantial number of years of life lost [33]. Green tea polyphenols have been studied for their cancer-preventing properties in various animal models of hepatocarcinogenesis, and these aspects will be addressed herewith. Seminal studies by Ding showed for the first time that administration of purified epicatechin complex (87% concentration) isolated from green tea was effective in inhibiting diethylnitrosamine (DEN)-initiated, phenobarbitol-promoted proliferation of precancerous liver cells [32]. Green tea (2.5%) administered to rats before and following DEN treatment effectively inhibited hepatocarcinogenesis by triggering antioxidant activity [34]. Green tea was effective in inhibiting hepatocarcinogenesis in mice with DEN-induced, pentachlorophenol-promoted hepatic carcinoma [35].

Subsequent studies have also shown that feeding catechin-containing green tea was effective in reducing deleterious effects of liver tumor promoters, induction of cytolethality, inhibition of gap junctional intercellular communication, and induction of cell proliferation [36]. Green tea extract prevented the induction of hepatocyte cytolethality by glucose oxidase, xanthine oxidase, and paraquat (all oxygen free radical inducers) in a dose-responsive manner [36]. Green tea extract prevented the inhibition of gap junctional-mediated intercellular communication by phenobarbital, lindane, and paraquat in a dose-dependent manner. The effect of green tea extract on hepatocyte DNA synthesis was examined in male mice containing preneoplastic liver lesions induced by diethylnitrosamine [36]. Green tea extract significantly decreased the labeling index in hepatic preneoplastic foci [36]. Mice administered green tea extract (1.25%, total 40 weeks treatment) prior to, during, and after DEN treatment showed a decrease in number of hepatic tumor cells, when compared to DEN-alone treated cohorts [37].

Green tea extract is also reported to possess an anticarcinogenic effect in DEN-initiated hepatocarcinogenesis in normal rats without chronic hepatocyte damage or liver cirrhosis [38]. Tea polyphenols and tea pigments have been observed to effectively reduce the GST-Pi expression at both transcription and translation level, and thus inhibition of carcinogen-induced expression of GST-Pi has been suggested as one of the mechanisms in the anticarcinogenic effect of phytochemicals in tea [39]. Tea polyphenols and pigments have been shown to modulate the phase detoxifying enzymes by inhibiting the overexpression of GST-Pi and promoting the expression of GST-alpha and GST-mu, thus inhibiting the occurrence and development of the precancerous lesions of rat liver [40]. Additionally, feeding epigallocatechin gallate was effective in preventing simultaneous development of liver and tongue carcinogenesis in mice. Mechanistic studies indicated that chemopreventive effects

were associated with modulation of cellular proliferation/apoptosis, Wnt/β-catenin, Hh/Gli1 pathways and their associated genes Cyclin D1, cMyc, EGFR and E-cadherin [41].

15 EFFECT OF TEA POLYPHENOLS ON FATTY LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) caused by lipid deposition within liver cells is a common clinicopathological condition with a range of conditions [42,43]. Despite the fact that it represents a serious and growing clinical problem, to date no approved drugs are available for the treatment of NAFLD [43]. In a double-blind, placebo-controlled, randomized clinical trial on patients with NAFLD, green tea extract supplement was administered (500 mg tablet for 90 days); activities of ALT and AST in serum decreased significantly, indicating a beneficial effect of green tea [43]. Additionally, animal studies also indicated that administering green tea extract (1%) for 6 weeks was effective in reducing the levels of hepatic lipids and serum ALT in obese mice [44]. Immunohistochemical observations indicated that steatotic livers from obese mice had extensive accumulation of tumor necrosis factor-α (TNF-α), whereas GTE at 1% decreased hepatic TNF-α protein and inhibited adipose TNF-α mRNA expression [44]. Administering green tea extract decreased the mRNA expression of adipose sterol regulatory element-binding protein-1c, fatty acid synthase, stearoyl CoA desaturase-1, and hormone-sensitive lipase and serum nonesterified fatty acid concentrations [44]. Feeding green tea extract corrected the levels of catalase, glutathione peroxidase activities, ALT, lipids, total glutathione, malondialdehyde, and Mn- and Cu/Zn-superoxide dismutase in the liver [44].

16 EFFECT OF TEA POLYPHENOLS ON OBESITY-INDUCED LIVER DAMAGE

Obesity is an established risk factor for many types of cancers, particularly for hepatocellular carcinoma, owing to its carcinogenic potential and the association with nonalcoholic fatty liver disease [45]. Mechanistically, obesity is associated with insulin resistance and type 2 diabetes, and proinflammatory status of the body is a potential risk factor for various metabolic diseases and liver cancer. Abnormal activation of the insulin-like growth factor (IGF)/IGF-1 receptor (IGF-1R) axis is also involved in obesity-related liver tumorigenesis. Tea phytochemical EGCG (0.1%, 34 weeks) administered to obese mice (db/db model of obese, type 2 diabetic mice) with DEN treatment, inhibited the phosphorylation of IGF-IR, ERK (extracellular signal-regulated kinase), GSK-3beta (glycogen synthase kinase-3beta) and c-Jun NH-terminal kinase in the liver. EGCG also decreased the serum levels of insulin, IGF-1, IGF-2, free fatty acid, and TNF-alpha and down-regulated the hepatic expression of mRNAs of TNF-α and interleukins. The antitumorigenic actions of EGCG have been suggested to be brought out by improving hyperinsulinemia and attenuating chronic inflammation [46].

17 CONCLUSIONS

Observations from the scientific studies carried out in the recent past have clearly shown that tea possesses hepatoprotective action against diverse xenobiotic agents and heptotoxic agents. Several mechanisms are likely to account for the observed pharmacological effects, the most important being the free radical scavenging, antioxidant, antiinflammatory, increase in the antioxidant enzymes, modulation of Phase I and II enzymes and possible antiviral effects. However, although considerable work has been done to exploit the hepatoprotective effects, countless possibilities for investigation still remain. Further in-depth mechanistic in vitro studies, relevant animal studies, especially with old mice, and rationally designed clinical trials are required. The outcomes of such studies may be useful for further clinical applications of tea in humans and may open up a new therapeutic avenue, especially in the geriatric population.

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CHAPTER

31

CAPE and Tympanosclerosis

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1 THE EFFECT OF CAFFEIC ACID PENETHYL ESTER ON TYMPANOSCLEROSIS

Propolis is a natural product, used for many years, consisting of plant resins, pollen, and saliva enzymes of bees [1,2].

It is used by bees to insulate hives from the outside, to narrow the entrance of holes, to repair cracks and damaged areas, and to mummify and disinfect harmful substances, such as microorganisms and insects that enter the hive [3–5].

More than 300 compounds have been identified in the chemical structure [6]. Its main components are phenols (flavanoids: flavones, flavonones, flavoneller) and related compounds (phenolic acids and esters, coumarins, ketones) and caffeic acid esters (caffeic acid benzyl ester, salicylic acid benzyl ester, cinnamic acid benzyl ester, and caffeic acid phenethyl ester) [5].

Propolis has been used for many years as a traditional therapeutic agent for antibacterial and antiinflammatory purposes. Recently, many scientific studies have shown the antimicrobial, antiinflammatory, antioxidant, immunomodulator, antimutagenic, and carcinostatic effects of propolis [3,7,8].

In research that investigated the antiinflammatory properties of propolis, it was shown to reduce the formation of leukotrienes and prostaglandin by suppressing the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism with in vitro and in vivo studies [5]. Öztürk and colleagues found that propolis decreases inflammatory cell infiltration as potently as dexamethasone [6]. In addition, the antioxidant effects of propolis have been particularly emphasized in recent years. It has been shown that the polyphenolic compounds in propolis have free radical scavenging properties [6,9].

The standardization of propolis is difficult because the content of propolis varies according to the ingredients the bees collect from the plants and also propolis has a large number of different compounds [3,8]. Therefore, the substances in the content have been purified and investigations have been conducted to determine which substances are responsible for the main effects. As a result of these investigations, it has been suggested that caffeic acid phenethyl ester (CAPE) is the main component that mediates certain effects such as antimicrobial, antiinflammatory, and antioxidant. Consequently, CAPE is one of the most studied research topics in recent years [5,10,11].

2 CAFFEIC ACID PHENETHYL ESTER

CAPE [phenethyl 3-(3-4 dihydroxyphenyl) acrylate] is an active component of propolis produced by honey bees [12]. In 1988, CAPE has been extracted and described through the efforts of Grunberger (a scientist from New York Columbia University) and his colleagues. At the beginning of the 1990s, Sigma-Aldrich produced commercial preparations for the market [13].

CAPE has two annular structures. One of these annular structures has two functional OH^- groups that show almost all chemical properties of the CAPE molecule [7,14]. These OH^- groups actively exchange electrons and exhibit oxidizing and reducing properties. These two hydroxyl groups provide strong antioxidant properties to the molecule. Also, it has a lipophilic property because of its long carbon groups in an aromatic and aliphatic structure. Therefore, when CAPE is administered intraperitoneally, it reaches an adequate blood

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concentration [12,14–16]. The molecular weight is 284.31 g/mol, and the molecular formula is $C_{17}H_{16}O_4$ [13].

