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# **PHYTOCHEMICALS – BIOACTIVITIES AND IMPACT ON HEALTH**

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**Edited by Iraj Rasooli**

## **Phytochemicals – Bioactivities and Impact on Health**

Edited by Iraj Rasooli

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## Preface

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Throughout the development of civilization, plants, plant parts, and derived oils and extracts have functioned as sources of food and medicine, as symbolic articles in religious and social ceremonies, and as remedies to modify behavior. Taste and aroma not only determine what we eat but often allow us to evaluate the quality of food and, in some cases, identify unwanted contaminants. The principle of self-limitation taken together with the long history in the use of natural flavor complexes in food suggests that these substances are safe under intended conditions of use. Based on a rich history of use of selected plants and plant products that strongly impact the senses, it is not unexpected that society would bestow powers to heal, cure diseases, and spur desirable emotions, in an effort to improve the human condition. The perception is that these products are “natural” which has, in part, mitigated the public’s need to know whether these products work or are safe under conditions of intended use. Herbs and spices have been used for many centuries to improve the sensory characteristics and extend the shelf-life of foods. As a result, considerable research has been carried out on the assessment of the biological activity of many herbs, spices and their extracts. Overwhelming scientific data, from epidemiological studies, indicate that diets rich in fruit, vegetables and grains are associated with a lower risk of several degenerative diseases, such as cancers and cardiovascular diseases. Free radicals were a major interest for early physicists and radiologists and much later were found to be a product of normal metabolism. Today, we know well that radicals cause molecular transformations and gene mutations in many types of organisms. Oxidative stress is well-known to cause many diseases, and scientists in many different disciplines became more interested in natural sources which could provide active components to prevent or reduce its impacts on cells.

Currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity. Plant products are also known to possess potential for food preservation. Oxidation of lipids, which occurs during raw material storage, processing, heat treatment and further storage of final products is one of the basic processes causing rancidity of food products, leading to their deterioration. Synthetic antioxidants have been used in the food industry since the 1940s, but trends in many health-related industries tend to shift preferences to natural sources. For these reasons,

consumers tend to be suspicious of chemical additives and thus the demand for natural and socially more acceptable preservatives has been intensified.

Originally added to change or improve taste, spices and herbs can also enhance shelf-life because of their antimicrobial nature. Due to undesirable influences of oxidized lipids on the human organism, it seems to be essential to decrease contact with products of lipid oxidation in food. Plant-derived natural products are highly abundant; many exhibit numerous biological activities. Therefore, investigation of natural antioxidants has been a major research interest for the past two decades as many research groups and institutions have been screening plant materials for possible antioxidant properties. Researchers have been interested in biologically active compounds isolated from plant species for the elimination of pathogenic microorganisms because of the resistance that microorganisms have built against antibiotics. Essential oils and extracts obtained from many plants have recently gained popularity and scientific interest. Many plants have been used for different purposes, such as food, drugs and perfumery. In this book, we were faced with the daunting task of making a superior collection of experiences backed by years of combined research and observations. One of our goals for this edition was to make the book more accessible to those engaged in herbal research. To accomplish this we focused on four specific areas: Phytochemicals, anticancer properties, nutritional value and antioxidative properties.

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# **Part 1**

## **Phytochemicals**



# Naturally Occurring Organic Sulfur Compounds: An Example of a Multitasking Class of Phytochemicals in Anti-Cancer Research

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## 1. Introduction

*Allium* plants, especially garlic (*Allium sativum*), have been cultivated since thousands of years all over the world not only as spicy food but also as medicinal plant. According to Block, the garlic plant was for the first time referred to in an Egyptian medical papyrus 1550 BC. In this Codex Ebers, 22 formulas of garlic were specified for the treatment of various disorders including heart problems, headache, bites, parasites and even tumors (Block, 1985). Today, the therapeutic value of garlic and other *Allium* vegetables is confirmed by multiple epidemiological and experimental studies. Especially prevention of cardiovascular diseases has been attributed to regular garlic consumption (Galeone et al., 2009; Kris-Etherton et al., 2002; Rahman & Lowe, 2006). Moreover, cholesterol lowering, hypoglycemic, immune-stimulatory, anti-microbial and even anti-cancer properties have been reported for garlic compounds (Agarwal, 1996; Amagase et al., 2001; Balkwill & Mantovani, 2001; Borrelli et al., 2007; Goncagul & Ayaz, 2010; Kalra et al., 2006). Epidemiological studies clearly show the correlation between moderate garlic intake and a low cancer incidence (Galeone et al., 2006; Kim, J.Y. & Kwon, 2009; Salem et al., 2011). A case-control study conducted in the 1980s in Italy revealed for example that people living in high-risk areas for gastric cancer consumed less garlic compared to people in low-risk regions where stomach and colon cancers were three times less frequent (Buiatti et al., 1989). The health beneficial effects of garlic and other *Allium* species make this plant family an extremely interesting research topic.

## 2. Bioactive chemicals and formulations from *Allium* vegetables

It appears that the biological activities of *Allium* plants are primarily attributed to organo-sulfur compounds (OSCs) (Bianchini & Vainio, 2001; Herman-Antosiewicz et al., 2007a; Jacob, 2006; Kalra et al., 2006; Powolny & Singh, 2008). Garlic and other *Allium* plants contain the highest amount of sulfur compounds described for common vegetables. In 1844, Wertheim provided evidence that OSCs are also causing the characteristic pungent garlic

odor (Lanzotti, 2006; Wertheim, 1844). Other pharmacologically interesting ingredients of *Allium* vegetables, on which we will not focus here, include saponins, saponins and flavonoids, the latter being mainly present in onion (Miean & Mohamed, 2001). Allixin and organo-selenium compounds also contribute to some biological effects (Corzo-Martinez M., 2007). It has been proposed that these non-sulfur compounds act together with the OSCs in a synergistic manner (Amagase, 2006). The composition of OSCs differs depending on the *Allium* species (Nencini et al., 2007), plant cultivation or storage conditions and processing methods (Verma S.K., 2008). Some OSCs are absent in the bulbs and require mechanical exposure like cutting, crushing or chewing to be formed. According to Verma et al., whole garlic bulbs contain 16 OSCs versus 23 OSCs after crushing (Verma S.K., 2008). In other reports 33 OSCs are reported in fresh garlic (Kalra et al., 2006). The cytoplasm of intact cloves contains biologically inactive  $\gamma$ -glutamylcysteine and S-alk(en)ylcysteinesulfoxides [(S-allylcysteinesulfoxide (alliin; 85%), S-methylcysteinesulfoxides (methiin; 10%) and S-trans-1-propenylcysteinesulfoxides (isoalliin; 5%) (Verma S.K., 2008)] that serve as precursors of volatile thiosulfinate (Kamel A., 2000; Lanzotti, 2006).  $\gamma$ -glutamylcysteine is hydrolysed and oxidized to S-alk(en)ylcysteinesulfoxides, mainly alliin (Corzo-Martinez M., 2007) or transformed into S-allylcysteine (SAC) by the action of  $\gamma$ -glutamyl transpeptidase. The latter reaction occurs in particular during wintering and sprouting of the garlic plant in order to ensure the production of sufficient alliin and isoalliin (Verma S.K., 2008). SAC can be oxidized to alliin (Kamel A., 2000; Lanzotti, 2006), which is then enzymatically transformed to diallylthiosulfinate (allicin) following the slicing of garlic cloves. This reaction is catalyzed by alliinase (also alliin lyase), an enzyme normally stored inside cytoplasmic microcompartments and released only after mechanical crushing (Weiner et al., 2009). Allylsulfenic acid, which is produced as short-lived intermediate, undergoes a spontaneous condensation reaction yielding allicin together with pyruvic acid and ammonium. Alliinase transforms cysteinesulfoxides to thiosulfinate in less than 60 seconds. The conversion of alliin to allicin is particularly rapid because enzyme and substrate appear in equal high amounts within the cell (Verma S.K., 2008). Due to their high instability, the volatile thiosulfinate are degraded within 24 hours into “second generation products” like oil-soluble mono-, di- and triallylsulfides (DAS, DADS, DATS) as well as vinyldithiins, thioacroleines and ajoene (Amagase, 2006; Kamel A., 2000; Munchberg et al., 2007), which still possess considerable biological activities and thus possibly represent the actual active compounds (Freeman F., 1995; Kamel A., 2000). Figure 1 summarizes the most important garlic-derived OSCs and their synthesis.

Higher polysulfides with four (DATTS) or more sulfur atoms are formed at higher temperatures (Lanzotti, 2006). According to data of Block reviewed by Kamel and Saleh, vinyldithiins are formed by dimerization of two thioacrolein molecules that arise from  $\beta$ -elimination of allicin whereas the generation of ajoenes is based on an initial S-thioallylation reaction of allicin (Block E., 1984; Kamel A., 2000). Further transformation leads to the formation of polysulfides that are present in multiple garlic preparations in substantial amounts and are comparatively stable. The most common allylsulfides are DAS, DADS, DATS and DATTS. Higher polysulfides up to heptasulfides are found less frequently and in low concentrations. Additional sulfur species can be generated from the interaction with intracellular thiols (cysteine, glutathione (GSH) or proteins) (Kalra et al., 2006; Weisberger & Pensky, 1958). Thus, S-allylmercaptocysteine (SAMC) arises from allicin whereas a similar reaction between DAS and cysteine gives rise to allylmercaptan (AM) (Kalra et al., 2006).

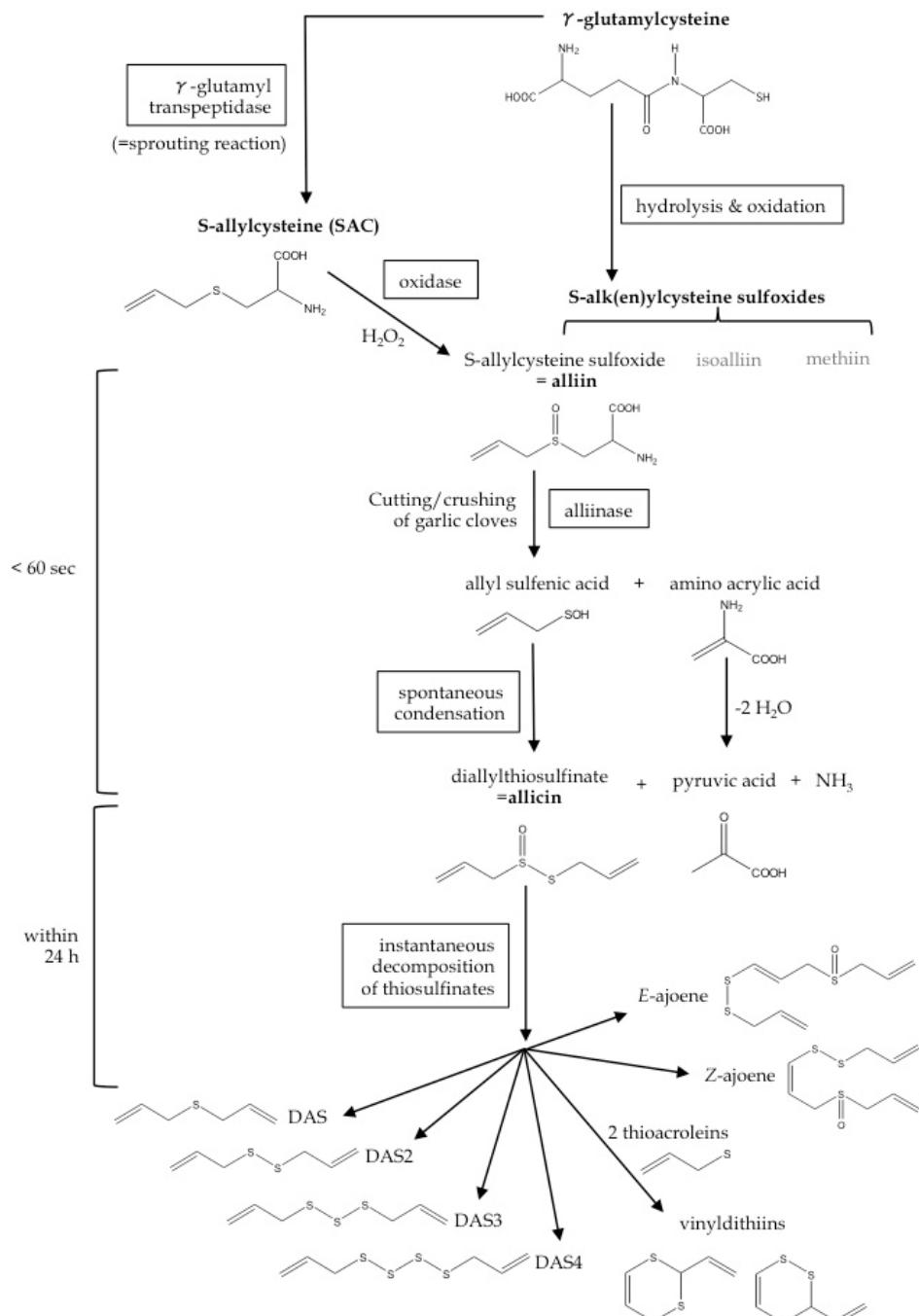


Fig. 1. Overview of the generation of different OSCs in garlic cloves

The different garlic formulations that are on the market vary significantly in their chemical composition. In view of clinical trials it is important to know about the components contained in diverse garlic preparations in order to correctly interpret the results. The most common garlic formulations include aqueous extract, garlic powder, garlic oil, oil macerate and aged garlic extract (AGE). For the aqueous extract garlic cloves are steeped in purified water. The primary compound in this extract is allicin (or alliin if alliinase is inactivated due to heating of the garlic extract). Further OSCs are allylmethylthiosulfonate, 1-propenylallylthiosulfonate and  $\gamma$ -glutamyl-S-alkylcysteine (Verma S.K., 2008). Garlic powder is produced by dehydration of crushed garlic cloves followed by pulverization. Thus, the composition of OSCs of the obtained powder is theoretically expected to correspond to the raw cloves. Nevertheless, the content of alliin (the major sulfur compound in raw and powdered garlic) and allicin varies considerably. Even if alliinase activity is comparable to fresh garlic, more than half of the alliin is lost during the dehydration process. Allicin is high in fresh garlic cloves, whereas the dehydrated powder is almost free of allicin, which might be explained by its instability (Amagase et al., 2001). Two types of garlic oil are generally synthesized: steam distilled oil or oil macerate. Garlic oil prepared by steam distillation of whole garlic cloves, ground in water, is completely free of hydrophilic OSCs and allicin (Amagase et al., 2001). According to Verma et al. it contains 57% diallylsulfides (DADS 26%, DATS 19%, DATTS 8% and lower concentrations of penta- and hexasulfides) in addition to 37% allylmethyl- and 6% dimethyl mono- to hexasulfides (Verma S.K., 2008). The available oil macerate products, consisting of mixtures of chopped garlic cloves homogenized and macerated in vegetable oil, are rich in allicin-derived OSCs including vinyldithiins, ajoenes and sulfides (Staba et al., 2001). Higher concentrations of dithiins and ajoenes are present in essential oil obtained by garlic extracted in organic solvents (Verma S.K., 2008). Another garlic preparation frequently used in *in vivo* studies or clinical trials is AGE. In contrast to aqueous extract, this extract prepared from sliced garlic cloves is aged for up to 20 months in a 15-20% ethanol solution. This aging process leads to a massive loss of allicin whereas water-soluble compounds like SAC and SAMC, which are odorless and stable, are enriched. AGE further contains some amounts of oil-soluble OSCs (Amagase et al., 2001).

### 3. Chemical properties of OSCs

#### 3.1 Relevance of sulfur atoms for the reactivity of OSCs

The complex chemistry of garlic is believed to confer the plant anti-microbial activity as self-defense mechanism (Schneider, T. et al., 2011b). Chemical properties of the various OSCs strongly rely on the presence of highly reactive sulfur atoms, which exert multiple reactions including nucleophilic substitutions or redox reactions (Jacob, 2006; Munchberg et al., 2007). In line with the importance of sulfur, recent reports provided evidence that the biological activity of polysulfides directly correlates with their number of sulfur atoms, even if this relationship is not linear (Anwar et al., 2008). Thus, we found DATTS being the most active diallylsulfide in inducing apoptosis in U937 lymphoma cells, followed by DATS, whereas DAS and DADS instead were rather inactive (Cerella et al., 2009). Similarly, DATTS showed the highest antibiotic activity against *Staphylococcus aureus* (Tsao & Yin, 2001). The general trend seems to be that DAS is hardly active, DADS shows some activity, which is strongly increased in polysulfides with three or four sulfur atoms. Instead, five or more sulfur atoms

do not improve the reactivity of polysulfides significantly and the pentasulfide had for example effects on yeast comparable to DATS (Jirousek L., 1956). Munchberg et al. explain these differences as follows. DADS is more active than DAS due to its oxidizing activity and the ability to generate thiols. The strong gain of activity of DAT(T)S might result from their reaction products, e.g. perthiols (RSSH). Even if perthiol formation is elevated for polysulfides with four to six sulfur atoms, no further improvement of activity can be observed for the penta- and hexasulfides because of their declined stability (Munchberg et al., 2007).

### 3.2 Interaction with intracellular thiols

The most remarkable property of OSCs, especially reported for allicin and to some lesser extent for allylsulfides, is their reactivity towards intracellular thiols such as cysteine, GSH or proteins (Kalra et al., 2006; Weisberger & Pensky, 1958). These thiolation reactions give not only rise to the formation of further sulfur species, as mentioned above. More crucially, they lead to the inactivation of the affected proteins/enzymes (Weisberger & Pensky, 1958) and thus importantly interfere with cellular functions. Allicin is known to interact rapidly with thiol groups leading to the formation of S-allyl derivatives. Thus, the antibiotic activity of allicin has been suggested to be a consequence of its interaction with cysteine residues of different peptides and proteins of the target cell finally inducing cell death (Munchberg et al., 2007). Rabinkov et al. studied the chemical interaction between allicin and L-cysteine (as -SH carrier) and identified SAMC as the reaction product *via* RP-HPLC and subsequent <sup>1</sup>H and <sup>13</sup>C NMR analysis (Rabinkov et al., 1998). They further investigated the interaction of allicin with thiol-containing enzymes and found a very rapid inactivation of papain and two alcohol dehydrogenases, from which NAD<sup>+</sup>-dependent alcohol dehydrogenase from horse liver was even irreversibly inactivated (Rabinkov et al., 1998).