3 CAPE AND OXIDATIVE STRESS

The compounds that contain at least one electron in the outer orbitals that do not form a pair are called free radicals. The unstable electrons are highly reactive and open to electron exchange with other molecules [17].

Reactive free oxygen radicals (especially peroxyl, the hydroxyl radical, and superoxide anion) are rapidly degraded, leading to oxidation of membrane lipids, protein denaturation, DNA degradation, and inactivation of the sulfhydryl enzyme. This increases membrane permeability, leading to protein and nucleic acid degradation and cell death. Free radicals may also induce apoptosis and inflammatory cytokine synthesis by affecting intracellular and nuclear signaling pathways [18].

Reactive oxygen species (ROS) are mainly produced from arachidonic acid metabolism, the xanthine oxidase system, and activated neutrophils [6]. Under normal conditions, there is a balance between the production and clearance of free radicals in the body. This balance is degraded in some cases leading to an increase of free oxygen radicals, thus leading to tissue damage by these radicals. Compounds with antioxidant properties can prevent this damage through enzymes or pathways that mediate the action of free radicals [15].

In experiments with propolis extracts, either containing or not containing CAPE, it was found that CAPE-containing compounds had a much stronger free radical scavenging effect, suggesting that CAPE is responsible for the antioxidant effect of propolis [15]. It has been proven that CAPE inhibits the production of oxygen radicals in the xanthine oxidase system in neutrophils [6].

It has been shown in rat experimental models that administration of CAPE has a protective effect against oxidative damage in various tissues such as kidney, liver, heart, lung, red blood cells, and neural structures [11].

The most striking feature of the biochemical and physiological consequences of the antioxidant effect of CAPE is that it is a very specific inhibitor of the nuclear transcription factor nuclear factor kappaB (NF-κB) in many cell types [10,13]. NF-κB is a transcription factor related to the Rel mutagen family. It activates many genes that play a role in the pathways of immunity and inflammation [10,19]. It is also an important agent in the apoptosis pathway [20].

NF-κB is normally inactivated in the cytoplasm by the inhibitor factor kappa B (iκ-B). However, mitogens, inflammatory cytokines (such as TNF-α, IL-1), bacterial products, protein synthesis inhibitors, oxidative stress caused by free oxygen radicals (such as H_2O_2), and stimuli such as ultraviolet rays cause the phosphorylation of iκ-B and consequently the translocation of NF-kB to the cell nucleus. As a result, they trigger production of reactive oxygen species and proinflammatory cytokines in the cells [16,21]. Impairment of NF-κB regulation plays a role in many pathologies, such as graft versus host disease, toxic shock, cancer, acute inflammatory diseases, and atherosclerosis [16]. CAPE specifically blocks the formation of many inflammatory agents, primarily tumor necrosis factor alpha (TNF-α), by blocking NF-κB [22].

In various experimental studies, it was found that increased free oxygen radical production causes ototoxicity and CAPE could suppress this process.

In a study, it was shown that abundant amounts of ROS are produced in the inner ear after aminoglycoside treatment, which triggers necrosis and apoptosis in the inner ear by activation of cell death pathways involving c-jun N-terminal kinases (JNKs), caspase cascades (especially caspases 3 and 9), nuclear translocation of endonuclease G, and activation of m-calpain [23].

In their study that investigated the efficacy of CAPE in the prevention of streptomycin-induced ototoxicity in rats, Bakır and colleagues have observed severe degeneration and increased apoptotic cells in cochlear hair cells in rats given streptomycin, whereas in the streptomycin +CAPE group, hair cells were mostly preserved. Furthermore, they have found significant differences between the streptomycin and the streptomycin+CAPE group in distortion product otoacoustic emissions (DPOAEs). In this study, caspase-3 immunoreactivity in the streptomycin group was also measured as high due to increased apoptotic events in the cochlea. In conclusion, they have suggested that CAPE may reduce damage of inner hair cells by antioxidant properties and may be used as a prophylactic agent for prevention of streptomycin-induced ototoxicity [11].

In a similar study conducted by Park and colleagues, CAPE has been shown to reduce apoptotic cell death and prevent mitochondrial damage in cochlear hair cells induced by neomycin in a zebrafish model, and these results are thought to be related to its antiapoptotic and antioxidative properties [24].

Carreño and colleagues have reported that the CAPE treatment has a severe antioxidant activity against hydrogen peroxide (H_2O_2)-induced oxidative stress via decreasing the amount of ROS and preserving cell integrity, viability, and morphology when compared with quercetin, ascorbic acid, and trolox treatment in both B-cell lymphoma and macrophage cell models [9].

Sun-Young Jo and colleagues, in their study which investigate the effect of CAPE on diesel exhaust particle induced inflammation of middle ear epithelial cells, were directly determined the antioxidative effects of CAPE by measuring the production of reactive oxygen species and were determined the antiinflammatory effects of CAPE by measuring the levels of tumor necrosis factor α and

nicotinamide adenine dinucleotide phosphate oxidase (NOX) 4 levels. Furthermore, the effects of CAPE were compared with those of *N*-acetyl-L-cysteine, which is known to have anti-oxidative and anti-inflammatory effects. As a result of the study, it was found that CAPE significantly inhibited tumor necrosis factor α and NOX4 expression caused by diesel exhaust particles in a dose- and time-dependent manner. The accumulation of reactive oxygen species has also been reduced. The anti-inflammatory and anti-oxidative effects of CAPE have been reported similar to *N*-acetyl-L-cysteine [25].

4 CAPE AND INFLAMMATION

One of the most important features of CAPE is antiinflammatory effect [10]. The antiinflammatory effect is more pronounced when compared to other propolis components because modulates the arachidonic acid cascade strongly. The antiinflammatory activity of CAPE was found to be equivalent to diclofenac and hydrocortisone in some studies [26].

The CAPE exhibites it's antiinflammatory effect via inhibiting the release of arachidonic acid from the cell membrane, inhibiting the activity of cyclooxygenases 1 and 2 enzymes (COX-1 and COX-2) [27] or via blocking the NF-κB activation that is stimulated by inflammatory cytokines TNF-α and IL-β [28]. While some researchers have suggested that inhibition of NF-κB signaling is associated with direct inhibition of nuclear translocation of p65 by NF-κB, others have implicated associated with indirect inhibition of IκB phosphorylation and degradation. Some researchers have suggested that the inhibition mechanism of NF-κB signaling by CAPE may differ between different cell types [10].

CAPE also inhibits the oxygenation of arachidonic acid and linoleic acid by 5'-lipoxygenase enzyme [12,24].

Li and colleagues, in their study conducted on human gingival fibroblasts (HGFs), was administered CAPE at different doses (10, 20, and 30 μM) for 1 h, then HGFs were subjected to lipopolysaccharide stimulation for 24 h. They have evaluated the production of IL-6, IL-8, iNOS, and COX-2, as well as the activation of TLR4-mediated NF-κB, PI3K/AKT, and MAPK signaling pathways. As a result, they have reported that CAPE inhibits LPS-induced IL-6, IL-8, iNOS and COX-2 production in a dose-dependent manner. Moreover, CAPE inhibits LPS-induced TLR4/MyD88 and nuclear factor kappa B (NF-κB) activation, while phosphatidylinositol 3 kinase (PI3K) and protein kinase B (AKT) phosphorylation are inhibited by CAPE. These results indicate that CAPE can be used in the treatment of inflammatory periodontal diseases [10].

Toyoda and colleagues have investigated the antiinflammatory effects of CAPE on a *Helicobacter pylori*-induced gastritis model. In *H. pylori*-stimulated AGS cells, NF-κB activation is significantly increased compared to uninfected control cells. CAPE has reduced NF-κB activation in a dose-dependent manner. Also, mRNA expression of inflammatory mediators such as IL-8, TNF-α, IL-1 β, INOS in CAPE-treated cells was significantly suppressed compared to control cells stimulated with *H. pylori*. Furthermore, *H. pylori* stimulation has increased p65 phosphorylation and inhibitor factor kappa B (iκ-B)-α degradation in AGS cells and CAPE has inhibited them in a dose-dependent manner. As a result it has been suggested that CAPE has inhibitory effects on *H. pylori*-induced gastritis through the suppression of NF-κB activation [29].

Although the NF-κB inhibition mechanisms of CAPE are not fully understood, the investigations in animal models have shown antiinflammatory, anticarcinogenic, and immunomodulatory effects of the compound. Further research is needed to confirm how CAPE affects many signal transduction cascades outside the NF-kappa pathway [29].

5 TYMPANOSCLEROSIS

5.1 Definition and Incidence

Tympanosclerosis, a sequel seen during the recovery period of middle ear infections, is characterized by the formation of hyaline plaques in the middle ear, in the eardrum, and rarely in the mastoid cavities [30–32]. The most common localization of calcification plaques is the tympanic membrane and it is defined as myringosclerosis if this process is limited to the tympanic membrane [31,33,34]. Other localizations include middle ear mucosa, ear ossicles, and occasionally the mastoid [35]. In the middle ear, it mostly affects the attic [36].

The incidence of tympanosclerosis in the community has been reported to be 10% by Plester [30].