An exchange reaction between a thiol (R'SH) and a polysulfide (RSxR) will lead to the formation of a mixed sulfide (RSxR') and a perthiol (RSSH) or other RSxH species (Munchberg et al., 2007). In the case of a tetrasulfide, two possible sites for a nucleophilic attack exist (at the central or the two terminal S-S bonds). Even if the formation of a trisulfide and perthiol seems to be the favored reaction, one cannot exclude that in addition disulfide (RSSR') and hydrogentrisulfide (RSSSH) generation takes place (Munchberg et al., 2007). For DATS, it is well documented that it targets specific cysteine residues within tubulin monomers (Hosono et al., 2008) thereby modulating tubulin conformation (Jordan et al., 1998) and consequently disturbing the microtubule (MT) network (Hosono et al., 2008). Hosono et al. suggested a mechanism by which DATS activates a thiol-disulfide exchange reaction through directly interacting with the thiol moiety of the cysteines C12 and C354 of β-tubulin. Finally, DATS binds covalently to tubulin *via* formation of SAMC-modifications (Hosono et al., 2005). A similar mode of action has also been reported for SAMC (Xiao et al., 2003). Li et al. likewise described an inhibitory effect of Z-ajoene on tubulin polymerization *in vitro* (Arora & Shukla, 2002). As it has been demonstrated that ajoene induces a rapid decrease of GSH (Scharfenberg et al., 1994), one may speculate that this OSC acts in a similar manner by directly targeting thiols. Such thiolation reactions on tubulin affect the formation of normal spindle microtubules during mitosis and thus trigger the induction of a cell cycle arrest (see Section 5.2). Accordingly, many studies showed that N-acetyl-cysteine (NAC), which is commonly used as antioxidant, counteracted the biological effects of OSCs (Wu,

X.J. et al., 2009; Xiao et al., 2005) (see Sections 5.2 and 5.5.3). The ability of NAC to increase the intracellular thiol pool has to be considered, which might quench the OSCs and thus prevent their interaction with thiol groups of potential target molecules. It is worth to mention that RSxH species arising from these thiolation reactions of polysulfides might also contribute to their biological activity as these hydrothiols are highly reactive and can for example act by reactive oxygen species (ROS) generation and as ligands for transition metal ions (Munchberg et al., 2007). Additional possible biochemical reactions related to polysulfides are reviewed by Munchberg 2007 in detail and include homolytic S-S cleavage, Sx transfer reactions and hydrophobic interactions with membranes and proteins (Munchberg et al., 2007). Antioxidant effects and metal binding ability are two further properties of OSCs that significantly contribute to their chemopreventive and chemotherapeutic activity and will be therefore discussed later on (see Section 5.3).

## 4. Chemopreventive/-therapeutic potentials of *Allium*-derived OSCs

### 4.1 Chemoprevention

#### 4.1.1 Epidemiological studies

The endemic abundance of the *Allium* species contributes to their worldwide consumption and availability. The procedures to extract active natural occurring molecules or to synthesize them *de novo* in large amounts require relative low cost. Besides, the millenary use of these plants for dietary purposes ensures that the derived active natural compounds possess low or null systemic toxicity. All these considerations make the clinical exploitation of such derivatives/extracts of these plants an attractive and favorable strategy for chemopreventive and therapeutic purposes. Under this view, many epidemiological studies have been first conducted to scientifically validate the multi-beneficial effects on health (Scherer et al., 2010). Several interesting reviews give a critical overview of epidemiological studies examining correlations between the intake of *Allium* derivatives, in form of vegetables or supplements, and the chemoprotection from cardiovascular diseases, diabetes, cholesterol level alterations, gallstone formation and specific chronic inflammatory diseases. Here, we focus our attention on the studies dealing with the potential anti-cancer properties of *Allium* derivatives, taking into account the current lack of anti-cancer chemopreventive agents, on one hand, and the continuous demand of new targeted anti-cancer therapeutics, on the other hand.

From various case-control studies, it emerges that cancers affecting the digestive tract and the prostate appear as the most impacted ones. A number of studies investigated any inverse correlations between the consumption of *Allium* species and the incidence of cancers affecting the esophageal and stomach tract. These studies have been performed on geographical areas located in different continents as in Asia (China (Takezaki et al., 1999; You et al., 1989) and Japan (Gao et al., 1999)), Europe (Italy (Pelucchi et al., 2009) and Netherlands (Dorant et al., 1996)), or America (Venezuela (Munoz et al., 2001), Uruguay (De Stefani et al., 2001) and Hawaii (Hirohata & Kono, 1997)). Overall, a protective effect was reported, despite the obvious genetic variance existing among the populations examined in the different studies. Similarly, a reduced cancer risk has been widely documented in the instance of colorectal and prostate (Galeone et al., 2006; Hsing et al., 2002) (Fleischauer et al., 2000) forms of cancer. A limited number of studies explored the impact of a regular intake of

*Allium* vegetable on the incidence of cancers affecting breast, endometrium and lungs (Challier et al., 1998; Galeone et al., 2009; Satia et al., 2009). In many instances, inverse correlations have been also reported. Remarkably, amongst the human malignancies, gastric, colorectal and prostate cancers are the best characterized and documented ones for their multiple pre-neoplastic stages. This aspect provides additional tools to evaluate the impact of natural occurring compounds specifically on tumor progression. Accordingly, a regular consumption of garlic has been associated with the reduction in the incidence of pre-neoplastic lesions occurring in the gastric mucosa of individuals infected by *Helicobacter pylori* (You et al., 1998). In parallel, studies analyzing the preventive effect of garlic extracts on colorectal cancer have evidenced their suppressive potential on the development and progression of colorectal adenomas (Tanaka et al., 2004; Tanaka et al., 2006). A population-based study analyzing the impact of a diet rich in *Allium* vegetables on the incidence of prostate cancer showed that the anti-cancer effects were more pronounced in men presenting localized rather than advanced forms (Hsing et al., 2002), thus implying major effects of *Allium* derivatives on pre-neoplastic steps.

The many published epidemiological studies seem to encourage large-scale and long-term clinical trials with *Allium* derivatives. Meta-analyses, however, have not always supported the same conclusions. A representative example is a study published in 2009 (Kim, J.Y. & Kwon, 2009) and evaluating the impact of garlic intake on the risk of different forms of cancer. The study consisted in a critical systematic review of the publications appearing in Medline and EMBASE databases in the period 1955-2007 and matching some selected keywords. The criterion adopted for the selection of the studies to be considered as relevant was the satisfaction of the US Food and Drug Administration's evidence-based review system for scientific evaluation of health claim (<http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodLabelingNutrition/ucm073332.htm>). The conclusions pointed out the lack of evidence that may support an actual chemopreventive effect of garlic. Other critical reviews of epidemiological studies remark moderate evidence (Fleischauer & Arab, 2001). Overall, these investigations highlight all the limits frequently affecting epidemiological studies. Most of all, the bias due to the interview system frequently adopted to screen the population, which does not provide rigorous information about the actual amount of product/s intaken; the deep heterogeneity in the form of administration/consumption of the vegetable of interest (as food or as supplement; if as food, cooked or raw or in form of extract). Therefore, these contradictory analyses prompt to select further diversified approaches to validate the anti-cancer effects of *Allium* species.

#### **4.1.2 Experimental studies**

In this view, an important point is the *in vivo* experimental investigation of the effects of *Allium* vegetables. Mainly based on murine models, similar studies permit to gain detailed insights. First of all, it is possible to select and test specific purified compounds deriving from the *Allium* species and by this way to identify the most active natural occurring compounds. Second, we have at our disposal several *in vivo* models to mimic tumor progression or to evaluate the therapeutic impact on different transplanted cancers. Their exploitation is a fundamental part in the assessment of the mechanisms implicated in the anti-cancer effects and in the establishment of the efficaciousness of new anti-cancer agents as well.

Many lines of evidence show an ability of garlic extracts to prevent pre-neoplastic lesions of the gastrointestinal tract. Azoxymethane is a carcinogenic agent that induces the development of precancerous lesions in the colon consisting of aberrant crypt foci (ACF). The regular administration of garlic extracts to rats exposed to this tumor promoter is able to reduce the size and the number of ACF (Sengupta et al., 2003, 2004b). The chemopreventive effects are associated with an attenuation of early pre-neoplastic events (*i.e.*, the expression of cyclooxygenase 2 (Sengupta et al., 2004a); and a reduction in lipid peroxidation (Sengupta et al., 2003). Moreover, the histological analysis suggested the triggering of apoptosis (Sengupta et al., 2004b). Similar evidence has been accumulated in experimental *in vivo* models of oropharyngeal, gastric and skin carcinogenesis (Arora & Shukla, 2002; Balasenthil et al., 2002; Bhuvaneswari et al., 2004; Kalra et al., 2006; Prasad et al., 2008; Tanaka et al., 2004; Velmurugan et al., 2005). Garlic extracts or purified OSCs generally lead to the expression of markers of apoptosis and the reduction of the pre-neoplastic lesions. A similar pattern of modulations has been frequently reconfirmed in the instance of xenograft mice models of hepatocellular carcinoma (Zhang, Z.M. et al., 2007) and refractory forms of prostate cancer (Howard et al., 2008; Singh et al., 2008).

#### 4.2 Therapeutic implications

Garlic extracts and isolated OSCs manifest a direct cytoidal activity. This point will be discussed in further detail in section 5.5. Here, we focus our attention on the chemoadjuvant properties of *Allium* derivatives. Preclinical studies have demonstrated the ability of garlic to modulate carcinogen metabolism, suggesting that its consumption could also influence drug intake. Interactions between garlic and drugs have been well described such as in the case of AIDS medication (*e.g.* interaction with saquinavir and darunavir metabolism) (Borrelli et al., 2007). Some pieces of evidence underline the ability of OSCs to chemosensitize cancer cells to chemotherapeutic treatment by modulation of cytochrome P450 isoforms. This, in turn, affects the pharmacokinetics of the corresponding drugs (see sections 6). *Allium* derived compounds have been reported to counteract the nuclear factor κB (NF-κB), which is frequently implicated in the resistance of cancer cells to chemotherapeutics. The modulation of both cellular targets may be implicated in the chemosensitization to the same treatment. Studies on docetaxel, a chemotherapeutic administrated to patients affected by hormonal or colon cancers, show that both mechanisms may be implicated in the ability of OSCs to modulate its activity (Ban et al., 2009; Cox et al., 2006; Howard et al., 2008). Cox *et al.*, however, have shown that co-administration of garlic and docetaxel did not significantly affect the drug disposition in breast cancer patients but a reduction of its clearance in specific cases cannot be excluded (Cox et al., 2006). Besides, two studies have shown that powder and AGE feeding to rats was able to prevent nephrotoxic and cardiotoxic side effects of both cisplatin and doxorubicin, two chemotherapeutic agents successfully used in cancer therapy (Alkreaty et al., 2010; Razo-Rodriguez et al., 2008). However, further investigations are needed to evaluate the actual potential interaction between garlic and chemotherapeutic agents. In addition, data suggested that immune-enhancing activity of garlic could take part in its anti-tumor effect. Indeed, garlic, and especially AGE, presented similar effectiveness to immunotherapy with bacillus Calmette-Guérin in transplanted bladder tumor mice. Garlic is able to promote the Th1 immune response by stimulating proliferation and tumor site

infiltration of macrophages and lymphocytes, increasing natural killers activity and enhancing the release of cytokines (e.g., IL-2, TNF- $\alpha$ , INF- $\gamma$ ) (Lamm & Riggs, 2001).

## 5. Mechanisms of chemopreventive/-therapeutic activities of *Allium*-derived OSCs

### 5.1 Modulation of detoxification of xenobiotics

#### 5.1.1 Modulation of the metabolism of carcinogens

The effectiveness of a chemopreventive compound can be evaluated by its ability to interfere with different stages of carcinogenesis: initiation, promotion and progression (Surh, 2003). It was found that garlic and some of its constituents prevent tumor initiation by inhibiting the activation of pro-carcinogens and by stimulating their elimination (for recent reviews, see (Herman-Antosiewicz et al., 2007a; Iciek et al., 2009; Melino et al., 2011)). Indeed, the metabolizing-process of carcinogens comprises two phases: bioactivation and detoxification. The pro-carcinogens are activated by phase I enzymes through different types of reactions such as oxidation, hydroxylation, hydrolysis, cyclization. These reactions are generally catalyzed by the superfamily of cytochrome P450-dependent monooxygenases (CYP450). In the second step, the reactive carcinogens obtained are inactivated by phase II enzymes (e.g., glutathione S-transferases, glutathione peroxidases, UDP-glucuronyl transferases, quinone reductases). These detoxication reactions require the conjugation with endogenous substrates (e.g. glucuronic acid, glutathione, sulfate) to enable the excretion of inactive products (Sheweita & Tilmisany, 2003).

Several studies have contributed to elucidate the mechanisms by which garlic prevents chemical-induced cancer in animal models. Opposite effects have been reported on the modulation of CYP450 family members, both up- and downregulation depending on the isoenzymes. CYP450 2E1, which is responsible for the activation of small polar molecules such as acetaminophens, benzene and nitrosamines, is the isoenzyme most frequently reported to be induced by garlic. For example, dietary intake of garlic oil or powder leads to the inhibition of the hepatic microsomal CYP450 2E1 activity in mouse and rat, respectively (Park et al., 2002; Zeng et al., 2009). Moreover, garlic constituents, especially DAS and allylmethylsulfide (AMS), are effective in reducing the hepatic CYP450 2E1 protein level and activity (Davenport & Wargovich, 2005; Wargovich, 2006). Brady et al. have investigated the mechanisms of action of DAS and showed that this compound could directly act as a competitive inhibitor of CYP450 2E1 enzyme (Brady et al., 1988). Moreover, DAS could act indirectly through the formation of the oxidized products, diallylsulfoxide (DASO) and subsequently diallylsulfone (DASO<sub>2</sub>), by CYP450 2E1 itself leading to the autocatalytic destruction of the enzyme (Brady et al., 1991; Jin & Baillie, 1997). Other CYP450 isoenzymes were modulated after garlic treatment. As example, DADS and DATS increased the transcript and the protein levels of CYP450 1A1/2, 2B1 and 2E1 in rats (Wu, C.C. et al., 2002). Other CYP450 isoenzymes seem to be implicated such as CYP450 2B1/2, 2B6, 2B10 and 3A11 (Davenport & Wargovich, 2005). Indeed, DAS is able to activate the constitutive androstane receptor (CAR), which is known to regulate the expression of those ones (Fisher et al., 2007; Sueyoshi et al., 2011). A consisting body of evidence was obtained through animal survey model but recently Ho et al. have confirmed the inhibitory effect of garlic on CYP450-metabolism in Fa2N-4 human hepatocytes. Exposure to garlic extract reduced the expression and the activity of CYP450 2C9 but exerted no effect on CYP450 3A4 (Ho et al., 2010).

The induction of phase II enzymes of the carcinogen metabolism by garlic was clearly described. Especially, many studies have reported that garlic treatment enhanced glutathione S-transferase (GSTs) activities. GSTs are important detoxifying enzymes, which stimulate the clearance of reactive compounds by conjugation with GSH. The group of Singh has shown that the chemopreventive effect of DADS, against benzo[a]pyrene-induced forestomach cancer in mice, was mainly mediated through the induction of the pi class mGSTM1-1 in liver and forestomach (Bose et al., 2002; Hu et al., 1996). However, different isoenzyme profiles could be obtained according to the target tissue considered. Thus, DADS administration can also upregulate the alpha and mu (mGSTM1, mGSTM4) classes in stomach and small intestine of mice fed with this compound whereas liver and colon GSTs were modulated to a lesser extent (Andorfer et al., 2004). More recently, Tsai and colleagues have shown that the induction of GSTPs at mRNA and protein levels in Clone 9 cells treated by DADS or DATS was dependent on JNK-AP-1 and ERK-AP-1 signaling pathways (Tsai et al., 2007; Tsai et al., 2005). In addition to GSTs upregulation, a study performed by Fukao et al. has shown that intraperitoneal administration of DADS and DATS in rats led to an increase of hepatic quinone reductase activity (Fukao et al., 2004). In 2004, Chen and coworkers have suggested that the activation of the antioxidant response element (ARE) and the increased level of the transcription nuclear E2-related factor 2 (Nrf2) were correlated with the induction of the detoxifying enzymes NAD(P)H :quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO1) in human hepatoma HepG2 cells treated with DAS, DADS or DATS (Chen, C. et al., 2004). The implication of Nrf2 in garlic chemopreventive effects has recently been confirmed (Fisher et al., 2007).

Finally, data concerning the modulation of phase II enzymes such as GSH peroxidase (GPX), superoxide dismutase (SOD), catalase, N-acetyltransferase are more disputed. For example, Singh et al. have reported that DATS induces GPX activity in the lung of A/J mice (Singh et al., 1997). Conversely, Chen et al. failed to show that DADS treatment modulates GPX or SOD activities (Chen, L. et al., 1999).