Tympanosclerosis is a well-known sequela of acute and chronic otitis. It can also develop secondary to ventilation tube application in the treatment of otitis media with effusion [37].

In different studies, the incidence of tympanosclerosis was reported to be 7%–33% and the frequency of myringosclerosis 24%–52% in patients with chronic otitis media [38].

While there is no hearing loss in myringosclerosis, fixation of the malleus head at the epitympanic region and the incus or the stapes at the oval window with tympanosclerotic plaques may result in transmission-type hearing loss in the tympanosclerosis. In addition, sensorineural hearing loss can also occur by affecting the otic capsule

[30,39]. Prevention of the development of tympanosclerosis is important because of the difficulties in surgical treatment and the high risk of serious morbidity such as hearing loss [40].

5.2 History

For the first time in 1734, limestone plaques in the eardrum were defined by Cassebohm [41–43]. Von Trolch first described sclerotic changes after middle ear infections in 1878, and then Walb revealed the histological structure [30]. Also, Toynbee in 1860 and Politzer in 1883 worked on the histopathology [44]. The first researcher who defined tympanosclerosis in the present sense was Politzer in 1883 [30]. Politzer was focused on the distinction between tympanosclerosis and otosclerosis [44]. In 1955–56, Zöllner and Beck made detailed examinations using ear microscopes and revealed pathologies caused by tympanosclerosis in the middle ear and bones [30]. The first to used the term *tympanosclerosis* was Zöllner in this period [34,45]. Studies of authors such as Weiss, Sheehy, Gordon, Friedman, Tos, and Bak-Pedersen have focused on tympanosclerosis and the use of technological developments has increased the work in this area [44,46].

5.3 Ethiopathogenesis of Tympanosclerosis

The etiological factors leading to tympanosclerosis are not yet fully illuminated [34,45]. It is thought that multiple factors play a role in the etiopathogenesis. It has been suggested that increased free oxygen radicals, immunological susceptibility, genetic predisposition, mechanical damage and trauma developed during procedures such as miringotomy and ventilation tube (VT) application, inflammatory reaction due to otitis, various chemical agents, and metabolic disorders may lead to tympanosclerosis [47,48].

5.4 Theories About Etiopathogenesis

5.4.1 Mechanical Theories

5.4.1.1 INFLAMMATION

Inflammation triggers the formation of tympanosclerosis. One reason is that it disrupts the movements of the eardrum, and the other is the increase in the production of free oxygen radicals by inflammatory cells. Vibrations in the inflamed eardrum can cause stress in the tissue, resulting in an increase in the number of fibers [49].

In addition, increased proinflammatory cytokines and peroxynitrite produced by tissues in the middle ear during inflammation, and free oxygen radicals produced by streptococci such as hydrogen peroxide, may cause cell damage and apoptosis in the tissue. As a result, fibrosis and calcification development at the lamina propria can be observed [49].

5.4.1.2 VENTILATION TUBE APPLICATIONS

Myringotomy and VT in the treatment of otitis media with effusion (EOM), which cannot be cured by medical treatment, is still the gold standard treatment method and is one of the most common surgical interventions in children [31,33]. According to many studies, myringosclerosis is the most common complication of this surgical procedure [31,41,50]. Even VT applications are the best-known factor in the role of tympanosclerosis etiology, since the eardrum is damaged during the application and the tube also causes repetitive trauma in the eardrum due to its weight for as long as it remains [30].

The mechanism of the VT application leading to tympanosclerosis has not been fully clarified. According to the general theory proposed for the mechanism, damage to the mucosal connective tissue occurs due to trauma caused by paracentesis and VT, and degeneration of collagen fibers develops in the healing period of this damage, resulting in fibrosis, calcification, and accumulation of hyaline in the middle ear [49,51]. It has been suggested that VT application reduces vibratory movements in the eardrum, which leads to hyalinization and calcification in the collagen layer, together with an increase in the number of collagen fibers [33].

According to Friedman and colleagues, it is also possible that the human body perceives the VT as foreign matter and reacts to it. The presence of granulation tissues and crusts alongside VTs strengthens this suggestion [41].

During VT application, the occurrence of bleeding is a factor that increases the likelihood of tympanosclerosis [48,49,52].

5.4.2 Immunological Theories

Schiff and colleagues have suggested that autoimmune reactions cause the development of tympanosclerosis [41].

The immune complex type (type-3) reaction develops due to trauma, infection, and inflammation, and these immune complexes initiate a sclerotic reaction leading to lamina propria damage [53]. The fact that the levels of HLA-B35 and HLA-DR3 in the sera of patients with tympanosclerosis are significantly higher than the normal population supports this theory [54].

Flodin has investigated the role of inflammatory mediators in the development of tympanosclerosis in animal models. In their study (an otit model of streptococcus pneumoniae type 3), after the inoculation of streptococcus pneumonia into the middle ear cavity, first macrophages entered the middle ear mucosa and differentiated into osteoclasts, which initiate sclerotic

processes by leading to bone remodeling [55]. In another study, they have shown that the factor responsible for differentiation into osteoclasts was IL-6 [56].

Guo et al. investigated tympanosclerosis formation and transforming growth factor β1 (TGF-β1) and matrix metalloproteinase-9 (MMP-9) expression after streptococcus pneumonia type 3 infusion in the middle ear. They stated that TGF-β1 and metalloproteinase-9 (MMP-9) expression increased with the formation of tympanosclerosis. TGF-β1 has been implicated in matrix deposition and tissue regeneration as well as collagen accumulation and fibrous degeneration in the inflammatory region. TGF-β1 may accelerate degradation of extracellular matrix (ECM) by increasing expression of matrix metalloproteinases (MMPs). Overexpression of matrix metalloproteinase-9 (MMP-9), which degrades native type IV collagen and gelatin, is directly related to degradation of ECM and may result in fibrosis formation. In conclusion, up-regulation of TGF-β1 or MMPs has been implicated in the incidence of otitis media and prognosis, thus establishing a possible link between fibrosis and TS [57].

Görür et al. reported that the level of plasma fibronectin may be a predictor of tympanosclerosis [58].

5.4.3 Hyperoxia Theory

Many experimental investigations suggest that free oxygen radicals produced by inflammatory cells and bacteria during otitis media play a role in the development of tympanosclerosis. It is important that *Streptococus pneumania*, the most frequent cause of middle ear infections, produces hydrogen peroxide (H_2O_2), which is an important free oxygen radical agent in the body [40].

Major free radicals are hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), and hydroxyl (OH) radicals, which are the oxygen metabolites that are formed after the chemical reduction of oxygen [40]. Under normal conditions, the antioxidant enzymes in the body prevent the damage caused by these radicals. However, when the enzymes are inadequate and the amount of ROS produced exceeds the elimination capacity, tissue damage begins [50]. Since ROS is produced significantly by macrophages in the connective tissue, the most important damage is in this region and the formation of tympanosclerosis is triggered [30,59].

For tympanosclerosis, the increase in the oxygen concentration in the environment also has an impact [60]. In healthy humans and animals, the O_2 concentration in the middle ear is between 5.5% and 12.1%. This concentration increases during paracentesis or when the VT is inserted and it reaches the normal O_2 level in the external environment, which is 21% [30]. This causes a relative O_2 surge in the middle ear. This excess of O_2 is thought to increase ROS production and trigger tissue damage [61].

In their study Mattson et al. divided the rats into four groups after myringotomy and housed the groups at 10%, 15%, 21%, 40% O_2 saturation, respectively. They observed more intense myringosclerosis in rats under high O_2 saturation. Since the amount of oxygen in the middle ear is elevated after myringotomy, it is thought that it triggers tympanosclerosis by increasing free O2 radical formation [62]. In another study, Mattson et al. have emphasized the importance of free O2 radicals in the etiology by showing that there is a significant reduction in the formation of myringosclerosis in the group to which they applied local antiinflammatory agents inhibiting free O2 radical production [63].

After similar studies it was thought that the role of free O2 radicals in the pathogenesis could be important.

5.4.4 Genetic Relationship

The incidence of tympanosclerosis in patients with coronary atherosclerosis is reported to be higher than in normal patients. The high incidence of coexistence of atherosclerosis and tympanosclerosis suggests that there may be a genetic familial predisposition to sclerotic degeneration in all body tissues [30].

However, there are also publications reporting the opposite; Çiçek et al. have reported that coronary heart disease and atherosclerosis were not significantly associated with the development of tympanosclerosis [64].

Also, according to some authors, gender is thought to have an effect on the development of tympanosclerosis. Studies conducted by Koç and Üneri have shown that 71% of boys and 31% of girls have developed myringosclerosis after VT insertion [65]. However, there are studies that indicate that there is no statistically significant relationship between male gender and myringosclerosis [31,46,66].

5.5 Histopathology of Tympanosclerosis

Histologically, tympanosclerosis is characterized by decreased vascularization in lamina propria, increased hyalinization, calcium accumulation, and formation of cartilage and/or bone-like plaques due to fibroblast infiltration secondary to collagen deposition in connective tissue [37]. Macroscopically, there are plaques in fibroelastic consistency and lamellar structure. Light microscopy shows abundant collagen fibers interrupted with submucosal plaques and fissures [49]. Examination with electron microscopy showed that plaques formed irregular collagen fibers and calcium phosphat [30,67].