### 5.1.2 Modulation of the efflux of carcinogens

In addition to the modulation of carcinogen metabolism, OSCs can also influence the activity of transporters such as P-glycoprotein (P-gp), which allow the efflux of xenobiotics from the cells. This activity is particularly interesting to improve the cancer response to chemotherapies in the case of multidrug resistance phenotypes. Indeed, the treatment of leukemia K562 cells resistant to vinblastine (K562R) with a non-cytotoxic dose of DAS enhanced the cytotoxic activity of vinblastine as well as other *Vinca* alkaloids. The authors showed that DAS reduced the protein level of P-gp in K562R cells at a level comparable to non-resistant K562 (Arora et al., 2004). Such beneficial effect was also described for ajoene which improves the chemotherapy-induced apoptosis of cytarabine and fludarabine in human acute myeloid leukemia cells (Hassan, 2004).

### 5.2 Cell cycle arrest

One fundamental feature of carcinogenesis is the uncontrolled proliferation of tumor cells. Under physiological conditions, cell cycle regulation involves sophisticated control systems to ensure the precise sequence of the different phases and to obtain two identical daughter cells (Vermeulen et al., 2003). Cell cycle progression occurs mainly by the sequential action of

cyclin-dependent kinases (Cdks). Cdks are positively regulated by interaction with their regulatory subunits cyclins. On the contrary, association with specific inhibitors (e.g., p21, p27, p57) negatively regulates their activities. Moreover, phosphorylation-dephosphorylation events are implicated. Thus, cell division cycle 25 (CDC25) phosphatases act as negative regulators by phosphorylating specific tyrosine and threonine residues on Cdks.

Cell cycle arrest is triggered in response to cellular stress such as DNA damage or MT network alterations through the activation of the cell cycle checkpoints (Sancar et al., 2004). The G1/S checkpoint prevents the replication of damaged DNA whereas in G2/M it avoids the cell to trigger mitosis until the replication is correctly achieved. The activation of these checkpoints involves several signaling pathways such as p53, p38 MAPK, ataxia telangiectasia mutated (ATM)/ATM and Rad3-related (ATR) and checkpoint kinase (Chk1/2).

Many studies have reported antiproliferative effects of garlic and OSCs in various cancer cell models, generally through the induction of cell cycle arrest in G2/M phase. In particular, detailed studies have been done on neuroblastoma (SH-SY5Y), prostate (PC-3, DU145) and colon (SW480) cancer cells (for a recent review see (Scherer et al., 2009)). The group of Milner was one of the first to highlight the importance of the modulation of Cdk1 activity in OSCs-induced cell cycle arrest. They have shown that DADS treatment of human colon HCT-115 cells led to G2/M phase arrest by suppressing Cdk1 activity (Knowles & Milner, 1998). DADS increased the expression of cyclin B1 but reduced the formation of the active cyclin B1/Cdk1 complex. The authors also reported the presence of inactive hyperphosphorylation of Cdk1, which seems to be related to the downregulation of CDC25C phosphatase (Knowles & Milner, 2000). Singh's team has extensively studied the molecular mechanisms involved the inhibition of CDC25C activity. On one hand, they have demonstrated that this inactivation was dependent on the phosphorylation of the serine residue S216 CDC25C, which is recognized as a binding site for the cytoplasmic protein 14-3-3 (Xiao et al., 2005). The activation of ATM/ATR and p38 MAPK signaling pathways, leading to Chk1 activation, were clearly involved in this process (Herman-Antosiewicz & Singh, 2005; Xiao et al., 2009b; Yuan et al., 2004). Indeed, Chk1 or ATR protein knockdown markedly attenuated the DATS-induced cell cycle arrest features in PC3 cells (Herman-Antosiewicz et al., 2007b). However, no clear evidence was provided about the importance of p53 status in ATM/ATR and Chk1/2 signaling pathway activation (Jo et al., 2008; Wang, H.C. et al., 2010a; Xiao et al., 2009b). On the other hand, Xiao and colleagues have reported a ROS-dependent destruction of CDC25C, which occurred independently of its phosphorylation. Implication of ROS generation, such as superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ), was confirmed by pretreatment in the presence of the antioxidant NAC, which significantly reduced the oxidation and the degradation of CDC25C in DU145 cells treated with DATS (Xiao et al., 2005). However, a recent study of the same group challenged the role played by CDC25C in the antiproliferative effect of DATS. Indeed, ectopic expression of CDC25C or the presence of its redox-insensitive mutant in DU145 cells failed to confer protection against DATS-induced G2/M phase arrest. This effect seems to be mainly related to differential kinetics of nuclear translocation between Cdk1 and cyclin B1 (Herman-Antosiewicz et al., 2010). Finally, a recent report suggested that both diallyl- and dipropyltetrasulfides (DPTTS) could act as irreversible inhibitors of CDC25C ( $IC_{50}$  about 1 $\mu$ M) (Viry et al., 2011).

Other mechanisms of action involved in OSCs-mediated cell cycle arrest have also been identified. In particular, Hosono et al. have demonstrated, through the use of an *in vitro* cell-free model, that DATS inhibited tubulin polymerization. Mass spectrometry analysis has demonstrated that DATS induced the oxidation of two cysteine residues in the  $\beta$ -tubulin (C12 and C354) (Hosono et al., 2005). These results were confirmed in another study by the use of reducing agent (2-mercaptoethanol and dithiothreitol), which abolished the MT-disrupting activity of allicin (Prager-Khoutorsky et al., 2007). In addition, it was also reported that OSCs could modulate the transcription of important regulators of the cell cycle. As example, the DADS-induced G2/M phase arrest of colon cancer Caco-2 and HT-29 cells was associated with the upregulation of p21, which appeared to be closely dependent on the acetylation status of its promoter (see section 5.4) (Druesne et al., 2004). Figure 2 summarizes the modulatory effects of OSCs on cell cycle.

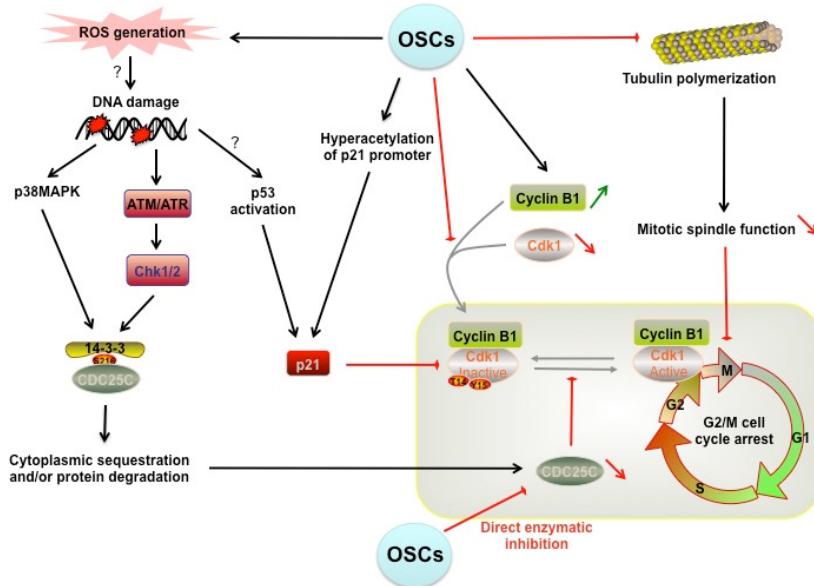


Fig. 2. Schematic overview of cellular targets of OSCs implicated in cell cycle arrest.

### 5.3 Redox modulation

#### 5.3.1 Antioxidant activity

Cellular damage by oxidation of macromolecules such as DNA, proteins or membrane lipids is an important process during early carcinogenesis (Borrelli et al., 2007; Valko et al., 2006). Antioxidant activities of phytochemicals that counteract ROS or free radicals are therefore highly desirable for potential anti-cancer compounds. Some OSCs derived from *Allium* have been reported to possess an antioxidant activity (Borek, 2001). Due to their sulfur atoms, which can appear in up to ten different oxidation states (-2 to +6), these compounds can undergo redox-reactions (Cerella et al., 2009). Nishimura et al. reported that *Allium*-derived organic sulfides and sulfoxides form *in vivo* sulfoxides and sulfones, respectively, by

scavenging free hydroxyl ( $\bullet\text{OH}$ ) or  $\bullet\text{OOH}$  radicals (Nishimura et al., 2000). Rabinkov and colleagues clearly demonstrated the radical scavenging ability of alliin and allicin by using the *in vitro* Fenton oxygen-radical generating system, in which the amount of  $\bullet\text{OH}$  radicals, measured with the spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO), was significantly reduced in response to both OSCs (Rabinkov et al., 1998). Alliin was described as stronger superoxide scavenger than allylcysteine or allyl disulfide (Okada et al., 2005). By direct ESR detection Okada et al. provided evidence that allicin is a potent scavenger of peroxy radicals (Okada et al., 2005). Structure-activity studies of the same authors revealed that the  $-\text{S}(\text{O})\text{S}-$  group in combination with an allyl group is particularly important for the antioxidant activity of thiosulfinate (Okada et al., 2005). Several studies performed on bovine pulmonary artery endothelial cells revealed that AGE and SAC inhibited  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$  formation as well as  $\text{H}_2\text{O}_2$ -induced lipid peroxidation (Yamasaki & Lau, 1997) (Wei Z., 1998). Particularly for the stable water-soluble sulfur species SAC and SAMC remarkable antioxidant properties are well documented. Thus, Imai et al. who investigated the antioxidant effects of AGE, a formulation especially enriched in both SAC and SAMC, provided evidence that these OSCs possess extraordinary radical scavenging activity (Imai et al., 1994). Counteracting ROS accumulation could also explain the beneficial effects of garlic in diseases different from cancer, in which ROS play a major role, for example cardiovascular and inflammatory diseases. In this respect, aqueous garlic extract, AGE and SAC are effective in the prevention of copper-induced LDL oxidation, a process implicated in the pathogenesis of atherosclerosis (Amagase et al., 2001; Galeone et al., 2006). AGE also protects rat liver microsomes from thiobarbituric acid-reactive substances (TBARS) formation (Horie et al., 1989). Other garlic-derived compounds, including lipophilic polysulfides and dithiins have also shown to act as antioxidants (Amagase et al., 2001), e.g. by preventing LOOH formation on human LDL (Nishimura et al., 2004). Nishimura et al. identified 3,4-dihydro-3-vinyl-1,2-dithiin as the compound with the most efficient antioxidant activity on human LDL at physiological concentration (Nishimura et al., 2004). Another strategy used by OSCs is the increase in the cellular antioxidant defense by upregulating/activating phase II detoxification enzymes (see Section 5.1.1) as well as alteration of the GSH redox cycle have been demonstrated in response to AGE and SAC (Geng, 1997). Oxidative stress is also linked to inflammation, while the latter is associated with carcinogenesis (Bartsch & Nair, 2006; Borrelli et al., 2007; Kundu & Surh, 2008). ROS are known to activate the NF- $\kappa\text{B}$  signalling pathway thereby causing aberrant expression of target genes, including pro-inflammatory cytokines and chemokines that are involved in tumorigenesis (Balkwill & Mantovani, 2001; Karin & Greten, 2005). Blocking of this pathway therefore plays a role in the antioxidant defense. Indeed, SAC was efficient in preventing NF- $\kappa\text{B}$  activation induced by both, tumor necrosis factor alpha and  $\text{H}_2\text{O}_2$  in human T lymphocytes (Geng, 1997). DADS acts on the NF- $\kappa\text{B}$  pathway by blocking activation and nuclear translocation of p65 and p50 (Pratheeshkumar et al., 2010).

On the other hand, there are some publications reporting a pro-oxidant activity of diallylsulfides and ajoene. These studies suggested that polysulfide/ajoene-induced apoptotic cell death is caused by ROS accumulation and peroxide production (Aquilano et al., 2010; Charlier et al., 2010; Dirsch et al., 1998; Wang, H.C. et al., 2010a). DADS, DATS and DATTS and to a lesser extent the corresponding propylsulfides have been identified as hemolytic agents, as they induce oxidative damage in erythrocytes by the formation of  $\text{H}_2\text{O}_2$  in the presence of GSH and haemoglobin (Munday et al., 2003).

### 5.3.2 Interaction with metalloproteins

Metalloproteins are indispensable for cells to achieve cancer prevention. Matrix metalloproteinases for example counteract carcinogenic processes like metastasis or angiogenesis by ensuring the consistency of the extracellular matrix, but may facilitate tumor invasion if overexpressed (Brew et al., 2000; Gomez et al., 1997; Luttun et al., 2000). Members of other metalloprotein classes including the cytochrome P450 family or proteins are involved in iron or drug/xenobiotic metabolism and activation of carcinogens (Sheweita, 2000; Shimada, 2006). The ability of garlic-derived OSCs to form complexes with metal ions might result in the inactivation of metallo-enzymes and therefore represents an important anti-cancer strategy of OSCs. Thiolates for example are exceptional ligands for various metal ions. Even if an experimental proof for direct binding of OSCs to metal ions is still missing, polysulfides are able to coordinate with several sulfur atoms at once and thus are likely to form metal complexes (Munchberg et al., 2007). In this respect, different OSCs are known to alter the activity of members of the cytochrome P450 family (see Section 5.1.1).

A family of zinc-dependent endopeptidases, named matrix metalloproteinases (MMPs) according to their primary function (Kessenbrock et al., 2010; Nagase & Woessner, 1999), is also affected by some garlic-derived OSCs (Ho et al., 2010; Meyer et al., 2004; Peng et al., 2010; Polette & Birembaut, 1998). Often, high expression levels of MMPs are detected in cancer cells, which further increase during tumor progression (Chambers & Matrisian, 1997). Such an imbalance, particularly of MMP9 (Egeblad & Werb, 2002) and MMP2 activity, is associated with cancer cell invasion and metastasis (Ahmed & Mohammed, 2011; Ellerbroek & Stack, 1999; Peng et al., 2010; Polette & Birembaut, 1998). MMPs therefore represent a crucial target in anti-cancer treatment. Meyer et al. found that DADS decreased MMP2 activity in a dose-dependent manner in HUVEC cells (Meyer et al., 2004). AM and SAC did not show any effect. DADS affected in addition MMP9 activity, whereas MMP1 and MMP3 activity/secretion were not altered by any of the OSCs. These results are in line with a study of Shin et al. who found DADS suppressing MMP2 and MMP9 in a time-dependent manner in prostate LNCaP cancer cells (Ho et al., 2010). As already mentioned above it has been speculated that perthiols arising from polysulfides might contribute to the biological activity of the latter. Similar to thiols, RSxH species serve as good ligands for zinc, copper, iron or other metal ions (Munchberg et al., 2007). Based on their low  $pK_a$  values, RSxH species are expected to strongly bind free or protein-bound metal ions (Munchberg et al., 2007). Instead of binding directly to the active site metal ion (Schneider, T. et al., 2011a), they might also form low molecular weight complexes in the cytosol. Due to these disturbances of metal homeostasis the pool of free (i.e. unbound) metal ions is diminished and impairs *de novo* synthesis of functioning metalloproteins (Munchberg et al., 2007). As free adventitious transition metal ions are also involved in oxidative stress (Bush, 2000), the OSC-induced reduction of the cytosolic metal ion pool contributes to the antioxidant effect of these sulfur compounds.

### 5.4 Modulation of histone acetylation

Recent studies have suggested the modulation of histone acetylation as one of the mechanisms involved in the anti-cancer activity of garlic (Druesne-Pecollo & Latino-Martel, 2011). In eukaryotic cells, histones are proteins responsible for the DNA condensation and thus play an important role in the regulation of gene expression. Histones are subjected to

post-translational modifications, including acetylation, which is a reversible process occurring on lysine residues in the N-terminal domain of the protein. In cancer cells, various genes are abnormally expressed due to epigenetic modifications of the chromatin, leading to the tumor phenotype. Controlling chromatin remodeling and gene expression is a relevant strategy for anti-cancer drug development (Espino et al., 2005). Few studies have described the ability of OSCs to increase histone acetylation. Lea et al. reported for the first time the increased acetylation of histones H3 and H4 in mouse erythroleukemia DS19 and human leukemia K562 after treatment with DADS and AM (Lea et al., 1999). Then, the same group has confirmed those effects *in vivo* as histone acetylation was induced in liver and Morris hepatoma 7777 in rats treated with DADS and AM (Lea & Randolph, 2001). Data suggested a direct link between histone hyperacetylation and the antiproliferative activity of some garlic components. First, increased histone acetylation was obtained for concentrations similar to those used to inhibit cell proliferation of DS19, human colon cancer Caco-2 cells and human breast cancer T47D cells ( $\mu\text{M}$  range for allicin and SAMC, mM range for allylphenylsulfone and SAC) (Lea et al., 2002). In this study, the authors failed to characterize the mechanism involved as no inhibitory effect was observed on histone deacetylase (HDAC) activity, enzymes which repress the histone acetylation. Conversely, Druesne et al. confirmed the ability of DADS and AM to inhibit HDAC activity in a cell-free assay (29 % and 92 % of HDAC inhibition in presence of 200 mM of each compound, respectively) (Druesne et al., 2004). Recently, *in vitro* assay and molecular modeling have led to the identification of AM as the most potent competitive inhibitor of HDAC ( $K_i=24 \text{ }\mu\text{M}$  for HDAC8). HDAC inhibition appeared to be responsible for the hyperacetylation of the p21 gene promoter (Nian et al., 2009; Nian et al., 2008).

Another important consideration is the chemopreventive activity of chromatin remodeling molecules. Indeed, it has been shown *in vivo* that DADS administration to rat effectively induced acetylation of histones H3 and H4 in non-tumor colonocytes. Results from microarray analysis indicated that DADS was able to modulate the expression of 49 genes (24 h after the end of DADS perfusion), mostly implicated in cell cycle regulation, DNA repair, cellular adhesion (Druesne-Pecollo et al., 2007). These results suggest a potential link between the modulation of gene expression, through histone hyperacetylation, and the chemopreventive activity of OSCs. Further investigations are needed to determine whether garlic components could be effective in histone acetylation, and thus in cancer prevention, at doses achievable in human diet.

## 5.5 Induction of apoptosis

### 5.5.1 Activation of the mitochondrial apoptotic pathway

A number of studies document the ability of OSCs to induce apoptosis. Apoptosis is an active form of cell death by which cells organize their self-elimination. It may be part of a programmed physiological event, including tissue remodeling and turn over; besides, irreversibly damaged cells following stress activate this mechanism. The apoptotic program is accomplished by the triggering different cascades of biochemical events. Two main intracellular routes take place (Coppola & Ghibelli, 2000). The intrinsic or mitochondrial pathway typically starts as a consequence of cellular damage. It requires the crucial involvement of pro- and anti-apoptotic members of the B-cell lymphoma (Bcl-2) family, which control the permeabilization of the outer mitochondrial membrane. The resulting

release of cytochrome c (and other additional factors) into the cytoplasm promotes caspase cleavage/activation, whose most upstream implicated member is caspase-9. The extrinsic or physiological pathway is mediated by the stimulation of specific death receptors on the plasma membrane. Once stimulated by their ligands, they unleash an ordered intracellular multi-step signaling, which includes the formation of the cell death domain and the activation/cleavage of caspase-8. The two pathways are interconnected.

The activation of the extrinsic or the intrinsic apoptotic pathways is the target of most chemotherapeutic agents. OSCs derived from *Allium* species induce apoptosis by activating the intrinsic apoptotic pathway. The release of cytochrome c from mitochondria, the decreased level of the anti-apoptotic protein Bcl-2 and the activation of caspase 9/3 are common hallmarks (Cerella et al., 2011; Scherer et al., 2009). The findings concern very heterogeneous cancer cell models, ranging from adherent (e.g., prostate, lung, colon, breast, cervical and thyroid) to non-adherent (e.g., various leukemia and lymphomas) cancer cell lines.

A large body of evidence shows a strong correlation between the number of sulfur atoms and the apoptogenic potential manifested by OSCs (see Section 3.1). Less defined, instead, is the contribution of the length of the sulfur chain in the anti-cancer role played by OCSs. Studies comparing the effects of diallylpolysulfides deriving from garlic, which bring an unsaturated (alkenyl) chain, with propylpolysulfides extracted from onion, which contain a saturated alkyl group, show marginal differences in their apoptogenic potential: generally, molecules containing the same number of sulfur atoms, show also a similar impact on different cancer cell models (Cerella et al., 2011). In contrast, Hosono and colleagues have provided evidence by using a colon cancer cellular model HT29 that the alkenyl group impacts intracellular targets (see also section below) much stronger than the corresponding compound with an alkyl group (Hosono et al., 2008). This result may lead in this cell system to a potential differential role also on apoptosis, which, however, was not yet investigated. Further studies are required to explain cell-type specific responses to OSC.

### 5.5.2 Mechanisms implicated in G2/M arrest

In all instances, apoptosis is preceded by alterations in the progression of the cell cycle, consisting in a G2/M arrest (section 5.2). The activation/up-regulation of the tumor suppressor p53 has been mentioned since very early studies on apoptosis as a crucial and very upstream event (Shen & White, 2001). The arrest in G2/M induced by OSCs may be compatible with an involvement of p53 to activate apoptosis. p53 expression has been effectively found upregulated in studies performed with garlic extracts (Hong et al., 2000) (De Martino et al., 2006), as well as in a number of investigations based on the use of purified sulfur compounds: DAS (Hong et al., 2000), DADS (Bottone et al., 2002; Hong et al., 2000; Pratheeshkumar et al., 2010; Song et al., 2009), DATS (Malki et al., 2009) and SAMC (Lee, 2008). p53 upregulation, however, seems to be a dispensable event in the apoptogenic activity of OSCs, since the use of p53-mutated or deleted cancer cell models has clearly demonstrated that OSC-induced apoptosis similarly takes independently of wild-type p53 (Busch et al., 2010; Lee, 2008; Xiao et al., 2009b).

A more careful observation of the cellular morphology after OSCs treatment suggests an arrest of the cell cycle at early steps of mitosis and more precisely during the pro-metaphase.

Accordingly, the nuclear chromatin partially condenses and acquires a dotted pattern. The pattern of modulations that OSCs produce on cell cycle-related proteins confirms these conclusions. The accumulation of cyclin B1 correlates with the down-regulation of CDC25B and 25C, which control the phosphorylation status and the activation of cyclin-dependent kinase (Cdk1) (see section 5.2). Besides, treated cells present a high positivity to the phosphorylated form of histone H3, at the level of ser 10 (H3P) (Cerella et al., 2009; Herman-Antosiewicz & Singh, 2005). The degradation of cyclin B1 and H3P is an indispensable event to progress during mitosis into the anaphase. Therefore, their accumulation indicates that OSCs prevent the transition to this step and, by this way, may trigger the mitochondrial apoptotic pathway.

### **5.5.3 Alterations induced by OSCs on the MT network as a causative event of apoptosis**

Arrest at early steps of mitosis may be the consequence of aberrations occurring during the DNA synthesis; alternatively, it occurs after alteration of the MT network. A few reports analyzed the potential of OSCs to cause DNA damage (Wang, H.C. et al., 2010a). In most instances, this is not an early event, but rather appearing tightly linked to the onset of apoptosis. Consequently, DNA damage is not generally considered as the causative event determining the OSC-induced apoptosis. A large body of evidence, conversely, indicates that OSCs induce strong alterations of MT re-arrangement (see figure 3 and section 3.2). This property is common to different natural occurring OSCs manifesting apoptogenic properties on the different cancer cell models (Aquilano et al., 2010; Hosono et al., 2005; Li et al., 2002a; Prager-Khoutorsky et al., 2007; Xiao et al., 2005; Xiao et al., 2003).

Despite the common observation that the MT network is affected by OSCs, there is no general consensus about the molecular events leading to these alterations. Canonically, stress-induced apoptosis has been always associated with early alterations in the cellular redox state (Coppola & Ghibelli, 2000). Changes in the intracellular GSH content and the generation of ROS are commonly assumed to act as crucial events in determining the commitment to intrinsic apoptosis. Previously published data already showed that a pretreatment with antioxidants, mainly with NAC, counteracts OSCs-induced apoptosis (Dirsch et al., 1998). Moreover, NAC prevents the G2/M arrest (Wu, X.J. et al., 2005). Similar results have provided the strongest evidence that the induction of apoptosis by OSCs effectively depends on the alterations of the cell cycle. Besides, the generation of ROS has been documented in some studies by the use of the fluorescent tool 2,7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) upon incubation of the cells with DADS and DATS (Filomeni et al., 2003; Kim, Y.A. et al., 2007; Sriram et al., 2008). Consequently, ROS have been considered by a number of studies as the determining event for cell cycle arrest and apoptosis induced by OSCs. As previously discussed, agents such as NAC may counteract OSCs-dependent cell cycle alterations and, therefore apoptosis, simply by acting as donors of thiols and thus competing with the intracellular thiols for the interaction with OSCs. Several pieces of evidence support this model. First, the direct ability of NAC to prevent the OSCs-induced tubulin depolymerization (see section 3.2). Second, ROS production has not always been detected, even if apoptosis was induced (De Martino et al., 2006). The debate, however, remains open for further discussion. The over-expression of the

enzyme SOD has been shown in the neuroblastoma SH-SY5Y cells to counteract apoptosis (Filomeni et al., 2003). Besides, pre-incubation of cells with catalase, which buffers H<sub>2</sub>O<sub>2</sub> production, counteracted apoptosis induced by DADS in the HL-60 leukemia cell line (Kwon et al., 2002) and in T24 human bladder cancer cells (Lu et al., 2004). Similarly, over-expression of catalase in the prostate cancer cell line DU145 protects against apoptosis induced by DATS (Xiao et al., 2004). A conceivable interpretation is that ROS species may be generated downstream of the formation of intracellular thiols after OSC treatment and play a determinant role depending on the cell-specific antioxidant reservoir.

#### 5.5.4 Modulation of Bcl-2 family members by OSCs

OSCs modulate several proteins belonging to the Bcl-2 family. The anti-apoptotic Bcl-2 protein has been described to be downregulated. This, in turn, increases Bcl-2-associated X protein (Bax)/Bcl-2 ratio, thus promoting apoptosis (Karmakar et al., 2007). The downregulation of Bcl-2 is more precisely the consequence of a protein cleavage. In our laboratory, we have shown that DATTs promotes Bcl-2 proteolysis (Cerella et al., 2009). This finding is in line with previous results collected with Z-ajoene (Li et al., 2002b) and DATS (Xiao et al., 2004). Bcl-2 cleavage is preceded by phosphorylation. Xiao and colleagues published that Bcl-2 phosphorylation occurs after treatment with DATS in human prostate cancer cells PC-3 and DU145 (Xiao et al., 2004). Similarly, the expression of the anti-apoptotic Bcl-xL protein is modulated by DADS in breast cancer cells (Nakagawa et al., 2001); in line with these results, we have described that the downregulation of B-cell lymphoma-extra large (Bcl-xL) protein upon treatment with DATTs in human leukemia cells (Cerella et al., 2009). Specific Bcl-2 homology domain 3 (BH3)-only proteins seem to be modulated by OSCs by post-translational modification or by intracellular redistribution. Unphosphorylated Bcl-2-associated death promoter (Bad) sequesters Bcl-xL and Bcl-2 thus favoring Bax and Bcl-2 homologous antagonist killer (Bak) activation (Yang et al., 1995). Dephosphorylation of Bad has been documented after DATTs treatment (Cerella et al., 2009). Besides, the translocation of Bad to the mitochondria occurs in prostate cancer cells upon treatment with DATS (Xiao & Singh, 2006). When breast cancer MDA-MB-435 cells were exposed to water-soluble extracts (Lund et al., 2005), expression level, phosphorylation and mitochondrial localization of different isoforms of the BH3-only protein BimEL were altered. Interestingly, BimEL in normal physiological conditions is anchored to (and sequestered by) the MT complex (Chen, D. & Zhou, 2004; Lund et al., 2005). This may provide interesting insights about how the MT network is disrupted and the subsequent activation of the mitochondrial pathway may be interconnected.

The permeabilization of the outer mitochondrial membrane is an event controlled by the two pro-apoptotic family members, Bax and Bak, which orchestrate the formation of specific channels (Antignani & Youle, 2006). OSCs activate Bax and Bak (Cerella et al., 2009; Kim, Y.A. et al., 2007; Xiao et al., 2006a; Xiao et al., 2009a). Interestingly, time-course analyses have evidenced that Bak might be activated before Bax (Cerella et al., 2009). The activation of Bax fits the activation of caspases and the appearance of massive apoptosis (Cerella et al., 2009), thus suggesting that its activation is crucial for the completion of the apoptotic program. In other instances, modifications in the levels of Bak and Bax have been detected, with Bak being affected much earlier than Bax (Kim, Y.A. et al., 2007). This evidence is in favor of non-redundant roles of Bax and Bak during OSCs-induced apoptosis. How Bax and

Bak may promote the formation of channels and the mitochondrial outer membrane permeabilization is still unknown. Similarly, differential roles played by Bax and Bak during apoptosis remain to be determined. Intriguingly, the chemotherapeutic agent vinblastine, which affects the MT network and induces mitotic arrest, also modulates Bak at early timepoints (Upreti et al., 2008). These results may suggest a specific interplay between Bak and Bax typically activated by tubulin-affecting agents. OSCs might conceivably represent an interesting tool to investigate and to clarify some aspects that belong to essential issues of apoptosis research.

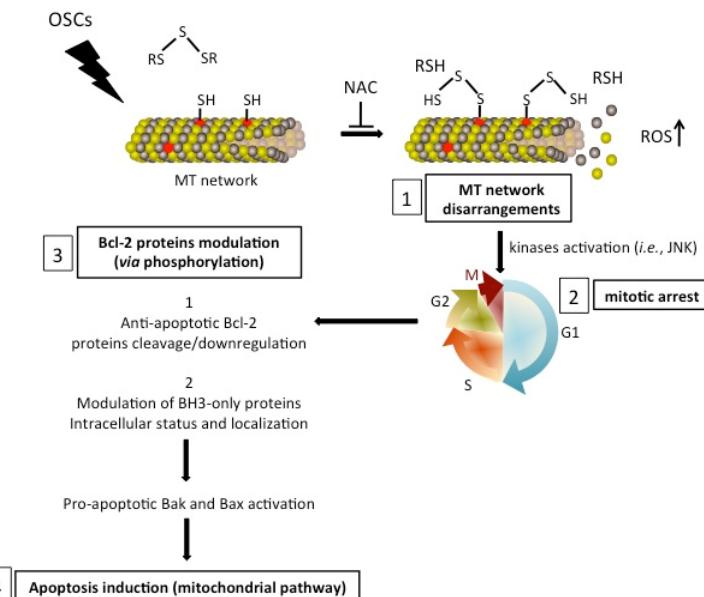


Fig. 3. Mechanisms involved in the apoptogenic properties of OSCs

The induction of apoptosis by OSCs occurs *via* a well-ordered set of events. The first target is the MT network whose modification leads to G<sub>2</sub>/M arrest of the cells in early mitosis. Kinases act as sensors of the stress occurring to MT. Once activated, they translate the stress signal to the Bcl-2 family proteins, by modulating anti-apoptotic and BH3-only proteins *via* phosphorylation. This impact on Bcl-2 modulatory proteins unleashes the pro-apoptotic Bak and Bax, which are now free to trigger the mitochondrial apoptotic pathway (modified from Cerella et al., 2009).

### 5.5.5 Involvement of kinases in OSCs-mediated apoptosis

Different Bcl-2 family members are modulated by OSCs *via* protein phosphorylation. In addition, different kinases are activated upon treatment with several OSCs. The activation of c-Jun N-terminal kinase (JNK) appears as the most relevant for the apoptogenic potential of OSCs. JNK activation has been commonly reported upon DADS treatment in neuroblastoma SH-SY5Y (Filomeni et al., 2003) and breast cancer MCF-7 cells (Lei et al., 2008); in prostate PC-3 and DU145 cells in response to DATS (Xiao et al., 2004); by SAMC in colon cancer SW480 cells (Xiao et al., 2003). JNK activation is required for the apoptogenic activity of

OSCs, since the specific JNK inhibitor SP600125 is able to exert a protective effect (Wu, X.J. et al., 2009; Xiao et al., 2003). JNK activation and Bcl-2 phosphorylation are events that accompany changes in the MT dynamics (Mollinedo & Gajate, 2003). This suggests an intermediate role of JNK as a transducer of the stress produced by OSCs between the MT network and Bcl-2 (and possibly to other Bcl-2 family members). Alternatively, JNK activation has been explained as a consequence of ROS production, which in turn, may impair the ability of the JNK guardian GST to sequester and prevent the activation of the kinase (Filomeni et al., 2003). Further MAP kinases are activated by OSCs. Phosphorylation of extracellular signal-regulated kinase (ERK) has been frequently reported (Xiao et al., 2004). Similarly, there is evidence of the activation of p38 mitogen-activated protein kinase (MAPK) (Das et al., 2007; Zhang, Y.W. et al., 2006) and Akt kinases (Wang, Y.B. et al., 2010b). The results, however, are not conclusive because their inhibition did not give univocal results. Figure 3 reports a model of interactions between kinases and the other factors/events modulated during OSCs treatment.

### 5.6 Inhibition of metastatic process and differentiation

Metastasis is a major cause of death in cancer patients. Tumor invasion is a multistep process involving the detachment of cancer cells at the site of the primary tumor, the entry into the systemic circulation and the invasion of new tumor sites. These different steps require multiple molecular events including loss of adhesion and motility of cancer cells, and stimulating angiogenesis (which appears to be essential for cancer cell spread). First, several *in vitro* studies have reported that garlic extracts and some of its components are able to affect cancer cell motility and invasiveness. In 2002, Hu and coworkers published a first report showing that AGE suppressed rat sarcoma cell migration *in vitro* (Hu et al., 2002). Referring to these results, the antimetastatic activity of purified OSCs (e.g., DADS, SAC) was investigated. Shin and colleagues demonstrated that DADS reduced the motility of the prostate LNCaP cancer cell model by increasing the tightness of the tight junctions and by reducing the activity, the mRNA and protein levels of MMP2 and 9 (Shin et al., 2010). SAC also altered breast tumor MDA-MB-231 cell adhesion and invasion through the induction of adhesion protein E-cadherin expression and the decrease of MMP2 activity (Gapter et al., 2008). Recently, Lai et al. have reported that the inhibition of MMP2, 7 and 9 in colo 2005 cells by DAS, DADS and DATS were mediated through the downregulation of phosphoinositide 3-kinase (PI3K), Rat sarcoma (RAS), MAP kinase kinase kinase (MEKK) 3, MAP kinase kinase (MKK7), ERK1/2, JNK1/2 and p38 MAPK (Lai et al., 2011). Moreover, suppression of angiogenesis, a process leading to the formation of new blood vessels from the pre-existing vascular network, has also been reported. This process is essential in cancer development as it takes part in primary tumor growth (*i.e.*, provision of oxygen and nutrients) and in spreading to distant sites, leading to metastasis. Tumor cells are known to promote angiogenesis through the production of several growth factors, which stimulate endothelial cell proliferation and migration (for a recent review, see (Makrilia et al., 2009)). In 2006, studies have shown that AGE and DATS inhibited the proliferation and the migration of endothelial cells as well as the capillary-like tube formation *in vitro* (Matsuura et al., 2006; Xiao et al., 2006b). For example, Xiao et al. reported that DATS exerted its antiangiogenic activity through the inhibition of vascular endothelial growth factor (VEGF) secretion, the reduction of VEGF receptor 2 expression and the inactivation of Akt signaling pathway in human umbilical vein endothelial cells (HUVEC) (Xiao et al., 2006b).