Pathology of tympanosclerosis can be examined in four stages:

5.5.1 Stage 1: Inflammation and Fibrogenesis

Proinflammatory mediators isolated in middle ear inflammation include lipopolysaccharides, bacterial components, cytokines, chemokines, and intercellular adhesion molecule-1 (ICAM-1). The cytokines identified in middle ear effusions are IL-1β, IFN-γ, IL-2, IL-6, IL-8, PAF, TNFα, and FGF [68].

In the early stages of inflammation, interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) are released, especially from inflammatory cells. These mediators stimulate macrophages to cause osteoclast transformation and play a role as an important osteotrophic factor. There are publications advocating that bone remodeling with this osteoclast activity is effective in the pathogenesis of tympanosclerosis [55,56].

After these reactions in the lamina propria, fibrogenesis starts. There is an increase in microfibrils due to increased fibroblast. The increased microfibrils combine to form the fibers, and the fibers combine to form the collagen. Collagenous bands form sclerotic plaques holding together around the protein polysaccharide complexes [30].

5.5.2 Stage 2: Cell Degeneration and Precipitation

This includes the stage of degenerating inflammatory cell placement on collagen fibers forming the primer calcification tract [49].

5.5.3 Stage 3: Calcification

Membrane-covered extracellular matrix vesicles, which store Ca+ and P+, play a role. Calcification begins with the formation of Ca+, P+ containing structures called calcospheric at the center of the vesicle. Calcospherules combine to form the calcification plaque by filling the gaps between the collagen fibers [49].

5.5.4 Stage 4: Decalcification and Recovery

Over time, calcium phosphate precipitates in the calcospheres decrease, so that the plaques may become smaller in diameter or may disappear completely [49].

5.6 Clinical Types and Characteristics of Tympanosclerosis

5.6.1 Classification

1. Clinical Classification
   - **a.** Histological tympanosclerosis:
     It is characterized by the formation of plaques at histological level chronic otitis media and cholesteatoma [49].
   - **b.** Clinical Tympanosclerosis:
     Myringosclerotic appearance of the ear membrane, hyaline degeneration findings, appearance of calcification plaques, secondary ossification classified in this group.
   - **c.** Surgical Tympanosclerosis:
     Characterized by involvement and fixation of the ear bones, impaired eardrum movement and hearing loss [46].
2. Classification According to the Status of the Eardrum
   - **a.** Open Tympanosclerosis: The ear membrane is perforated and therefore the middle ear is open to external ambulation. Constitute about 80%–90% of tympanosclerosis.
   - **b.** Closed Tympanosclerosis: The ear membrane is not perforated and the middle ear is not open to external mediation.
3. Classification by Location
   - **a.** Miringoskleroz: Located in the eardrum (24%–51%).
   - **b.** Timpanoskleroz: Located in the tympanic cavity.

Bluestone has staged tympanosclerosis:

Stage 1: TS is limited to tympanic membrane and hearing is not affected.
Stage 2: TS is limited to tympanic membrane but hearing loss due to TS is observed.
Stage 3: TS affects middle ear but hearing loss is not observed.
Stage 4: TS affects middle ear and hearing loss is observed.
Stage 5: TS affects the tympanic membrane and middle ear but no hearing loss.
Stage 6: There are dense TS, middle ear and tympanic membranes affected, and hearing loss is observed [69].

5.6.2 Diagnosis

In general, anamnesis, physical examination, and audiologic examination are used for diagnosis. The contribution of imaging modalities is limited, but well-documented tomography may be useful in the presence of mature plaques. Findings of the oppositional ear are important. Sometimes the diagnosis can be made only as an operative finding. Myringosclerotic cases are noticed randomly, while white calcificated plaques are easily seen during otomicroscopic examination [34].

In cases with tympanosclerosis, if the bones are affected, hearing loss develops [70]. Hearing loss is a transmission type of hearing loss that can produce air-bone gap of about 25–45 dB, usually evident at low frequencies. Sometimes it can gain mixed character by adding damage of the cochlea. It is also thought that aggressive types of tympanosclerosis may cause sensorineural hearing loss by affecting the otic capsule [30,70].

5.6.3 Differential Diagnosis

In the differential diagnosis, otosclerosis, other pathologies of the bone chain, especially malleus fixation or cholesteatoma, should be considered [49].

5.6.4 Treatment

There is no curative treatment of tympanosclerosis, and relapses are frequent after surgery; because of this, the most effective treatment is the prevention of development [71].

6 CAPE AND TYMPANOSCLEROSIS

Because of the role played by inflammation and the fact that increased production of free oxygen radicals in the pathogenesis of tympanosclerosis has been demonstrated in various studies, experimental ITT (intention to treat) research has been concentrated on antiinflammatory and antioxidant drugs.

Previously, some antioxidant and antiinflammatory substances, such as L-carnitine, vitamin E, ascorbic acid, superoxide dismutase, *N*-acetyl cysteine, selenium, *N*-nitro L-arginine methyl ester (L-NAME), and ginkgo biloba extract, have been tested on rats and humans for the treatment of tympanosclerosis and the development of sclerotic plaque has been prevented or reduced by these agents. They have shown these effects via reducing the production of reactive oxygen radicals by neutrophils in response to inflammation after myringotomy [50,51,62,63,71–76].

Tympanic membrane thickness, middle ear inflammation grade, and sclerotic plaques are the most important parameters used to evaluate the formation of tympanosclerosis. Because of this, the studies on tympanosclerosis usually evaluate these parameters.

There are a few studies in the literature on the effect of CAPE on tympanosclerosis, showing proof of its antioxidant and antiinflammatory effects.

In a study conducted by Jae Jung Song et al., they investigated the antiinflammatory and antioxidant effect of CAPE on H_2O_2 induced inflammation and oxidative stress in human middle ear epithelial cells. Proinflammatory cytokine (tumor necrosis factor (TNF-α)), and COX-2 levels, as well as reactive oxygen radical (ROS) production were measured directly by flow cytometric analysis and indirectly by Western blot analysis that shows superoxide dismutase (SOD) expression. The effect of CAPE was compared to *N*-acetyl cysteine, which is known to have antioxidant and antiinflammatory effects. It has been determined that CAPE decreases the production of proinflammatory cytokines (tumor necrosis factor (TNF)-α and COX-2) in a dose and time-dependent manner and inhibits free oxygen radical accumulation. The study also showed that increased endogenous superoxide dismutase after administration of H_2O_2 decreased after administration of CAPE, and this effect was attributed to the antioxidant effect of CAPE [77].

In a study performed by Song et al. investigating the effects of CAPE on myringosclerosis, 35 rats divided into four groups and myringotomy was performed. After myringotomy, for 2 weeks, group 1 (10 rats) were not treated, group 2 (10 rats) were injected with intraperitoneal saline, group 3 (10 rats) were injected with 10 mg/kg intraperitoneal CAPE, and group 4 (5 rats) were used as a control group. On the 15th day, before sacrificing, otomicroscopic examination was performed for all rats, the presence of myringosclerosis on the pars tensa of the tympanic membrane was evaluated, and the groups were compared. Significant reductions in sclerotic plaque formation were detected in the CAPE-treated (group 3) rats compared to untreated and IP saline injected rats (groups 1 and 2). They have suggested that the protective effect of CAPE might be related to several mechanisms, but most predominantly emphasized that CAPE inhibits NF-κB and down-regulates the release of several proinflammatory enzymes and cytokines. Song and colleagues also found that the tympanic membrane thickness was 0.75 ± 0.22 mm in the CAPE group, 1.17 ± 0.49 mm in the untreated group, and 1.19 ± 0.39 mm in the saline group. This difference was statistically significant [60].

In a similar study, rats were divided into four groups: comparison group ($n=4$), untreated group ($n=7$), saline (control) group ($n=7$) (2.5 mL/kg/day, intraperitoneal), and caffeic acid phenethyl ester group (10 μmol/kg/day, intraperitoneal). All rats except the comparison group had myringotomy on the right ear. After 15 days of treatment, the MS formation was assessed histopathologically. The severity of myringosclerosis in the CAPE group was significantly lower than that of the other groups and these results were related to the antioxidant and antiinflammatory effects of CAPE. Also in the study, the tympanic membrane thickness in the CAPE group was found to be significantly lower than the saline and the untreated group. But the thickness of the tympanic membrane in the CAPE group was found to be significantly greater than that of the tympanic membrane in the healthy group and it was indicated that long-term results of CAPE should be investigated after miringotomy. In conclusion, they have suggested that caffeic acid phenethyl ester has potential protective effects againest myringosclerosis development and ventilation tube placement [78].