*In vivo* studies have confirmed the evidence supporting the antimetastatic properties of garlic *in vitro*. Oral administration of SAMC to mice bearing prostate tumors reduced the number of pulmonary metastasis by 85% and completely abolished their presence in adrenal cells (Howard et al., 2007). In another study, Singh and coworkers have shown that oral administration of DATS to TRAMP mice decreased the development of pulmonary metastasis (about 50% compared with the control mice) without inhibiting angiogenic features at the tested dose (Singh et al., 2008). Despite the different evidence obtained *in vitro*, the molecular events implicated in the antimetastatic activity of OSCs remain to be clarified.

Cancer cells are characterized by the loss of growth control mechanisms and thus remain in a less differentiated, immature state. One important therapy approach is therefore the induction of differentiation in order to restrain the cancer cell proliferation. In this respect, garlic oil was described to induce differentiation in human gastric cancer BGC-823 (Brew et al., 2000) and HL-60 promyelocytic leukemia cells (Seki et al., 2000). The ability to induce cancer cell differentiation could be linked to DADS and SAC. Studies on DADS found that upregulation of p21(WAF) and acetylated histones H3 and H4 is associated with the induction of differentiation in HL-60 cells (Ling et al., 2006) while in MGC803 gastric cancer cells alterations of the ERK1/2 signaling pathway are involved in this process (Chu et al., 2006). Abnormal expression of cytokeratins (marker of epithelial cell differentiation), which is connected to malignant progression, was restored by SAC administration in hamster buccal pouch carcinogenesis in Syrian hamsters (Balasenthil et al., 2003). Other studies reported that SAC upregulated E-cadherin expression in MDA-MB-231 breast tumor and PCa prostate cancer cells (Chiang et al., 2006; Gapter et al., 2008). On the other hand, in former studies, no differentiation markers could be detected in neuroblastoma or melanoma cells upon SAC treatment (Takeyama et al., 1993; Welch et al., 1992).

## 6. Bioavailability and pharmacokinetics of OSCs

Little is known from preclinical and clinical studies about the bioavailability, the metabolism and the excretion of garlic ingredients after consumption. Guo and coworkers have reported that pure alliin is absorbed *in vivo* as it can be detected in stomach (7.2%), intestine (22.4%) and liver (2.5%) after oral administration to mice. In this study, neither allicin nor other degradation products, such as DAS, DADS, vinyldithiins, were found. This clearly indicates that alliin itself is never metabolized, in absence of alliinase, even by liver enzymes (Guo et al., 1990). *Ex vivo* experiments, on isolated perfused rat liver, have suggested that allicin is not a biologically active component of garlic. After infusion at low concentrations, allicin bound to liver epithelium, reached the hepatic first-pass and was rapidly metabolized into DADS and AM, both compounds detectable in hepatic tissue and bile; allicin instead was not found (Egen-Schwind et al., 1992b). Another study of the same authors confirmed that the metabolism of allicin occurred in liver as it was rapidly transformed in presence of liver homogenate (Egen-Schwind et al., 1992a). Experiments using intraperitoneal injection of [<sup>35</sup>S]-labeled DADS in mice revealed that a maximum of radioactivity (70% of the total amount) was detected in the liver 90 min after administration. Actually, mostly [<sup>35</sup>S]-labeled sulfate (80% of the radioactivity of the liver) was detected suggesting that the metabolism of DADS occurs in this organ (Pushpendran et al., 1980). Germain and coworkers have reported that DADS was mainly transformed into AM, AMS,

allylmethylsulfoxide and allylmethylsulfone when orally administrated to rats (Germain et al., 2002). Pharmacokinetics of vinyldithiins have also been studied in animal models. Both 2-vinyl-4H-1,3-dithiin and 3-vinyl-4H-1,2-dithiin were found in serum, kidney and fat tissue after oral administration to rats, whereas only 2-vinyl-4H-1,3-dithiin was found in liver. The latter was more rapidly eliminated probably due to its lower lipophilicity (Egen-Schwind et al., 1992a). Bioavailability of SAC, a main constituent of AGE, was more extensively studied in animal and human. SAC achieved the intestinal first-pass and occurred in plasma, liver and kidney. Its bioavailability was close to 100% when administrated to rat, mouse and dog. Interestingly, concentrations detected in blood correlated well with the doses of oral intake, suggesting SAC as a biological active component of garlic. Then, SAC was excreted in urine as N-acetylated metabolites, indicating a metabolizing-process mediated through N-acetyl transferases (Nagae et al., 1994). Comparable results have been obtained from clinical studies (Jandke & Spiteller, 1987; Rosen et al., 2001).

## 7. Ongoing clinical trials

At the moment, few clinical trials are ongoing (information available on [www.clinicaltrials.gov](http://www.clinicaltrials.gov)). These studies aim at elucidating the bioavailability of garlic in humans and evaluating its benefits in chemoprevention and as supplement in cancer treatment. A study with healthy patients deals with the bioavailability of allicin from garlic supplements (powder) and garlic clove (raw, cooked, processed). Concerning the chemopreventive benefit of garlic consumption, some investigators are recruiting healthy volunteers to evaluate the way by which garlic reduces cancer risk (analysis of a panel of cancer biomarkers in control *vs.* treated groups). Finally, two studies are conducted to assess the potential benefits of co-administration of garlic and conventional chemotherapeutics in cancer patients. As example, we can cite a clinical trial studying how garlic intake affects docetaxel treatment in locally advanced or metastatic breast cancer patients. Another phase II study, gathering 45 patients with aggressive follicular lymphoma (stage III/IV), has been started to assess the ability of garlic extract to impact apoptosis and cancer cell proliferation.

## 8. Conclusions

A large body of evidence supports the anti-cancer potential of OSCs from *Allium* species. The huge amount of data is consistent with a future dual potential clinical application of selected compounds, in chemoprevention as well as in chemotherapy. The sulfur component is mostly responsible of their chemical properties. The fact that many enzymes and proteins are regulated by modifications occurring at the level of redox-sensitive cysteine residues accounts for their versatility and multitasking potential. So far a good part of the path in the elucidation of the mechanisms responsible for OSCs anti-cancer activities has been covered.

Some important challenges need still to be faced. First, the impact of OSCs on cell cycle raises the question if purified agents from *Allium* species may be nevertheless toxic for non-malignant proliferating cells, at the concentrations tested *in vitro*. In this sense, indepth investigations confirming the differential effects of OSCs on cancer *vs.* healthy cells are absolutely required. Second, the fact that cancer of the digestive tract and prostate appear as the most sensitive ones to the chemopreventive effects of *Allium* vegetables intake may

reflect a problem of their stability. Tissues/organs, which are in inner connection with the digestive/urinary systems, are obviously exposed to higher concentrations of biologically active products deriving from the degradation/catabolism of any ingested food or supplements. This encourages any efforts aimed at increasing the stability of these compounds, hopefully without increasing also their toxicity. Finally, the conjugation of molecules specifically recognizing and binding to cancer cells together with the enzyme alliinase could generate high concentrations of alliin right at the tumor site and may represent an interesting attempt to overcome both limiting factors (Appel et al., 2011).

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## Phytochemicals in Soy\* and Their Health Effects

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### 1. Introduction

Consumption of soy foods has been associated, at least in part, with lower incidences of a number of chronic diseases indicated by epidemiological studies (1-4). Soy-based foods have been consumed in Asian countries such as China, Japan and Korea for many centuries. The lower rates of several chronic diseases in Asia, including cardiovascular diseases and certain types of cancer, have been partly attributed to consumption of large quantities of soy foods (5, 6).

Soy-based food was first introduced on a large scale to the general U.S. population as a source of high quality protein. A significant increase in soy food consumption during the last decade of the 20<sup>th</sup> century occurred because of the health benefits soy food might offer independent of their nutrient content (7). In the last few decades, extensive efforts have been made towards identifying bioactive components in soy foods that are responsible for the health benefits. Among them, isoflavones and soy proteins are the two major groups of components that have received the most attention (8-11). Isoflavones belong to a broad group of plant-derived compounds that have structural and functional similarities to estrogens, which has led to the term phytoestrogens (12, 13). Indeed, more than half of the soy-related papers are related to isoflavones (7). The analysis, bioavailability and the health effects of isoflavones have been extensively studied and frequently reviewed (12, 14-21). Consumption of isoflavones has been suggested to have multiple beneficial effects in a number of chronic diseases and medical conditions (4, 22, 23). However, accumulating evidence has also suggested that isoflavones only reflected certain aspects of the health effects associated with soy consumption. Other components in soy, such as soyasaponins, phytic acid or plant sterols, display a wide range of bioactivities, including anti-cancer, anti-oxidative, anti-viral, cardiovascular protective effects, and hepatoprotective actions (24).

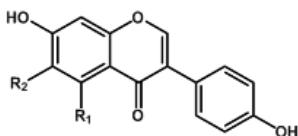
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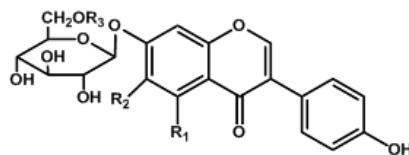
In this chapter, we summarize the structural characterization of major phytochemicals in soybean and soy-based foods. Potential health benefits of these phytochemicals, especially the non-isoflavone phytochemicals and their preventive effects of chronic and lifestyle-related diseases, are also briefly discussed.

## 2. Phytochemicals in soybean and their health effects

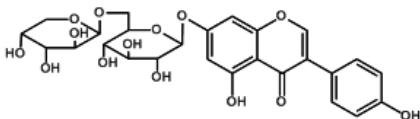
In this section, the structural characterizations of major phytochemicals that naturally exist in soybean are firstly summarized. Composition and contents of phytochemicals in soybean vary dramatically depending on the variety and growing environment. In general, the



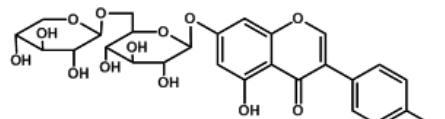
1.  $R_1 = R_2 = H$  daidzein
2.  $R_1 = OH, R_2 = H$  genistein
3.  $R_1 = H, R_2 = OCH_3$  glycitein



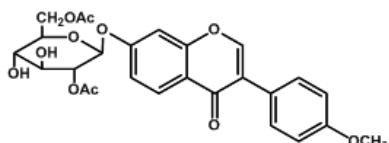
4.  $R_1 = R_2 = R_3 = H$  daidzin
5.  $R_1 = OH, R_2 = R_3 = H$  genistin
6.  $R_1 = H, R_2 = OCH_3, R_3 = H$  glycitein
7.  $R_1 = R_2 = H, R_3 = COCH_3$  6"-O-acetyl daidzin
8.  $R_1 = OH, R_2 = H, R_3 = COCH_3$  6"-O-acetyl genistin
9.  $R_1 = H, R_2 = OCH_3, R_3 = COCH_3$  6"-O-acetyl glycitein
10.  $R_1 = R_2 = H, R_3 = COCH_2COOH$  6"-O-malonyl daidzin
11.  $R_1 = OH, R_2 = H, R_3 = COCH_2COOH$  6"-O-malonyl genistin
12.  $R_1 = H, R_2 = OCH_3, R_3 = COCH_2COOH$  6"-O-malonyl glycitein



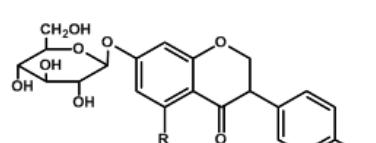
13. 6"-O- $\beta$ -D-arabinose-genistin



14. 6"-O- $\beta$ -D-xylose-genistin



15. 2",6"-O-diacetylgenistin



16. R= H dihydrodaidzein  
17. R= OH dihydrogenistein

Fig. 1. The chemical structures of isoflavones in soy.

contents of major phytochemicals from high to low in soybean are: phytic acid (1.0-2.2%) (25), sterols (0.23-0.46%) (26), saponins (0.17-6.16%) (27), isoflavones (0.1-0.3%) (23), and lignans (0.02%) (28). Secondly, the potential health effects of the major phytochemicals are outlined. For many years, the studies of health effects of soybean phytochemicals primarily focused on isoflavones. However, other phytochemicals in soy, such as soyasaponins, phytosterols, lignans, phytic acid, and oligosaccharides, have also been found to exert biological activities. These may contribute to overall health effects observed with soy consumption.

## 2.1 Isoflavones

### 2.1.1 Chemical characteristics of isoflavones

Isoflavones have been known to exist in plants for over 100 years. And soy, including the foods derived from this legume, is considered as a richest dietary source of isoflavones (7). Isoflavones (3-phenyl-4H-1-benzopyran-4-one) are a subclass of more ubiquitous flavonoids, while they differs from flavone (2-phenyl-4H-1-benzopyran-4-one) in that the phenyl group (B ring) is connected to position 3 instead of position 2 (**Figure 1**) (29). Soy mainly contains three isoflavones, namely daidzein (7,4'-dihydroxyisoflavone) (1), genistein (5,7,4'-trihydroxyisoflavone) (2) and glycitein (6-methoxy-7,4'-dihydroxyisoflavone) (3). Genistein and daidzein have been found in relatively high concentrations in soybean and most soy-based foods. In soybean and non-fermented soy foods, they are generally present as one of the following three  $\beta$ -glucoside conjugates: a) the corresponding glucosides: daidzin (daidzein 7-O- $\beta$ -D-glucoside) (4), genistin (genistein 7-O- $\beta$ -D-glucoside) (5) and glycitin (glycitein 7-O- $\beta$ -D-glucoside) (6); b) the corresponding acetylglucosides: 6"-O-acetyldaidzin (7), 6"-O-acetylgenistin (8) and 6"-O-acetylglycitin (9); and c) the corresponding malonylglucosides: 6"-O-malonyldaidzin (10), 6"-O-malonylgenistin (11) and 6"-O-malonylglycitin (12) (29). In addition to  $\beta$ -glucoside conjugates, isoflavones conjugated with other sugar moieties, including 6"-O- $\beta$ -D-arabinose-genistin (13) and 6"-O- $\beta$ -D-xylose-genistin (14). Other isoflavones, including 2",6"-O -diacetylolininin (15), and two dihydro-isoflavanones, dihydrodaidzin (16) and dihydrogenistin (17) were reported recently in the last ten years (30, 31).

### 2.1.2 Health effects of isoflavones

Health effects of isoflavones were initially thought to be related to their estrogenic activity (23). The molecular structures of isoflavones, especially genistein (2), are similar to that of 17 $\beta$ -estradiol (**Figure 2**). Isoflavones can bind to both  $\alpha$  and  $\beta$  isoforms of estrogen receptor (ER), but their binding affinity to ER $\beta$  is about 20 times higher than that to ER $\alpha$  (11). However, compared to physiological estrogen such as 17 $\beta$ -estradiol, isoflavones have approximately 100 times weaker affinities (32). Estrogen-like effects have been proposed as one of the major mechanism of action of isoflavone related to their health effects (8). A second mechanism of action of isoflavones, particularly of genistein, was discovered that genistein is a protein tyrosine kinase (PTK) inhibitor (8). Since then, isoflavones have been shown to affect a diverse array of intracellular signaling pathways (7). For instance, genistein and other isoflavones were found to interact with the peroxisome proliferator activated receptors, PPAR $\alpha$ / $\gamma$ . These nuclear receptors are activated by fatty acids (PPAR $\alpha$ ) and prostaglandins (PPAR $\gamma$ ) and serve as transcription factors (32). As polyphenols, isoflavones also have antioxidant activities,

which were proposed as another important mechanism of action of isoflavones. However, isoflavones are not strong antioxidant may not be able to scavenge oxidants directly. They therefore are considered as antioxidants because of their effects on gene expression of enzymes that enhance antioxidant defenses (32). In addition to what has been discussed above, several other mechanism have been proposed for the activities of isoflavones, including stimulation/inhibition of enzyme activities involved in steroid synthesis and metabolism, targeting thyroid peroxidase, and inhibiting cancer metastasis (32).

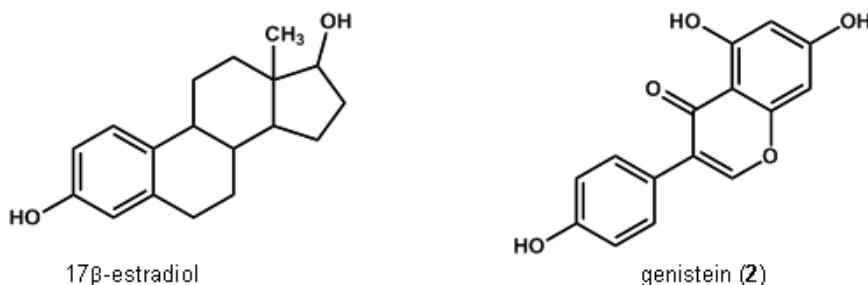


Fig. 2. The chemical structures of genistein and 17 $\beta$ -estradiol.

Consumption of isoflavones has been suggested to have multiple beneficial effects in a number of chronic diseases and medical conditions, including certain types of cancer (33-35), heart disease (36-38), bone functions (39-42) and most recently, prevention of obesity (43, 44). Many excellent reviews regarding the different aspects of health effects of soy isoflavone can be found in the literatures, thus, only a brief outline of the health effects of isoflavones is provided in this chapter.

### 2.1.2.1 Isoflavones and prevention of cancer

The incidences of breast and prostate cancers are much higher in the United States and European countries compared to Asian countries such as Japan and China. One of the major differences in diet between these populations is that the Japanese and the Chinese consume a traditional diet high in soy products (35). Epidemiological evidence together with preclinical data from animal and *in vitro* studies strongly supported a correlation between soy isoflavone consumption and protection towards breast and prostate cancers (45-47). However, clinical studies assessing soy consumption and risk of breast cancer have yielded inconsistent results. In a most recent meta-analysis of prospective studies suggested that soy isoflavones intake is associated with a significantly reduced risk of breast cancer incidence in Asian populations, but not in Western populations. Further studies are warranted to confirm the finding of an inverse association of soy consumption with risk of breast cancer recurrence (48).

Except for breast and prostate cancer, isoflavones also showed inhibitory effects on other hormone-related (e.g. endometrial, ovarian cancer) or hormone-independent cancers (e.g. leukemia and lung cancer) (49).

### 2.1.2.2 Isoflavones and prevention of cardiovascular diseases

A number of cardioprotective benefits have been attributed to dietary isoflavones including reduction in LDL cholesterol, inhibition of pro-inflammatory cytokines, cell adhesion

proteins and inducible nitric oxide production, potential reduction in the susceptibility of the LDL particle to oxidation, inhibition of platelet aggregation and an improvement in vascular reactivity (50). There are not randomized trials investigating the action of isoflavones on the incidence of clinical events. A few recent, well-designed studies have suggested an association of the ingestion of isoflavones with a reduction in the atherosclerotic burden, as indicated by the measurement of the intima-media thickness in carotid vessels (51).

#### **2.1.2.3 Isoflavones and bone health**

Observational studies have suggested that populations in Asia with a high dietary soy intake have a lower incidence of osteoporosis-related fractures when compared to Western populations. Isoflavones were suggested to prevent bone loss associated with menopause. Though extensive research using animal models has provided convincing data to indicate a significant improvement in bone mass or other end points following feeding with soyabean, results from intervention studies are still controversial (52). Additional research is needed to determine if isoflavones are an effective alternative to hormone replacement therapy for the prevention and treatment of osteoporosis (42).

#### **2.1.2.4 Isoflavones and prevention of obesity**

The prevalence of obesity and related diseases has increased rapidly in the Western world. Obesity is a disorder of energy balance and is associated with hyper-insulinemia, insulin resistance, and abnormalities in lipid metabolism, and it is one of the most important risk factors in the development of Type II diabetes, cardiovascular disease, atherosclerosis, and certain cancers (44). In recent years, evidence is emerging that soy isoflavones play a beneficial role in obesity and diabetes. Nutritional intervention studies in animals and humans indicate that consumption of soy isoflaovne or soy protein containing isoflavones reduces body weight and fat mass by lowering plasma cholesterol and triglycerides as well as by other mechanisms (43, 44, 53). Though the published results suggest a beneficial effect of soy isoflavone on obesity in human, these results also suggest that the effect may be dependent on whether the isoflavones are consumed in combination with soy protein (44).

## **2.2 Soyasaponins and soyasapogenols**

#### **2.2.1 Chemical characteristics of soyasaponins and soyasapogenols**

Saponins are sterol or triterpene glycosides that occur in a wide variety of plants. Soy-based foods are primary dietary sources of saponins (54). Chemical studies of saponins in soy track back to the 1930's (55, 56). From the 1980's to 1990's, a number of papers, especially those published by Japanese researchers, significantly enriched our knowledge about chemical structure and diversity of this type of compounds in soy (57-65).

Saponins in soy are often referred to soyasaponins. They differ from each other by the types of aglycones and the position where the sugar chain is attached. Generally, saponins are classified into four major groups, on the basis of their aglycone structures: group A, B, E and DDMP. Group A saponins have a hydroxyl group at the C-21 position, and group B saponins have a hydrogen atom at the same position. Group E saponins differ from group A and B by having a carbonyl group at C-22. Group B saponins may contain a DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) moiety at C-22 position, which are

denoted as DDMP saponins (66) (**Figure 3**) . DDMP soyasaponins and the acetylated soyasaponins of group A are sometimes considered as the genuine forms of groups A and B in soybeans. Group E soyasaponins are also considered to be phyto-oxidation products of group B soyasaponins (62, 67, 68). All four groups of soyasaponins are glycosides of oleanan triterpene aglycones known as soyasapogenols. The oleanan aglycone contains one or more hydroxyl groups, and carboxylic groups and double bonds may also be present. The sugar moieties are generally attached at the C-3 position and sometimes at the C-22 position of the aglycones. Group A soyasaponins have two sugar chains, separately attached to C-3 and C-22 positions of soyasapogenol A, except for A3 (33), which has only one sugar chain at C-3 (**Table 1**). C-3 sugar chain consists of two or three sugar residues, starting with a glucoronol residue (glcUA) (67). C-22 side chain consists of two sugar residues, starting with an anarabinosyl, and ending with a xylosyl or glycosyl residue (58, 63, 65, 69) (**Figure 3**). Group B soyasaponins have one sugar chain attached to the C-3 position of soyasapogenol B, with three exceptional compounds that have two sugar chains at C-3 and C-22 positions (41-43) (57, 59, 69-71) (**Figure 3**). Most recently, a new soyasaponin Bh (40) was identified to bear an unique five-membered ring containing a hemiacetal functionality (**Figure 3**) (72). Group E soyasaponins contain only one sugar chain at the C-3 position. They are considered to be formed by photo-oxidation at C-22 of group B (63, 69) (**Figure 3**). DDMP soyasaponins are categorized under B type soyasaponins by some researchers (27). Their structures were characterized as having DDMP group conjugated to group B at C-22 (67, 68, 73, 74) (**Figure 3**). Similar to B type soyasaponins, DDMP soyasaponins only contain one sugar chain attached to C-3 position. There are about 36 soyasaponins identified in soybean. Their structures and the related references are listed (**Tables 1-4**).

NO.	name	C-3 sugar chain	C-22 sugar chain	molecular formula	ref
18	Aa (acetyl A <sub>4</sub> )	glc(1→2)gal(1→2)glcUA(1→3)	2,3,4-tri-O-acetyl-xyl(1→3)ara(1→22)	C <sub>64</sub> H <sub>100</sub> O <sub>31</sub>	65
19	Ab (acetyl A <sub>1</sub> )	glc(1→2)gal(1→2)glcUA(1→3)	2,3,4,6-tetra-O-acetyl-glc(1→3)ara(1→22)	C <sub>67</sub> H <sub>104</sub> O <sub>33</sub>	64
20	Ac	rha(1→2)gal(1→2)glcUA(1→3)	2,3,4,6-tetra-O-acetyl-glc(1→3)ara(1→22)	C <sub>67</sub> H <sub>104</sub> O <sub>32</sub>	63
21	Ad	glc(1→2)ara(1→2)glcUA(1→3)	2,3,4,6-tetra-O-acetyl-glc(1→3)ara(1→22)	C <sub>66</sub> H <sub>102</sub> O <sub>32</sub>	63
22	Ae (acetyl A <sub>5</sub> )	gal(1→2)glcUA(1→3)	2,3,4-tri-O-acetyl-xyl(1→3)ara(1→22)	C <sub>58</sub> H <sub>90</sub> O <sub>26</sub>	65
23	Af (acetyl A <sub>2</sub> )	gal(1→2)glcUA(1→3)	2,3,4,6-tetra-O-acetyl-glc(1→3)ara(1→22)	C <sub>61</sub> H <sub>94</sub> O <sub>28</sub>	64
24	Ag (acetyl A <sub>6</sub> )	ara(1→2)glcUA(1→3)	2,3,4-tri-O-acetyl-xyl(1→3)ara(1→22)	C <sub>57</sub> H <sub>88</sub> O <sub>25</sub>	65
25	Ah (acetyl A <sub>3</sub> )	ara(1→2)glcUA(1→3)	2,3,4,6-tetra-O-acetyl-glc(1→3)ara(1→22)	C <sub>60</sub> H <sub>92</sub> O <sub>27</sub>	64
26	Ax	glc(1→2)ara(1→2)glcUA(1→3)	2,3,4-tri-O-acetyl-xyl(1→3)ara(1→22)	C <sub>63</sub> H <sub>98</sub> O <sub>30</sub>	69
27	A <sub>1</sub>	glc(1→2)gal(1→2)glcUA(1→3)	glc(1→3)ara(1→22)	C <sub>59</sub> H <sub>96</sub> O <sub>29</sub>	64
28	A <sub>2</sub>	gal(1→2)glcUA(1→3)	glc(1→3)ara(1→22)	C <sub>53</sub> H <sub>86</sub> O <sub>24</sub>	64
29	A <sub>3</sub>	ara(1→2)glcUA(1→3)	glc(1→3)ara(1→22)	C <sub>52</sub> H <sub>84</sub> O <sub>23</sub>	64
30	A <sub>4</sub>	glc(1→2)gal(1→2)glcUA(1→3)	xyl(1→3)ara(1→22)	C <sub>58</sub> H <sub>94</sub> O <sub>28</sub>	64
31	A <sub>5</sub>	gal(1→2)glcUA(1→3)	xyl(1→3)ara(1→22)	C <sub>52</sub> H <sub>84</sub> O <sub>23</sub>	64
32	A <sub>6</sub>	ara(1→2)glcUA(1→3)	xyl(1→3)ara(1→22)	C <sub>51</sub> H <sub>82</sub> O <sub>22</sub>	64
33	A <sub>3</sub>	rha(1→2)gal(1→2)glcUA(1→3)		C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	58

Table 1. The structures and molecular formulas of group A soyasaponins

NO.	name	C-3 sugar chain	C-22 sugar chain	molecular formula	ref
34	soyasaponin Ba or V	glc(1→2)gal(1→2)glcUA(1→3)		C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	64
35	soyasaponin Bb or I	rha(1→2)gal(1→2)glcUA(1→3)		C <sub>48</sub> H <sub>78</sub> O <sub>18</sub>	59
36	soyasaponin Bc or II	rha(1→2)ara(1→2)glcUA(1→3)		C <sub>47</sub> H <sub>76</sub> O <sub>17</sub>	59
37	soyasaponin Bb' or III	gal(1→2)glcUA(1→3)		C <sub>42</sub> H <sub>68</sub> O <sub>14</sub>	59
38	soyasaponin Bc' or Bx	glc(1→2)ara(1→2)glcUA(1→3)		C <sub>47</sub> H <sub>76</sub> O <sub>18</sub>	69
39	soyasaponin IV	ara(1→2)glcUA(1→3)		C <sub>41</sub> H <sub>66</sub> O <sub>13</sub>	57
40	soyasaponin Bh	rha(1→2) gal(1→2)glcUA(1→3)		C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	72
41	3-O-{α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)-β-D-galactopyranosyl(1→2)]-β-D-glucuronopyranosyl(1→2)-α-L-arabinopyranosyl}3β,22β,24-trihydroxyolean-12-ene	rha(1→2)[glc(1→3)gal(1→2)] glcUA(1→3)	glc(1→2)ar a(1→22)	C <sub>65</sub> H <sub>106</sub> O <sub>32</sub>	70
42	3-O-[α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→2)]-β-D-glucuronopyranosyl-22-O-[α-L-rhamnopyranosyl(1→2)- α-L-arabinopyranosyl]3β,22β,24-trihydroxyolean-12-ene	rha(1→2)gal(1→2)glcUA(1→3)	rha(1→2)ar a(1→22)	C <sub>59</sub> H <sub>96</sub> O <sub>26</sub>	71
43	3-O-[α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→2)]-β-D-glucuronopyranosyl-22-O-[α-L-rhamnopyranosyl(1→2)- β-D-glucopyranosyl]3β,22β,24-trihydroxyolean-12-ene	rha(1→2)gal(1→2)glcUA(1→3)	rha(1→2)gl c(1→22)	C <sub>60</sub> H <sub>98</sub> O <sub>27</sub>	71

Table 2. The structures and molecular formulas of group B soyasaponins

NO.	name	C-3 sugar chain	molecular formula	ref
44	soyasaponin Bd (sandosaponin A)	glc(1→2)gal(1→2)glcUA(1→3)	C <sub>48</sub> H <sub>76</sub> O <sub>19</sub>	63
45	soyasaponin Be (dehydrosoyasaponin I)	rha(1→2)gal(1→2)glcUA(1→3)	C <sub>48</sub> H <sub>76</sub> O <sub>18</sub>	63
46	soyasaponin Bf	glc(1→2)ara(1→2)glcUA(1→3)	C <sub>47</sub> H <sub>74</sub> O <sub>18</sub>	69
47	soyasaponin Bg	rha(1→2)ara-(1→2)glcUA(1→3)	C <sub>47</sub> H <sub>74</sub> O <sub>17</sub>	69

Table 3. The structures and molecular formulas of group E soyasaponins

NO.	name	C-3 sugar chain	C-22 DDMP	molecular formula	ref
48	soyasaponin $\alpha g$	glc(1→2)gal(1→2)glcUA(1→3)	DDMP(22→2')	C <sub>54</sub> H <sub>84</sub> O <sub>22</sub>	68
49	soyasaponin $\alpha a$	glc(1→2)ara(1→2)glcUA(1→3)	DDMP(22→2')	C <sub>53</sub> H <sub>82</sub> O <sub>21</sub>	74
50	soyasaponin $\beta g$ or VI	rha(1→2)gal(1→2)glcUA(1→3)	DDMP(22→2')	C <sub>54</sub> H <sub>84</sub> O <sub>21</sub>	68
51	soyasaponin $\beta a$	rha(1→2)ara(1→2)glcUA(1→3)	DDMP(22→2')	C <sub>53</sub> H <sub>82</sub> O <sub>20</sub>	68
52	soyasaponin $\gamma g$	gal(1→2)glcUA(1→3)	DDMP(22→2')	C <sub>48</sub> H <sub>74</sub> O <sub>17</sub>	68
53	soyasaponin $\gamma a$	ara(1→2)glcUA(1→3)	DDMP(22→2')	C <sub>47</sub> H <sub>72</sub> O <sub>16</sub>	68

Table 4. The structures and molecular formulas of group DDMP soyasaponins

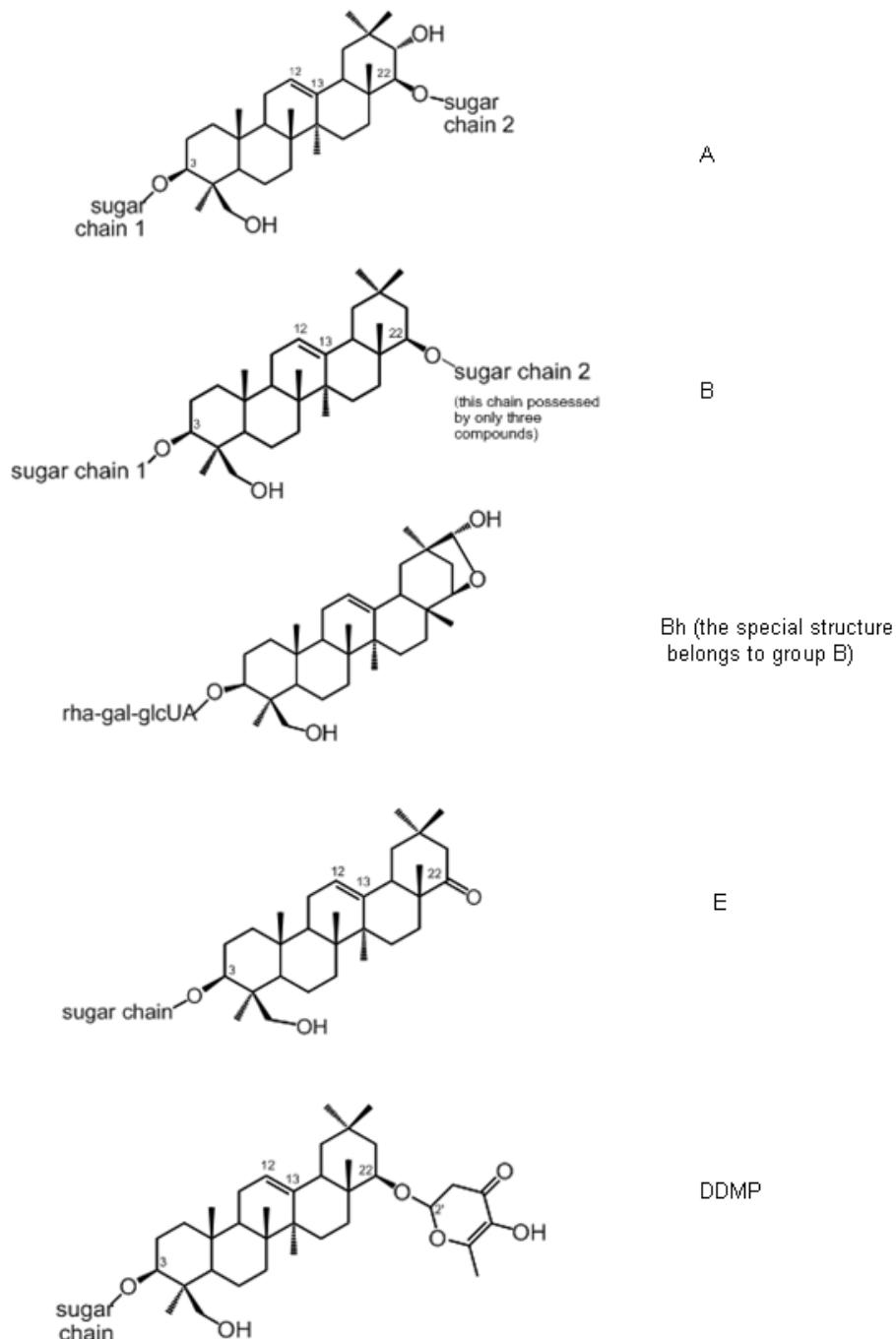


Fig. 3. The chemical structures of groups A, B, Bh, E and DDMP soyasaponins in soy.

Soyasapogenols are aglycones of soyasaponins. They can be obtained by acid or alkaline hydrolysis of soyasaponins. Though soyasapogenols don't exist in soy bean naturally, they may exist in certain soy products through food processing. Five soyasapogenols A (**54**), B (**55**), C (**56**), D (**57**) and E (**58**) have been found through hydrolysis of soyasaponins (59, 62, 75) (Figure 4). Among them, soyasapogenol A (**54**) has two hydroxyl groups at C-21 and C-22 position, which is considered as corresponding aglycone to bis-desmoside soyasaponin group A. Soyasapogenol B (**55**) has only one hydroxyl group at C-22 position. It is considered to be aglycone of both monodesmoside soyasaponin groups B and DDMP. Soyasapogenol E (**58**) is regarded as the corresponding aglycone to soyasaponin group E. Soyasapogenols C (**56**) and D (**57**) are considered by some researchers not to be genuine aglycones of soyasaponins, but as the acid hydrolysis products of soyasapogenol B (**55**) (76).