The severity of inflammation in the middle ear mucosa is an important factor in the evaluation of tympanosclerosis, but has not been assessed in any of the mentioned studies. In a study performed by İbrahim et al., rats were divided into four groups: CAPE group (10 rats received 10 mmol/kg/day intraperitoneal CAPE), alcohol group (10 rats received 2 mL 10% ethyl alcohol solution/day, intraperitoneal), control group (10 rats untreated), and normal group (4 rats not subjected to any procedure). Myringotomy was performed on all rats except the normal group and through myringotomy incision type 3 streptococcus pneumoniae strain at a dose of 0.03 mL 2.5×107 colony/mL was injected into the middle ear to

trigger middle ear inflammation by causing otitis media with effusion (EOM). Repeated miringotomies were performed once a week to induce tympanosclerosis for 5 weeks. Drug administration was continued on a daily basis for 5 weeks and at the end of this period, the prevalence of myringosclerosis in the tympanic membrane was evaluated semiquantitatively by an otomicroscope under general anesthesia. After that, the rats were sacrificed and the bullae of the rats were removed for the histopathological evaluation. At the end of the study, while no severe myringosclerosis was observed in the CAPE group, severe and moderate myringosclerosis was observed in 2 rats, and mild sclerosis was observed in 16 rats. They indicated that the presence of myringosclerosis in all rats in the CAPE group in their study, with nonobservance of myringosclerosis in some rats in similar studies, might be related to triggering of myringosclerosis by a single myringotomy in other studies and brevity of the study period. The study also found that the middle ear mucosa inflammation score was significantly lower in the CAPE group when compared to the alcohol and control groups, but inflammation was not completely prevented. It has been stated that there may be other factors that cannot be controlled by antioxidant and antiinflammatory effects in sclerosis formation. In this study the tympanic membrane thickness in the CAPE group was significantly greater than normal tympanic membrane thickness; however, when compared to the alcohol and control group, it was significantly thinner. In conclusion, CAPE has been shown to reduce the severity of myringosclerosis after myringotomy in a statistically significant manner, consistent with other studies on the effect of CAPE on myringosclerosis [79].

Since there is not yet a method that can treat tympanosclerosis after its development, CAPE can be used to reduce the risk of tympanosclerosis in patients with otitis media and VT. However, there are no studies for the use of CAPE in humans.

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Further Reading

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CHAPTER

32

The Polyphenolic Compound Hesperidin and Bone Protection

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Abbreviations

BMC	bone mineral content
BMD	bone mineral density
BV/TV	bone volume fraction
Conn.Dn	connectivity density
Ct.Ar/Tt.Ar	cortical area fraction
E.Pm	endosteal perimeter
MA	medullary area
ORX	orchidectomy
OVX	ovariectomy
Tb.N	trabecular number
Tb.Sp	trabecular separation
Tb.Th	trabecular thickness
μCT	microcomputed tomography

1 INTRODUCTION

1.1 Bone

Bone can be classified into two types, dense cortical bone and porous trabecular bone, and is a multifaceted tissue that provides many significant roles in our everyday life. Specifically, bones facilitate movement by serving as attachment sites for muscles and provide mechanical support for the body, protect vital organs, serve as a reservoir for minerals, and are also a site for hematopoiesis [1,2]. Constituents of bone include minerals, collagenous and noncollagenous proteins, lipids, and water. During mineralization, hydroxyapatite is crystallized within the collagen fibers, providing strength and rigidity to bones. Type I collagen is the basic building block of bone matrix, while noncollagenous proteins play roles in the organization of the extracellular matrix and regulating the mineralization process [3]. There are three types of bone-specific cells: osteoblasts, osteocytes, and osteoclasts. Osteoblasts are responsible for building bone and secreting proteins and factors involved with mineralization. When osteoblasts become embedded in the matrix they produce, they become osteocytes that serve a mechanosensory role to regulate bone formation or resorption [4]. Osteoclasts degrade bone through the release of acid and lysosomal proteases [5]. Thus, in order to maintain the structural integrity of bone throughout life, these three different cell types work together such that formation and resorption processes continually reshape or replace bone [6]. In early life stages, including fetal life and throughout growth, osteoblast activity exceeds osteoclast activity to yield a steady accumulation of bone mineral and an increasingly stronger bone structure [7].

Maternal and early life diet can influence bone development and potentially set a trajectory for stronger or weaker bones at adulthood. Previous research has manipulated the maternal diet to investigate the effects of food and food components on bone development of offspring through nutritional programming using well-characterized mouse models [8–12]. Nutritional programming of bone development can be defined as the ability for diet during early life to set a trajectory for better or compromised bone health at adulthood [13]. In humans, bone development occurs until approximately 20 to 30 years of age when peak bone mass, defined as the maximal amount of bone mass attained during the lifespan, is attained [14]. Following the attainment of peak bone mass, bone loss due to aging is inevitable in both males and females as a result of bone resorption exceeding formation. A decline in bone mineral density (BMD) may be accelerated in women after menopause [15] and can result in the development of osteoporosis, characterized by a compromised bone strength and an increased risk of fragility fracture [16]. Men can also develop osteoporosis during aging [17]. The onset of osteoporosis in men may occur due to a decline in testosterone

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levels with aging [18] as well as poor nutrition [19] and/or a lack of physical activity [20]. Thus, attainment of a maximal peak bone mass is a potential preventive strategy for reducing the risk of osteoporosis during later life. Another approach is to use diet to help prevent or attenuate a loss of mineral and deterioration of bone structure during aging, and specifically after menopause in women. While there are drug interventions that help attenuate destruction of bone tissue after menopause and/or during aging, and importantly, reduce the risk of fragility fractures, there are potential side effects [21–23]. Thus, nutritional strategies may serve as a safe and supportive approach for achieving optimal bone health throughout the lifespan. Moreover, such strategies may allow drug interventions to have greater efficacy and this aspect requires future study.

2 NUTRITION AS A STRATEGY FOR MAINTAINING HEALTHY, STRONG BONES

2.1 Citrus Fruits

Calcium and vitamin D are well-established as key nutrients for optimal bone health and function. There are specific dietary reference intakes for both these nutrients based on their critical role in bone health. The dietary reference intakes for calcium and vitamin D were based on measures of bone health: bone accrual, maintenance and loss throughout life [24]. Furthermore, protein is increasingly recognized for its importance in maintaining BMD and bone structure [25,26]. Moreover, in the context of adequate calcium consumption, higher protein intakes have been associated with a higher BMD [27,28] and a reduced risk of hip fracture [29,30]. Other dietary components that may have favorable, bone-specific effects include flavonoids. While flavonoids cannot be synthesized by humans and animals and therefore must be consumed in the diet [31], they are currently not considered essential nutrients and thus do not have a dietary recommended intake. Flavonoids are polyphenolic compounds produced by plants for a defense mechanism and to regulate their growth [32] and thus are naturally present in food and beverages of plant origin. This includes fruits, vegetables, tea, cocoa, and wine [33]. Flavonoids are classified as flavones, flavonols, flavanones, flavanonols, isoflavones, or flavan-3-ols [34]. Flavanones are comprised of three main aglycones, including hesperetin, naringenin, and eriodictyol, with citrus fruits as a particularly rich source.

Citrus fruits are often distinguished by their vibrant color, sweet or sour taste, acidity, and the presence of nutrients such as vitamin C, folate, and potassium, as well as the flavonoids hesperidin and naringin [35]. Some but not all epidemiological studies suggest a positive association between the intake of fruit and higher bone mineral content (BMC) and/or BMD in women and men [36–41]. In a cross-sectional study from China, fruit intake was associated with higher BMC and BMD of the total body, lumbar spine, hip and femur neck in postmenopausal women [36]. Although, Prynne et al. observed a positive association between higher fruit intake and higher BMD of the lumbar spine, while there was no association at the hip or femur neck [37]. A systematic review of studies in women age 45 years and over concluded that the association between fruit and vegetable consumption and bone health—assessed using outcomes such as fractures, BMD, bone turnover markers—requires further study [41]. This chapter specifically focuses on hesperidin as it is a predominant flavonoid in citrus fruits and, to date, is the most studied citrus flavonoid for its potential beneficial effect on BMD, bone structure, and bone strength using rodent models.

2.1.1 Hesperidin

Hesperidin ($C_{28}H_{34}O_{15}$) is chemically classified as a flavanone glycoside, containing hesperitin (the aglycone form) and sucrose. Hesperitin ($C_{16}H_{14}O_6$) is passively absorbed across the intestine into the circulation [42]. However, citrus fruits contain very low levels of hesperitin and most flavanones exist in the glycoside form (i.e., hesperidin). Thus, hesperidin is not readily available to be absorbed across the intestine and is hydrolyzed to hespertitin by microflora β-glucosidase in the colon prior to absorption [43]. Within 24 h of consumption, hesperitin is excreted in the urine [44].

While whole citrus fruits such as oranges, limes, and lemons, contain the highest levels of hesperidin, the amount varies between fruit type [45,46] (Table 32.1). As hesperidin is the dominant flavanone within oranges, there is a range of levels established within and between sweet oranges, tangerines, and sour oranges [45], although hesperidin is present in highest concentration in the peel. Additionally, naringin ($C_{27}H_{32}O_{14}$, the aglycone form of naringenin) is a citrus flavonoid found

TABLE 32.1 Concentration Range of Hesperidin and Naringin in Oranges [45], Limes [46], Lemons [46], and Grapefruits [46]

Food source (g/kg fresh weight)	Hesperidin	Naringin
Sweet orange	0.0256–0.393	0.000–0.017
Tangerine	0.043–0.471	0.000
Sour orange	0.000–0.005	0.061–0.341
Lime	0.052–0.430	0.000
Lemon	0.019–1.422	0.000–0.016
Grapefruit	0.000–0.18	0.000–0.48

abundantly in grapefruit [46], providing the bitter flavor [47]. Similar to hesperidin, naringin is most concentrated within the grapefruit peel. A few of the studies discussed in this chapter have used a combination of these two flavonoids.