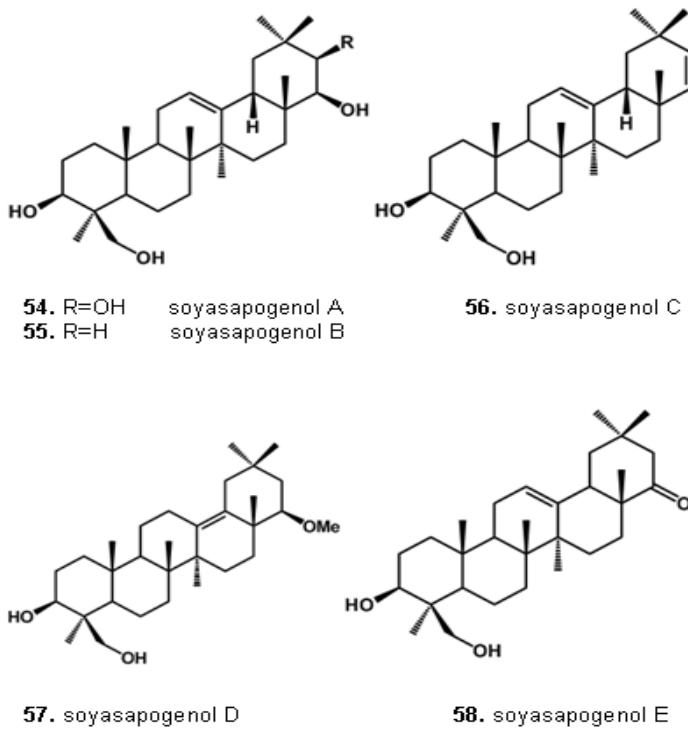


Fig. 4. The chemical structures of soyasapogenols A-E (hydrolysis products of soyasaponins).

### 2.2.2 Health effects of soyasaponins and soyasapogenols

Plant saponins as a whole have been reported to have a wide range of biological activities, which were summarized with a long list in a recent review (77). Soyasaponins and soyasapogenols have been reported to display diverse health effects (78), including anti-cancer, cardiovascular protective effects, anti-virus, hepatoprotective actions and antioxidant activities (24, 27, 79, 80). Their health effects largely depend on their chemical structures (81). Since this class of soy compounds are so poorly absorbed, their bioactivity maybe caused by indirect actions within the GI tract.

### 2.2.2.1 Anti-carcinogenic activities

Epidemiological studies have linked soy consumption to lower incidences of various types of cancer (82). As a group of major phytochemicals in soy, soyasaponins may be partially responsible (27). The evidence for soyasaponins having anti-carcinogenic effects in animal models is limited. Nevertheless, soyasaponins and soyasapogenols have been shown to have the anti-carcinogenic effects in a number of carcinoma cell lines. Before summarizing the data, we must keep in mind that since soy saponins are poorly absorbed, *in vitro* studies of these compounds in cell lines may not be able to provide much meaningful indication regarding their *in vivo* bioactivities.

Most studies related to the anti-carcinogenic activities of soyasaponins and soyasapogenols have been performed in human colon cancer, liver cancer or breast cancer cell lines (83-88). The proposed mechanisms of anticarcinogenic properties of soyasaponins and soyasapogenols include direct cytotoxicity, induction of apoptosis, anti-estrogenic activity, inhibition of tumor cell metastasis, anti-mutagenic activity effect, bile acid binding action and normalization of carcinogen-induced cell proliferation (**Table 5**) (89). From these studies done in different cancer cell lines, we've learned that there are at least three major factors that may affect the observed effects of soyasaponins and soyasapogenols. The first factor is the testing materials. Some researchers used crude extracts while others used purified saponins. It is possible that components of the crude extract interact synergistically, thus inducing effects not observed with pure saponins. Therefore, investigations with purified saponins are indispensable for matching a result to the molecular action of a specific saponin (90). The second factor is the type of cell lines. It is clearly shown that different cancer cell lines respond differently to soy saponins. For instance, soyasaponin I (35) had no effect on HT-29 colon cell growth (81), but it can decrease the migratory ability of B16F10 melanoma cells (91). The third factor is the dose. In another study, soyasaponin III (37) showed significant growth suppression at the highest concentration tested (50 ppm), and no significant effects from concentrations 6 to 25 ppm.

### 2.2.2.2 Cardiovascular protective effects

Soyasaponins showed cardiovascular protective effects through several different mechanisms.

The hypocholesterolemic effects of soy saponins have long been recognized (92-95). Two mechanisms by which saponins can affect cholesterol metabolism were suggested (96): 1) some saponins with particularly defined structural characteristics form insoluble complexes with cholesterol. When this complex-forming process occurs in the gut, it inhibits intestinal absorption of both endogenous and exogenous cholesterol. 2) Saponins can interfere with the entero-hepatic circulation of bile acids by forming mixed micelles. The re-absorption of bile acids from the terminal ileum is effectively blocked (92).

In animal models, soyasaponins were found to significantly reduce serum total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), triglycerides (TG) concentrations, and to increase high-density lipoprotein-cholesterol (HDL-C) levels in rats (97). 24-Methylenecycloartanol (59), in combination with soysterol greatly reduced plasma cholesterol and enhanced cholesterol excretion in rats (98). Hamsters fed group B soyasaponins had significantly lower plasma TC (by 20%), non-HDL-C (by 33%), TG (by 18%)

materials	cell line	dose	observation	mechanism	ref
Total soyasaponins	Human HT-1080 fibrosarcoma cell	Inhibit the metastasis of HT-1080 cells dose-dependently (100-300 µg/ml) after 24 h.	Inhibit the invasion of HT-1080 cells through a Matrigel-coated membrane.	Suppress MMP-2 and MMP-9 productions and stimulate TIMP-2 secretion.	86
Total soyasaponins	Human HT-29 colon cancer cell	Decrease HT-29 cell growth in a dose dependent manner at concentrations of 150, 300, and 600 ppm after 72 h.	Decrease HT-29 cell growth.	Suppress inflammatory responses. COX-2 and PKC expressions were significantly down-regulated.	87
Total soyasaponins	Human HepG2 liver hepatoma cell	Inhibit AFB <sub>1</sub> -DNA adduct formation in HepG2 liver hepatoma cells by 50.1 % at a concentration of 30 µg/ml after 48 h.	Inhibit AFB <sub>1</sub> -DNA adduct formation in HepG2 liver cells.	Induce membrane permeability change.	85
B-group soyasaponins	Human SNB 19 glioblastoma cell	Induce SNB 19 glioblastoma cell apoptosis dose-dependently (25-75 µM) after 48 h.	Induce apoptosis in SNB 19 glioblastoma cells.	Stimulate cytochrome-c release and activate caspase cascade.	88
B-group soyasaponins	Human HCT-15 colon cancer cell	Suppress HCT-15 colon cancer cell proliferation dose-dependently at concentrations of 25-500 ppm, and induce macroautophagy at concentration of 100 ppm after 24 h	Suppress HCT-15 colon cancer cell proliferation and induce macroautophagy.	Delay S-phase cell cycle.	83
Soyasaponin I (35)	Highly metastatic B16F10 melanoma cell	Decrease migratory ability of B16F10 melanoma cell dose-dependently (25-75 µM) after 12 h.	Decrease the migratory ability of cells, enhance cell adhesion to extracellular matrix proteins.	Inhibit the expression of α-2,3-linked sialic acids of B16F10 melanoma cell.	91
Soyasaponin I (35)	Human MCF-7 breast cancer cell	Significantly enhance MCF-7 cells to adhere the extracellular matrix at 50 µM after 24 h.	Enhance the adhesion of MCF-7 cells to the extracellular matrix proteins.	Alter MCF-7 breast cancer cell surface α2,3-sialylation pathway.	84
Soyasaponin III (37)	Human HT-29 colon cancer cell	37 and soyasapogenol B monoglucuronide show HT-29 colon cancer cell growth suppression at 50 ppm after 72 h.	They all suppress the growth of HT-29 colon cancer cell	The cell growth suppression of soyasaponins and soyasapogenols increased with increased lipophilicity.	81
Soyasapogenol B monoglucuronide	colon cancer cell	54 and 55 show a dose-dependent growth suppression from 6- 50 ppm after 72 h.			
Soyasapogenol A (54)					
Soyasapogenol B (55)					

Table 5. The anti-carcinogenic activities of soyasaponins and soyasapogenols

and a lower ratio of total to HDL cholesterol (by 13%) in hamsters fed group B soyasaponins, compared to those fed casein. Possible mechanisms involved greater excretion of fecal bile acids and neutral sterols (99).

The anti-thrombogenic action of soyasaponins was evaluated in a model of disseminated intravascular coagulation (DIC) induced by infusion of endotoxin or thrombin in rats. Total soyasaponins prevented the decrease of blood platelets and fibrinogen, and the increase of fibrin degradation products during DIC induced by infusion of endotoxin or thrombin in rats. *In vitro* experiments, total soyasaponins, soyasaponins I (35), II (36), A<sub>1</sub> (27), and A<sub>2</sub> (28) inhibited the conversion of fibrinogen to fibrin (100).

Total soyasaponins decreased elevated blood sugar and LPO levels, and reversed the decreased levels of SOD in streptozocin-induced diabetic rats (101). In an  $\alpha$ -glucosidase inhibitory assay, groups B, E and DDMP soyasaponins were shown to have potent inhibitory activities with IC<sub>50</sub> values of 10-40  $\mu$ mol/L (102).

#### 2.2.2.3 Anti-viral activities

Soyasapogenols A (54), B (55), E (58), and soyasaponin I (35), a major constituent of group B saponins, completely inhibited HIV-induced cytopathic effects 6 days after infection at concentration greater than 0.25 mg/mL, but had no direct effect on HIV reverse transcriptase activity. Soyasaponin I (35) also inhibited HIV-induced cell fusion in the MOLT-4 cell system (103).

Total soyasaponins showed significant inhibitory effect on the replication of HSV-1 and CoxB3. A soyasaponin cream was used in the treatment of patients suffering from herpes labialis. The treatment was found to be highly effective with a cure rate of 88.8% for the disease (104). Soyasaponin II (36) is more potent than soyasaponin I (35) as shown by a reduction of simplex virus type 1 (HSV-1) production. Soyasaponin II (36) was also found to inhibit the replication of human cytomegalovirus and influenza virus. This action was not due to inhibition of virus penetration and protein synthesis, but may involve a virucidal effect (105). In a structure–activity relationship study, the activity of soyasapogenol A (54) was less than 1/20 of that of soyasapogenol B (55), and the hydroxylation at C-21 seemed to reduce anti-HSV-1 activity (106).

#### 2.2.2.4 Hepatoprotective actions

The group B soyasaponins I (35), II (36), III (37), and IV (38) all exhibit hepato-protective actions towards immunologically induced liver injury in primary cultured rat hepatocytes. The action of soyasaponin II (36) is almost comparable with that of soyasaponin I (35), whereas soyasaponins III (37) and IV (38) are more effective than soyasaponins I (35) and II (36). This suggests that the disaccharide group shows greater action than the trisaccharide group. Furthermore, the soyasaponins having a hexosyl unit shows a slightly greater action than that of the pentosyl unit in each disaccharide group or trisaccharide group. Structure–activity relationships suggest that the sugar moiety linked at C-3 may play an important role in hepato-protective actions of soyasaponins (107).

Also in *in vivo* experiments, total soyasaponins inhibit the elevation of liver transaminases when administered orally to rats with peroxidized corn oil. The liver injury caused by peroxidized salad oil is inhibited by the addition of soyasaponin A<sub>1</sub> (27) during peroxidation (108).

### 2.2.2.5 Antioxidant activities

One mg of DDMP saponin/mL scavenges superoxide at a degree equivalent to 17.1 units of superoxide dismutase/mL by the ESR spin trapping method. The scavenging superoxide activity of DDMP soyasaponins is caused by the DDMP moiety attached to the triterpene aglycon since soyasaponin I (35), which is derived from soyasaponin  $\beta$ g (50). Lack of this group didn't show the scavenging activity (109).

## 2.3 Triterpenes and sterols

### 2.3.1 Chemical characteristics of triterpenes and sterols

Triterpenes and sterols are found in soybean oil unsaponifiable matter. They are both present in minor quantities.

Three triterpenes have been identified in soy. They are 24-methylenecycloartenol (59) (98), cycloartenol (60) (98) and bacchara-12,21-dien-3 $\beta$ -ol (61) (Figure 5) (110), respectively. Compounds 59 and 60 belong to the type of lanostane triterpene. In addition to having the basic structural characters of lanostane, they bear an extra tri-atomic ring in their structures that are formed by cyclization between methyl at C-18 and methine at C-9. Lanostane triterpenes in soy were believed to be the original materials for the biosynthesis of soy sterols. Bacchara-12,21-dien-3 $\beta$ -ol (61) is a natural occurring of baccharane-type triterpene which has all six-membered tetracyclic skeleton, and its side chain is at position C-17. This type of triterpene is extremely rare in nature and no more than 20 compounds of this type have been separated and identified.

Cholesterol and thirteen so-called plant sterols or phytosterols were found in soybean seed or the shoots (111) (Figure 6). Unlike animals, plant membranes generally contain little or no cholesterol and instead contain several types of phytosterols. Phytosterols are also steroid alcohols, whose chemical structures are similar to that of cholesterol, but with the presence of one or two carbon, saturated or unsaturated, substituents in side chains at C-24 differing from that of cholesterol (62) (Figure 6) (26, 111). The most abundant phytosterols are sitosterol, campesterol and stigmasterol (112). Phytosterols identified from soy so far include cholesta-5,24-dien-3 $\beta$ -ol (63),  $\beta$ -sitosterol (64) (113), stigmasterin (65) (24), sitostanol (66) (114), citrostadienol (67) (113), isofucosterol (68) (111), campesterol (69) (115), 24-epicampesterol (70) (115), 7-dehydrocampesterol (71) (115), campestanol (72) (111), obtusifoliol (73) (111), 24-methylenelophenol (74) (111), and 14 $\alpha$ -methyl-5 $\alpha$ -ergost-8-en-3 $\beta$ -ol (75) (111). The most abundant soy phytosterol is  $\beta$ -sitosterol (64), followed by campesterol (69) (112). These sterols can be divided into four groups based on their backbones, cholesterol (62), cholesta (63), stigmasta (64-68) and ergosta (69-75) (Figure 6). Compounds 62 and 63 have the same backbone except for the type of bond between C-24 and C-25. The former is single bond while the latter is double bond (Figure 6). Stigmasta and ergosta also share similar structures with the only difference for stigmasta being that it has one more methyl group at C-28 (Figure 6). However, nutritionists prefer to divide the phytosterols into two categories of " $\Delta^5$ -sterols", indicating a double bond at position C-5, and "stanol", indicating 5 $\alpha$ -reduction of that double bond (114). According to this principle, compounds 62-65 and 68-71 are  $\Delta^5$ -sterols; 66-67 and 72-75 are stanols (Figure 6).

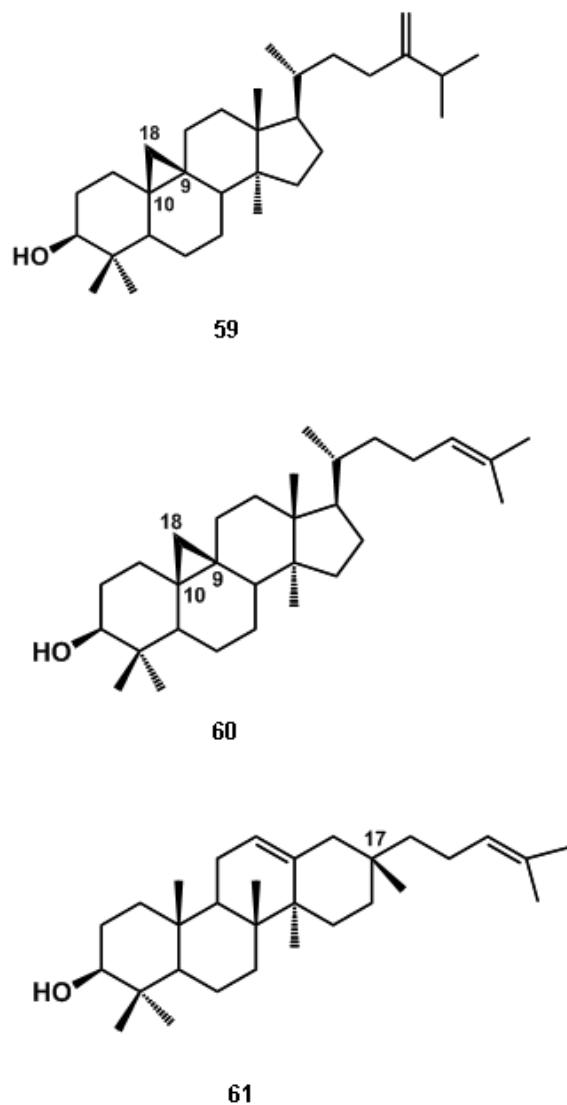
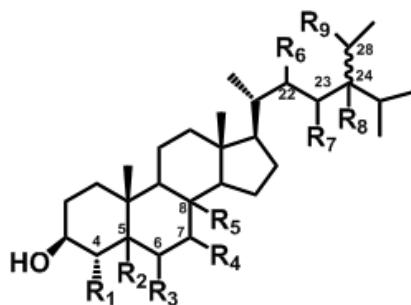
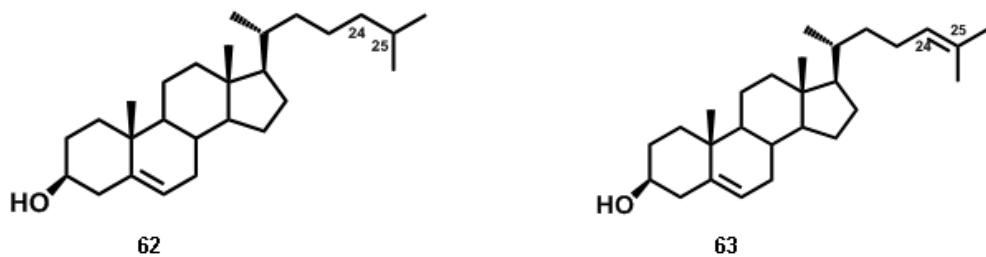


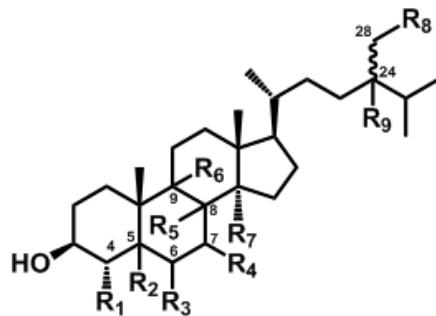
Fig. 5. The chemical structures of triterpenes in soy.