3 HESPERIDIN CONSUMPTION AND ITS EFFECT ON OUTCOMES OF BONE PROTECTION

3.1 Findings From Studies in Humans

Human consumption of hesperidin occurs through the diet, and in some cases, through supplement use. The bioavailability of hesperidin in men has been studied by Manach et al. [44]. Participants consumed either 0.5 or 1 L of commercially available orange juice, containing 0.444 g/L of hesperidin. Orange juice is reported to contain 0.2 to 0.59 g hersperidin/L. [48] The peak plasma concentration of hesperitin occurred 5 to 7 h after consumption, returning to baseline levels within 24 h [44]. Thus, due to the relatively fast turnover, it is likely that citrus or other sources of hesperidin would need to be consumed on a regular basis for maximal health benefits.

To date, only one study has investigated the effect of hesperidin on an outcome relating to bone health in humans [49]. Specifically, bone calcium retention was measured in a group of postmenopausal women (>4 years postmenopause) using a ^{41}Ca isotope tracer method. Hesperidin was studied alone and in combination with a calcium supplement (Calcilock) to determine if there was an added benefit—greater retention of calcium—by providing supplemental Ca with hesperidin. The study was a crossover design such that each woman received each of the three interventions (500 g hesperidin alone or in combination with calcium (0.5 g), or placebo) for 50 days, with a washout period between each intervention. There was a 5.5% greater retention of calcium when the combination of hesperidin and calcium was consumed, but interestingly, this effect was not evident when hesperidin was consumed alone [49]. This finding suggests that calcium itself may have mediated the positive effect on calcium retention or that the interaction of hesperidin and calcium is required. Because a calcium alone group was not studied, further investigation is required to more fully understand how hesperidin may modulate calcium retention.

3.2 Findings From Studies Using Rodent Models

Studies investigating the effects of hesperidin on outcomes of bone health have primarily used rodent models (Tables 32.2 and 32.3) to provide insight into potential effects on bone health in humans. The accelerated

TABLE 32.2 Summary of Bone Outcomes Measured Longitudinally in vivo as Well as ex vivo in Male and Female Mice Whose Mothers Were Fed Hesperidin and Naringin From Prepregnancy and Through End of Lactation [50,51]

(A)	Male offspring			Female offspring		
Age (months)	**2**	**4**	**6**	**2**	**4**	**6**
Proximal Tibia						
BMD	–	–	–	–	↓	–
Trabecular structure						
BV/TV	–	↑	–	↓	↓	–
Tb.Th	–	↑	–	–	–	–
Tb.N	–	↑	–	↓	↓	–
Tb.Sp	–	–	–	↑	↑	–
Conn.Dn	–	↑	–	↓	↓	–
Cortical structure						
Ct.Ar/Tt.Ar	↑	↑	↑	–	–	–
Ct.Th	↑	↑	↑	–	–	–
E.Pm	–	–	–	–	–	–
MA	↓	–	–	–	–	–

(B)	Male offspring	Female offspring
Whole Femur		
BMD	–	–
Femur Neck		
Trabecular structure		
BV/TV	–	–
Tb.Th	–	–
Tb.N	–	–
Tb.Sp	↓	–
Peak load	–	–
Femur Midpoint		
Cortical structure		
Ct.Ar	–	–
Ct.Th	–	–
Peak load	–	–
Distal Femur		
Trabecular structure		
BV/TV	–	–
Tb.Th	–	–
Tb.N	–	–
Tb.Sp	–	–

Continued

TABLE 32.2 Summary of Bone Outcomes Measured Longitudinally in vivo as Well as ex vivo in Male and Female Mice Whose Mothers Were Fed Hesperidin and Naringin From Prepregnancy and Through End of Lactation [50,51]—cont'd

(B)	Male offspring	Female offspring
	Lumbar Vertebrae 1–3	
BMD	–	–
Lumbar Vertebra 2		
Peak load	nm	–
Trabecular structure		
BV/TV	–	–
Tb.Th	–	–
Tb.N	–	–
Tb.Sp	–	–
Cortical structure		
Ct.Ar	–	–
Ct.Th	–	–

↑ represents that the bone outcome was significantly greater compared to offspring whose mothers were fed control diet.
↓ represents that the bone outcome was significantly lower compared to offspring whose mothers were fed control diet.
– represents no significant difference compared to offspring whose mothers were fed control diet.
nm = not measured.

lifespans of rodents allow researchers to conduct studies that capture significant aspects of the lifespan in a period of months rather than years, and thus are particularly useful to studying changes in BMD and bone structure in a shortened period of time. While timing of developmental changes in trabecular and cortical bone can vary among species and strains, it has been reported that the amount of trabecular bone peaks at 2 to 3 months of age while the amount of cortical bone continues to increase until later ages in Sprague-Dawley rats [56] and CD-1 mice [57]. Fig. 32.1 provides a summary of the studies in which hesperidin has been studied in female and male rodents in relation to stage of life.

Before discussing specific findings regarding the effects of hesperidin on bone outcomes in rodent studies, it is useful to review key measures that are reported in these studies. Ultimately, intervention studies aim to improve bone strength. Because bone strength is dictated by both the quantity of mineral and the quality of the bone structure, commonly measured outcomes include BMD measured by dual-energy X-ray absorptiometry (DXA) or microcomputed tomography (μCT), while three-dimensional bone structure is measured using μCT. BMD is expressed as the amount of mineral per area (g/cm^2) or volume (g/cm^3) of bone, depending on whether it is assessed by DXA or μCT, respectively. Some studies will report BMD for the whole bone as well as for specific sites that are more representative of trabecular (distal or proximal region of a

TABLE 32.3 Summary of the Effect of Hesperidin on BMD and Bone Structure in Rodent Models

Species	Age (months)	Intervention groups (g/kg diet)	Bone outcomes	Results
Maternal Exposure—Female & Male Offspring				
Female CD-1 mice (Dams) (n = 17)	In utero to age 6 months: exposure during in utero life through to weaning (age 21 days). From 21 days to 6 months of age all female offspring fed control diet	Dams fed control diet or control diet containing 5 g hesperidin + 2.5 g naringin from prepregnancy through end of lactation	Proximal tibia trabecular and cortical structure: BMD, BV/TV, Tb.Th, Tb.N, Tb.Sp, Conn.Dn, Ct.Ar/Tt.Ar, Ct.Th, E.Pm, MA	See Table 32.2
Female offspring (n = 16–18) [50]		Female offspring fed control diet from weaning (age 21 days) through to age 6 months	Femur trabecular and cortical structure: BMD, BV/TV, Tb.Th, Tb.N, Tb.Sp, Ct.Ar, Ct.Th L2 trabecular and cortical structure: BMD, BV/TV, Tb.Th, Tb.N, Tb.Sp, Ct.Ar, Ct.Th Biomechanical strength: Peak load at L2, femur midpoint and femur neck	
Female CD-1 mice (Dams) (n = 18)	In utero to age 6 months: exposure during in utero life through to weaning (age 21 days). From 21 days to 6 months of age all male offspring fed control diet	Dams fed control diet or control diet containing 5 g hesperidin + 2.5 g naringin from prepregnancy through end of lactation	Proximal tibia trabecular and cortical structure: BMD, BV/TV, Tb.Th, Tb.N, Tb.Sp, Conn.Dn, Ct.Ar/Tt.Ar, Ct.Th, E.Pm, MA	See Table 32.2

TABLE 32.3 Summary of the Effect of Hesperidin on BMD and Bone Structure in Rodent Models—cont'd

Species	Age (months)	Intervention groups (g/kg diet)	Bone outcomes	Results
Male offspring ($n=18$) [51]		Male offspring fed control diet from weaning (age 21 days) through to age 6 months	Femur trabecular and cortical structure: BMD, BV/TV, Tb.Th, Tb.N, Tb.Sp, Ct.Ar, Ct.Th L2 trabecular and cortical structure: BMD, BV/TV, Tb.Th, Tb.N, Tb.Sp, Ct.Ar, Ct.Th Biomechanical strength: peak load at tibia midpoint, femur midpoint and femur neck	
Growth—Females				
Female Wistar rats ($n=40$) [52]	3 to 6	SH control SH+5 g hesperidin OVX control OVX+5 g hesperidin	Femur BMD: T-total, M-middle, D-distal Biomechanical strength: ultimate load at femur midpoint	*SH+Hesperidin*: ↑ T-, M-, D-BMD – Femur strength *OVX+Hesperidin*: ↑ T-, M-, D-BMD ↑ Femur strength
Ovariectomized—Females				
Female ddY mice ($n=40$) [53]	2 to 3	SH control OVX control OVX+5 g hesperidin OVX+7 g α-glucosylhesperidin	Femur BMD: T-total, P-proximal, M-middle, D-distal Distal femur trabecular structure: BV/TV, Tb.Th, Tb.Sp	*OVX+Hesperidin*: ↑ T-, P-, D-BMD *OVX +α-Glucosylhesperidin*: ↑ T- and M-BMD ↓ Tb.Sp
Female Wistar rats ($n=40$) [52]	6 to 9	SH control SH+5 g hesperidin OVX+control OVX+5 g hesperidin	Femur BMD: T-total, M-middle, D-distal Biomechanical strength: ultimate load at femur midpoint	*SH+Hesperidin*: – BMD ↑ femur strength *OVX+Hesperidin*: ↑ T-, M-, D-BMD ↑ Femur strength
Orchidectomized—Males				
Male ddY mice ($n=35$) [54]	2 to 3	SH control ORX control ORX+5 g hesperidin ORX+7 g α-glucosylhesperidin ORX+0.13 g sinvastatin	Femur BMD: T-total, P-proximal, M-middle, D-distal Distal femur trabecular structure: BV/TV, Tb.N, Tb.Sp	*ORX+Hesperidin*: ↑ T-, P-, M-, D-BMD ↑ BV/TV ↑ Tb.N ↓ Tb.Sp *ORX +α-Glucosylhesperidin*: ↑ T-, P-, D-BMD ↑ BV/TV ↑ Tb.N ↓ Tb.Sp
Aging—Males				
Male Wistar rats ($n=48$) [55]	20 to 23	Control 5 g hesperidin 5 g naringin 2.5 g hesperidin+2.5 g naringin	Femur BMD: T-total, P-proximal, M-middle, D-distal Distal femur trabecular structure: BV/TV, Tb.Th, Tb.N, Tb.Sp Biomechanical strength: ultimate load at femur midpoint	*Hesperidin*: ↑ T-, P-, M-, D-BMD ↑ BV/TV – Femur strength *Naringin*: ↑ D-BMD – Femur strength *Hesperidin+Naringin*: ↑ T-, P- M-, D-BMD ↑ Tb.Th – Femur strength

SH+hesperidin vs. SH Control; OVX+hesperidin vs. OVX Control; ORX+hesperidin vs. ORX Control: ↑ represents significantly greater bone outcome compared to the control; ↓ represents significantly lower bone outcome compared to the control; – represents no significant difference compared to the control group.

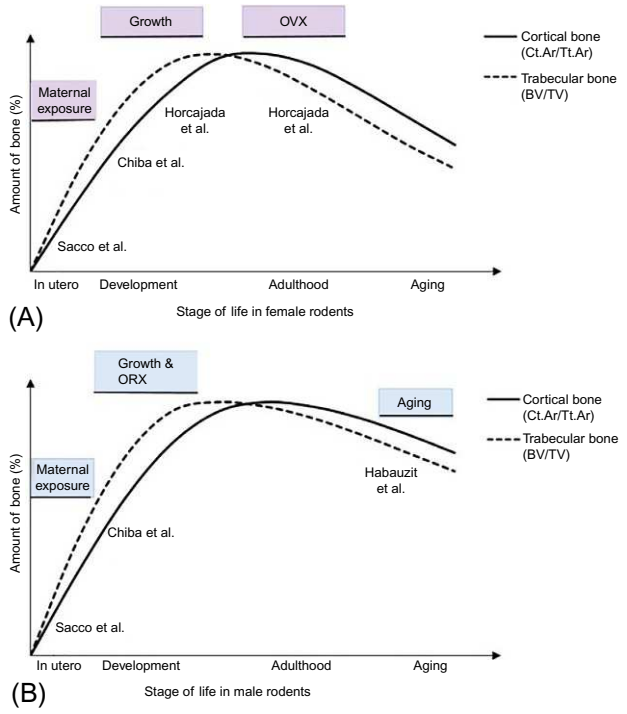

FIG. 32.1 Shaded areas demonstrate the stages of life when the effect of hesperidin on cortical (Ct.Ar/Tt.Ar) and trabecular (BV/TV) bone and/or BMD have been studied using (A) female and (B) male rodent models. Within growth and aging, OVX (females) or ORX (males) have sometimes been used to accelerate a loss of bone structure and mineral. The solid black line and hatched line approximate the changes in cortical and trabecular bone, respectively, and thus BMD throughout life. Although the overall changes throughout the lifespan are similar between sexes, males typically have greater peak trabecular and cortical bone and thus BMD throughout life.

long bone) or cortical (middle section of a long bone) bone. For measurement of bone structure, μCT acquires three-dimensional images that are reconstructed based on hundreds of X-ray projection images taken incrementally at multiple angles. Multiple measures of trabecular and cortical bone outcomes can be measured and insightful measures of trabecular bone morphology include bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) and connectivity density (Conn.Dn). For cortical bone morphology, important measures include cortical area fraction (Ct.Ar/Tt.Ar), cross-sectional thickness (Ct.Th), medullary area (MA), and endosteal perimeter (E.Pm) [58,59]. Together these measures provide a comprehensive assessment of how an intervention, such as hesperidin, alters bone strength in rodent models. Some studies have included measures of bone strength that are obtained through physical fracture tests at specific skeletal sites, and thus these measures can only be obtained at an endpoint of a study in an excised bone rather than being performed longitudinally, as can be done using in vivo μCT. Specifically, peak load or ultimate load is measured as the maximal force a skeletal site can withstand before fracture. Femur midpoint, femur neck, and lumbar vertebra are sites that are tested to evaluate the strength of cortical (femur midpoint) or a mixture of cortical and trabecular bone (femur neck, lumbar vertebra).

3.2.1 Nutritional Programming of Bone Development by Maternal Diet

As discussed earlier and building from the previously mentioned studies in which maternal and/or early postnatal diet has been shown to nutritionally program bone development [8–12], the effect of maternal consumption of hesperidin and naringin on bone development in offspring was studied [50,51] (Tables 32.2 and 32.3). Female CD-1 mice were fed the standard control diet (AIN-93G rodent diet) or the control diet supplemented with 5 g hesperidin + 2.5 g naringin/kg diet prior to pregnancy and during pregnancy and lactation. At weaning, female [50] and male [51] offspring were fed the control diet until 6 months of age. At age 2 and 4 months, female offspring whose mothers were fed hesperidin and naringin had compromised bone microarchitecture at the proximal tibia, as demonstrated by lower BV/TV, Tb.N, Conn. Dn and BMD and an increase in Tb.Sp. [50] These differences did not persist at 6 months of age, suggesting no long-term detriment to bone structure by 6 months of age, representing early adulthood (Table 32.2A). Furthermore, at 6 months of age, there were no differences in bone microarchitecture or strength of the femur neck or midpoint, or lumbar vertebra [50] (Table 32.2B). These outcomes were measured *ex vivo* at the study endpoint. Interestingly, male siblings responded differently, and this finding highlights the importance of measuring potential sex-specific effects. At 4 months of age, males of mothers fed hesperidin and naringin had better bone structure as shown by greater BV/TV, Tb.Th, Tb.N, and Conn.Dn. Also, cortical outcomes differed at the proximal tibia; MA at 2 months of age was lower and Ct.Ar/Tt.Ar and Ct.Th at 2 through 6 months of age were higher due to maternal exposure to hesperidin and naringen (Table 32.2A). Furthermore, at age 6 months, there was a decrease in Tb.Sp at the femur neck, a favorable effect, with no differences in peak load at the femur neck [51] (Table 32.2B). Peak load at femur midpoint and lumbar vertebra was also unaltered at 6 months of age [51]. The long-lasting benefits in male offspring demonstrated that there was a window of opportunity for these flavanone glycosides to program bone development, but that this occurred in a skeletal-site specific manner. Further study is required to understand the mechanisms by which these sex specific responses occurred and the specific contribution of hesperidin versus naringin.

3.2.2 *Effect of Hesperidin in Growing Female Rats With and Without Ovariectomy*

In young female Wistar rats (age 3 months), Horcajada et al. investigated whether hesperidin intervention for 3 months could preserve BMD after ovariectomy (OVX) [52]. While OVX is a surgical procedure used to cause estrogen deficiency and mimic the decline in BMD and deterioration of bone structure with aging in females, it is sometimes performed during an active phase of bone growth as was done in this study. Unlike many other studies using the ovariectomized rat model, this study also included sham-operated rats that received the intervention, and findings from this study provide insight into the effect of hesperidin during growth. Half of the females were ovariectomized while the other half were sham-operated (ovaries were intact) and were randomized to hesperidin (5 g hesperidin/kg diet) or no intervention. Rats were fed their respective diet for 3 months, until age 6 months. At 6 months of age, hesperidin intervention was shown to protect against the loss of BMD that occurs post-OVX. Total BMD as well as regional assessment of BMD at the metaphyseal and diaphyseal region along with ultimate load at the femur midpoint was higher in ovariectomized rats fed hesperidin compared to the ovariectomized group that was fed the control diet. Interestingly, rats that were sham-operated and fed hesperidin also had higher BMD at all three sites measured compared to sham-operated rats that received no hesperidin, but a higher ultimate load at femur midpoint was not observed [52]. Thus, beneficial effects were demonstrated in the context of growth as well as OVX during this relatively early stage of development, 3 to 6 months of age.

3.2.3 *Effect of Hesperidin in Supporting Bone Health After Ovariectomy or Orchidectomy*

Chiba et al. studied 2-month-old ovariectomized ddY female mice to represent postmenopausal osteoporosis [53]. Mice were fed a control diet (AIN-93G diet) or control diet containing 5 g hesperidin/kg diet or 7 g α-glucosylhesperidin/kg diet, a soluble form of hesperidin that is bound to glucose. An additional group was fed 17β-estradiol, to serve as a positive control. At 1 month following OVX, hesperidin and α-glucosylhesperidin protected against the loss of BMD. Hesperidin intervention resulted in full protection of BMD for total femur BMD and at the distal and proximal sites of the femur; α-glucosylhesperidin also protected against the loss of total BMD as well as at the proximal and middle sites but not the distal site. However, effects on bone structure were modest, with only α-glucosylhesperidin resulting in a significant decrease in Tb.Sp compared to the ovariectomized control group [53]. There were no other benefits of either hesperidin or α-glucosylhesperidin to other measures of trabecular bone structure such as BV/TV and Tb.Th. However, both hesperidin and α-glucosylhesperidin did result in a lower ratio of osteoclasts per bone surface, a favorable effect that may have had benefits to bone structure over a longer study period. A 1-month intervention is relatively short and given the young age of the mice it would be interesting to study the effect of these interventions over a longer time period.

Another study also showed a protective effect of hesperidin on femur BMD in ovariectomized rats as well as intact rats studied from 6 to 9 months of age. Six-month-old rats were ovariectomized or sham-operated and fed a control diet or diet containing 5 g hesperidin/kg diet for 3 months, until 9 months of age [52]. Among ovariectomized groups, hesperidin intervention resulted in a higher metaphyseal, diaphyseal, and total BMD of the femur compared to ovariectomized rats fed the control diet. Ovariectomized rats fed hesperidin also had a higher ultimate load at the femur midpoint compared to the OVX-alone group [52]. When hesperidin was fed to intact female rats for three months, from age 6 to 9 months, BMD was not higher than females fed the control diet. This finding contrasts with the observation in younger, intact rats in which higher BMD was observed with hesperidin intervention during growth (discussed in Section 3.2.2). However, among these rats with intact ovaries, hesperidin did result in a higher ultimate load at the femur midpoint, suggesting that bone strength benefits even in an estrogen-replete state at 6 through 9 months of age.

Similar to OVX, orchidectomy (ORX) can be performed in males to induce androgen deficiency and a loss of BMD and deterioration of bone structure. Consistent with previously discussed findings in ovariectomized females, hesperidin has been shown to attenuate a loss of BMD and bone structure after ORX. A study in 2-month-old ddY male mice investigated whether hesperidin intervention preserved BMD and bone structure after ORX [54]. Within the orchidectomized group, mice were fed one of four diets for a 1-month period: control diet (AIN-93G diet) or control diet containing 5 g hesperidin/kg diet, 7 g α-glucosylhesperidin/kg diet or 0.13 g simvastatin/kg diet. Simvastatin was of interest due to its potential to have favorable effects on bone metabolism (although this effect is not a consistent finding among studies) and the fact that hesperidin has been shown to inhibit HMG-CoA reductase, a rate-limiting enzyme in cholesterol synthesis. In orchidectomized ddY male mice, hesperidin and α-glucosylhesperidin protected against loss of BMD observed in orchidectomized mice not fed the interventions. Total femur BMD as well as BMD at the proximal, midsection, and distal region of the femur were significantly higher with both hesperidin and α-glucosylhesperidin after a 1-month intervention. Femur structure at the distal metaphysis was also shown

to respond favorably to both hesperidin interventions: BV/TV and Tb.N were both higher while Tb.Sp and number of osteoclasts per bone surface area was lower [54]. Simvastatin had a similar favorable effect on bone structure and decreasing the number of osteoclasts, and for total BMD as well as BMD at the distal and proximal region of the femur. Due to the fact that serum levels of C-terminal peptide of type 1 collagen—a marker of bone resorption—was lower with both hesperidin interventions suggests that bone formation was slowed. As well, the lack of change in serum osteocalcin—a marker of bone turnover—suggests that the benefits to BMD and bone structure were likely through an antiresorptive mechanism.

3.2.4 Effect of Hesperidin for Supporting Bone Health in Male Rats During Aging

While OVX and ORX are used to mimic the aging of bone, there is a paucity of research investigating the effect of hesperidin in a true aging model. Such studies are important as they more closely mimic the physiological changes of human aging. Habauzit et al. studied aging male mice and provided hesperidin or naringin alone, or in combination with 20-month-old gonad-intact male Wistar rats [55]. Rats were randomized to one of four intervention groups for 3 months: control diet or control diet containing 5 g hesperidin, 5 g naringin or 2.5 g hesperidin + 2.5 g naringin/kg diet. At 23 months of age, femur BMD and microarchitecture were analyzed. Rats fed hesperidin alone or the combination had greater BMD (total as well as regional BMD at the diaphyseal and metaphyseal site) while naringen alone (5 g/kg diet) was not consistently higher than the control group. Hesperidin alone also resulted in a higher BV/TV of the femur than the control group and the combination of hesperidin and naringen resulted in a higher Tb.Th of the femur while hesperidin alone was intermediary between control and the combination [55]. Thus when consumed alone or in combination, hesperidin has bone-promoting properties and prevents against bone loss throughout life. Further study of these combinations is warranted, and at different doses to elucidate potential combined benefits of these flavonoids.

4 SUMMARY

In summary, the bone-supporting properties of hesperidin have been demonstrated in rodent models used to mimic the human situation. OVX and ORX in rodents can assist in elucidating potential benefits of hesperidin at attenuating the loss of BMD and deterioration of bone structure during estrogen and androgen deficiency, respectively. However, studying the natural aging response in combination with dietary supplementation of hesperidin warrants further investigation. Studies to date using rodent models suggest that maternal exposure to hesperidin (and in combination with naringen) programs bone development in a sex-specific manner, but there is little evidence of sex-specific responses with respect to BMD and bone structure when hesperidin is fed from 2 months of age onwards. Moreover, future investigation in humans is needed to confirm the findings obtained from rodent models and to elucidate dose-specific and sex-specific responses of bone, particularly whether fracture risk is reduced with higher intakes of hesperidin.

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Glossary

Bone mineral density (BMD)
Amount of mineral in a defined area of tissue, unit in g/cm^2 (DXA) or g/cm^3 (μCT).

Bone volume fraction (BV/TV)
Ratio of the bone volume to the total volume of the region of interest, measuring of the amount of trabecular bone in given area, unit in %.

Connectivity density (Conn.Dn)
Degree of connectivity of trabeculae per total volume measured, unit in $1/mm^3$.

Cortical area fraction (Ct.Ar/Tt.Ar)
Ratio of the cortical bone area to the total cross-sectional area, it is a measure of the amount of cortical bone in a given area, unit in %.

Cortical thickness (Ct.Th)
Thickness of cortical bone excluding the vertical surfaces between pixels of adjacent cross-sections, unit in mm.

Endosteal perimeter (E.Pm)
Average endosteal perimeter of the region of interest, unit in mm.

Medullary area (MA)
Cross-sectional area of the medullary cavity, unit in mm^2.

Osteoblasts
Derived from mesenchymal stem cells and are responsible for bone formation and mineralization.

Osteoclasts
Derived from hematopoietic stem cells and are responsible for bone resorption.

Osteocytes
Mature osteoblasts that have become embedded in the bone. These cells are mechanosensory and assist with regulation of bone metabolism.

Orchidectomy (ORX)
Surgical procedure in which testicles are removed. Used to mimic aging in men.

Ovariectomy (OVX)
Surgical procedure in which ovaries are removed. Used to mimic deterioration of bone tissue that occurs after menopause.

Peak bone mass
Maximal amount of bone mineral attained in a lifespan.

Trabecular number (Tb.N)
Average number of trabeculae per unit length, unit in 1/mm.

Trabecular separation (Tb.Sp)
Mean distance between the trabeculae, unit in mm.

Trabecular thickness (Tb.Th)
Mean thickness of the trabeculae, unit in mm.

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Polyphenols: Mechanisms of Action in Human Health and Disease

Second Edition Volume 1

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Polyphenols are a unique group of phytochemicals that are present in all fruits, vegetables, and other plant products. They are a very diverse and multi-functional group of active plant compounds with powerful antioxidant properties. They exhibit remarkable chemical, biological and physiological properties, including antioxidant, cancer prevention, and cardio-protective activities. *Polyphenols: Mechanisms of Action in Human Health and Disease*, Second Edition, describes the mechanisms of polyphenol antioxidant activities and their use in disease prevention. Chapters highlight the anti-inflammatory activity of polyphenols on key dendritic cells, how they modulate and suppress inflammation, and how they are inactivated or activated by metabolism in the gut and circulating blood. Polyphenols have proven effective for key health benefits, including bone health, organ health, cardiac and vascular conditions, absorption and metabolism, and cancer and diseases of the immune system.

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- Expands coverage on green tea, cocoa, wine, cumin and herbs
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