### 2.3.2 Health effects of phytosterols

Phytosterols (not restricted to soy-based sources) that have long been known to reduce intestinal cholesterol absorption, lead to decreased blood LDL-cholesterol levels and lower cardiovascular disease risk. However, other biological activities for phytosterols have also been reported, including anti-cancer and immune modulatory effects.



64.  $R_1 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9 = H$ ,  $R_2 = R_3 = \Delta^{5(6)}$  24(R)  
 65.  $R_1 = R_4 = R_5 = R_6 = R_8 = R_9 = H$ ,  $R_2 = R_3 = \Delta^{5(6)}$  24(S)  
 66.  $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9 = H$  24(R)  
 67.  $R_1 = CH_3$ ,  $R_2 = R_3 = R_6 = R_7 = H$ ,  $R_4 = R_5 = \Delta^{7(8)}$  24(R)  
 68.  $R_1 = R_4 = R_5 = R_6 = R_7 = H$ ,  $R_2 = R_3 = \Delta^{5(6)}$  24(S)



69.  $R_1 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9 = H$ ,  $R_2 = R_3 = \Delta^{5(6)}$  24(R)  
 70.  $R_1 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9 = H$ ,  $R_2 = R_3 = \Delta^{5(6)}$  24(S)  
 71.  $R_1 = R_6 = R_7 = R_8 = R_9 = H$ ,  $R_2 = R_3 = \Delta^{5(6)}$ ,  $R_4 = R_5 = \Delta^{7(8)}$  24(R)  
 72.  $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9 = H$  24(R)  
 73.  $R_1 = CH_3$ ,  $R_2 = R_3 = R_4 = H$ ,  $R_5 = R_6 = \Delta^{8(9)}$ ,  $R_7 = CH_3$ ,  $R_8 = R_9 = \Delta^{24(28)}$   
 74.  $R_1 = CH_3$ ,  $R_2 = R_3 = R_6 = R_7 = H$ ,  $R_4 = R_5 = \Delta^{7(8)}$ ,  $R_8 = R_9 = \Delta^{24(28)}$   
 75.  $R_1 = R_2 = R_3 = R_4 = R_8 = R_9 = H$ ,  $R_5 = R_6 = \Delta^{8(9)}$ ,  $R_7 = CH_3$  24(S)

Fig. 6. The chemical structures of sterols in soy.

### 2.3.2.1 Phytosterols and cholesterol absorption

In the 1950's, phytosterols from soybeans were found to lower serum cholesterol level (116). Since then, the cholesterol-lowering effects of phytosterols have been extensively demonstrated in both humans and animals (117-120). A meta-analysis of 41 clinical trials showed that intake of 2 g/d of stanols or sterols reduced low-density lipoprotein (LDL) by 10%, with little additional reduction at higher doses (121). The U.S. National Cholesterol Education Program has recommended adding 2.0 g/day of phytosterols to the diet of adults to reduce LDL cholesterol and coronary heart disease risk (122).

Because phytosterols are not systemically absorbed, they are thought to act primarily in the intestinal lumen. As cholesterol analogs phytosterols compete for cholesterol in absorptive micelles resulting in reduced solubility of cholesterol (118). However, recent evidence suggests that the mechanism of action of phytosterols may be more complicated than originally thought (119, 123). As summarized in a recent review (123), since phytosterols/phytostanols do not need to be present in the intestinal lumen simultaneously with cholesterol to inhibit its absorption (120), other studies have suggested that phytosterols/phytostanols may exert an unknown molecular action inside enterocytes and hepatocytes. In line with this, injected phytosterols/phytostanols reduced plasma cholesterol levels in hamsters (124). To gain insight into the phytosterol/phytostanol affects on circulating cholesterol concentration via mechanisms independent of the luminal incorporation of cholesterol into mixed micelles, the effects of these plant compounds on intestinal LXR-mediated targets involved in sterol absorption have been evaluated. Conclusive studies using ABCA1 and ABCG5/G8-deficient mice demonstrated that the phytosterol-mediated inhibition of intestinal cholesterol absorption is independent of these ATP-binding cassette (ABC) transporters. Other reports have raised questions as to whether phytosterols/phytostanols regulate cholesterol metabolism in intestinal and hepatic cells through independent-LXR pathways. A number of studies have proposed a phytosterol/phytostanol action on cholesterol esterification and lipoprotein assembly (ACAT, apoB), cholesterol internalisation (NPC1L1, ANXA2), cholesterol synthesis (HMG-CoA reductase, C-24-reductase) and removal of apoB100-containing lipoproteins (LDLr). However, the impact of phytosterol/phytostanol intake on these physiological processes *in vivo* remains unclear (123).

In one study specifically using soybean-derived phytosterols (125), it was found that consumption of phytosterol-supplemented ground beef lowered plasma TC and LDL-cholesterol concentrations and TC:HDL cholesterol from baseline by 9.3%, 14.6%, and 9.1%, respectively.

### 2.3.2.2 Anti-cancer effects of phytosterols

In addition to their cholesterol-lowering actions, mounting evidence suggests that phytosterols possess anti-cancer effects against a number of different types of cancers (126-132). Phytosterols seem to act through multiple mechanisms of action, including inhibition of carcinogen production, cancer-cell growth, angiogenesis, invasion and metastasis, and through the promotion of apoptosis of cancerous cells. Phytosterol consumption may also increase the activity of antioxidant enzymes and thereby reduce oxidative stress. In addition to altering cell-membrane structure and function, phytosterols probably promote apoptosis by lowering blood cholesterol levels (127).

### 2.3.2.3. Physterols and immune function

Several reports in the literature suggest that phytosterols may have some immunological activity as highlighted in animal models of inflammation or even in *in vitro* and *in vivo* models of cancer (colorectal and breast cancer). Their direct immune modulatory activity on human lymphocytes has been proven, and the mechanism of action in cancer cells has been elucidated (133, 134).

## 2.4 Lignans

### 2.4.1 Chemical characteristics of lignans

In addition to isoflavones, lignans are considered another main group of phytoestrogens in soy, based on their chemical structures. Lignans are defined as dimeric phenylpropanoid (C6-C3) compounds, mostly linked at 8-8' (**Figure 7**) (135, 136). They are seven lignans identified from soy, namely, anhydrosecoisolariciresinol (76), isolariciresinol (77), secoisolariciresinol (78), matairesinol (79), lariciresinol (80), pinoresinol (81) and syringaresinol (82) (**Figure 7**) (28, 137).

### 2.4.2 Health effects of lignans

#### 2.4.2.1 Antioxidant activity

Pinoresinol (81) has been reported frequently as an antioxidant, using thiocyanate antioxidant, Cu<sup>2+</sup>-induced low density lipoprotein oxidation, lipid peroxidation in rat liver, DPPH radical, and peroxy radical assays (138). Syringaresinol (82) has also been demonstrated as antioxidative in Cu<sup>2+</sup>-induced low density lipoprotein oxidation and DPPH radical assays (138). Lariciresinol (80) has a high radical scavenging capacity compared to the well-known antioxidant Trolox. The trapping capacity (mmoles peroxy radicals scavenged per gram of compound) of lariciresinol (80) (7.3 mmol/g) is higher than that of trolox (6.8 mmol/g) (139). Secoisolariciresinol (78) showed strong antioxidant activity against DPPH with an IC<sub>50</sub> of 0.017±0.001 mM. It is about two times stronger than the standard antioxidant, 2,6-di-(*tert*-butyl)-4-methylphenol (BHT), IC<sub>50</sub> 0.031±0.001 mM (140). Isolariciresinol (77) showed 86.2 % inhibition of lipid peroxidation (LPO) at 25 µg/ml (141).

#### 2.4.2.2 Anticancer activity

Estrogenic enterolignans, END or ENL are the major metabolites of lignans in the mammalian gut. Because estrogens were linked to some cancers, especially breast cancer, enterolignans could affect some cancer risk. To our knowledge, the estrogenic or anti-estrogenic effects of enterolignans in humans are not very clear (142). For example, while weak estrogenic activity of ENL was demonstrated the anti-estrogenic activity of ENL was disclosed through depression of estrogen-stimulated rat uterine RNA synthesis (143).

The lignan ENL was found to have a bi-phasic effect on DNA synthesis in human breast cancer MCF-7 cells, showing induction at 10-50 µM and inhibition at higher concentrations, with an IC<sub>50</sub> of 82.0 µM (144). END and ENL are weak and moderate inhibitors of aromatase enzyme activity in a pre-adipose cell culture system (143). In human colon tumor cell

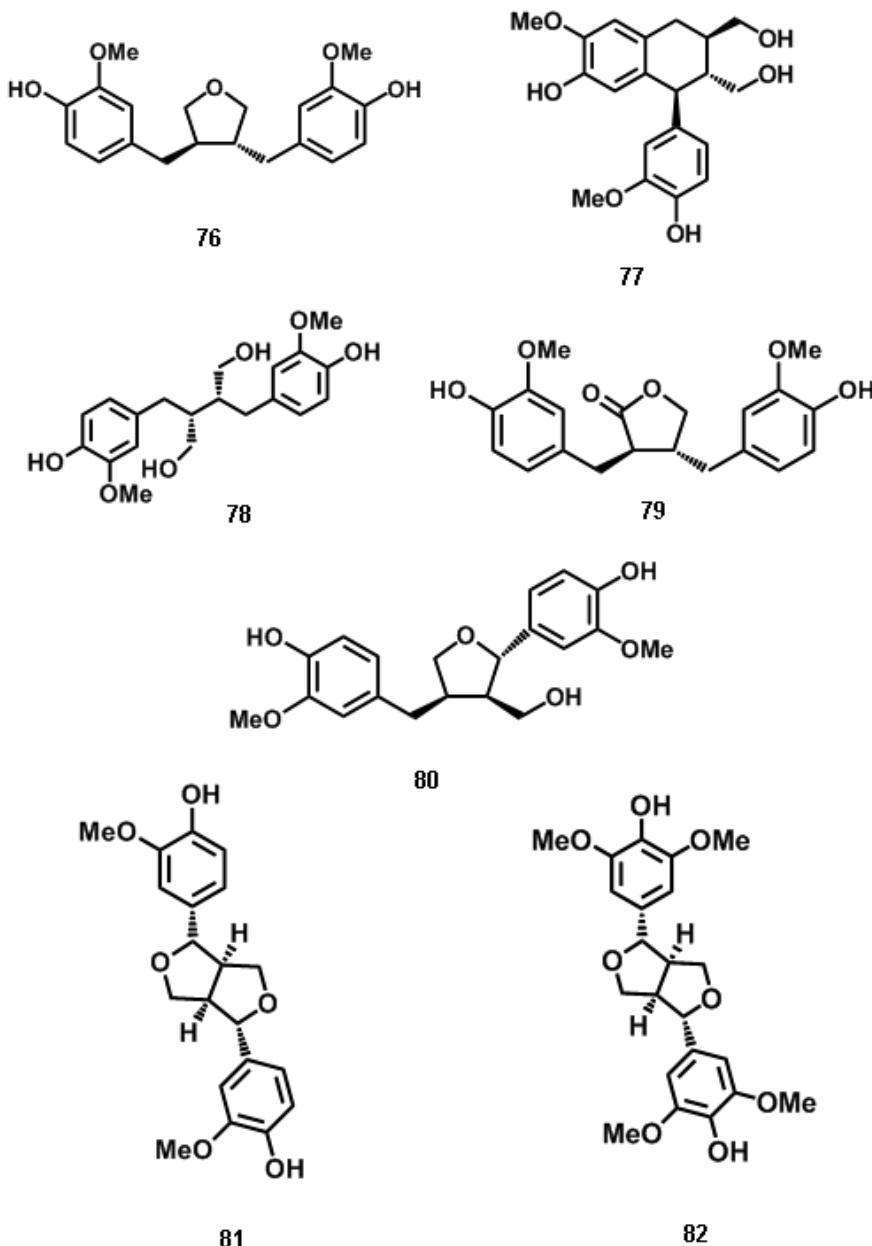


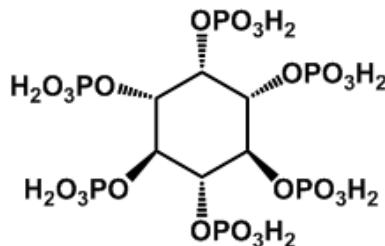
Fig. 7. The chemical structures of lignans in soy.

lines (LS174T, Caco-2, HCT-15, T-84), the same concentration of END and ENL ( $100 \mu\text{M}$ ) significantly reduce the proliferation of all cell lines, though ENL was more than twice as effective as END (145).

## 2.5 Phytate

### 2.5.1 Chemical characteristics of phytate

Phytate (83), the salt of phytic acid (myo-inositol-(1,2,3,4,5,6)hexakisphosphate, IP-6, InsP-6) (Figure 8), is a naturally occurring polyphosphorylated carbohydrate. It is widely distributed in the plant kingdom. Phytate serves as a storage form of phosphorous and minerals and contains ~75% of total phosphorous of the kernels (25). It is the major source of phosphorus in soy (79). Phytate is considered as a strong chelator of important minerals such as calcium, magnesium, iron, and zinc, and can contribute to mineral deficiencies in people. However, recent studies demonstrate that this “antinutrient” effect of IP6 is only manifested when large quantities of IP6 are consumed in combination with an oligoelements-poor diet (146).



83

Fig. 8. The chemical structures of phytic acid in soy.

### 2.5.2 Health effects of phytic acid

#### 2.5.2.1 Anti-cancer activities

Phytic acid (83) plays an important role in signal transduction, cell proliferation and differentiation (147). Recently phytic acid (83) has received much attention for its role in cancer prevention and control of experimental tumor growth, progression, and metastasis (148). A great majority of the studies were done in animals and showed that phytic acid had anti-neoplastic properties in breast, colon, liver, leukemia, prostate, sarcomas, and skin cancer (149, 150). The results and proposed mechanism of anti-carcinogenic activities of IP6 in various cell lines are summarized in Table 6 (151-154).

In animal studies, phytic acid can increase blood NK cell activity in DMH-induced colon tumors in rats, suppress rhabdomyosarcoma cell growth in a xeno-grafted nude mouse model (155), inhibit tumor growth and metastasis in rats (156), prolong survival of tumor-bearing mice, and reduce the numbers of pulmonary metastases (157). Phytic acid can also inhibit DMBA-induced mouse skin tumor development and this inhibitory effect is likely, by modulating proliferation, differentiation, or apoptosis (158).

#### 2.5.2.2 Other health effects

For a long time IP6 has been recognized as a natural antioxidant. In addition, IP6 possesses other significant benefits for human health, such as the ability to enhance the immune

cell line	dose	observation	mechanism	ref
Human Caco-2 colon cancer cell	Decrease the expression of TNF- $\alpha$ and TNFII in a dose dependent manner (1, 2.5 and 5 mM) after 12 h.	Decrease the expression of TNF- $\alpha$ and TNFII in Caco-2 cells.	Regulate cytokine production including TNF- $\alpha$ , to modulate immune response and cell death activation.	147
Human HT-29 colon cancer	Inhibit proliferation of HT-29 cells at 8 mmol/L and 13 mmol/L after 12 h.	Inhibit proliferation of HT-29 cells	Affect special cell cycle regulators, reduce over-expression of mutant P53, and prevent PCNA-dependent cellular proliferation.	154
Human MCF-7/Adr MDA-MB 231 and MCF-7 breast cancer cells	MCF-7/Adr - IC <sub>50</sub> 1.26 mM MDA-MB 231 - IC <sub>50</sub> 1.32 mM MCF-7 - IC <sub>50</sub> 4.18 mM after 96 h.	Inhibit the growth of three breast cancer cells	Change cell cycle distribution	153
Human HepG2 liver hepatoma cell	Inhibit the growth of HepG2 liver hepatoma cell in a dose-dependent fashion (0.25-5 mM) after 6 days.	Inhibit the growth of HepG2 liver hepatoma cell	Modulate cell signal transduction pathways	155
Human LNCaP prostate cancer cell	Inhibit the growth of LNCaP prostate cancer cell dose dependently (0.5-4 mM) after 24 h.	Inhibit the growth of LNCaP prostate cancer cell	Inhibit Akt activation, cause CDKI accumulation and induce LNCaP cell cycle arrest.	151
Human DU145 prostate cancer cell	Inhibit the growth of DU145 prostate cancer cell dose dependently (0.25-2 mM) after 24 h.	Inhibit the growth of DU145 prostate cancer cell	Induce G1 arrest in DU145 cell cycle progression.	152

Table 6. The anti-carcinogenic activities of IP

system, prevent pathological calcification and kidney stone formation, lower elevated serum cholesterol, and to reduce pathological platelet activity (148). IP6 inhibited the replication of HIV-1 in a T cell line and in a freshly isolated strain in peripheral blood mononuclear cells, possibly acting on HIV-1 early replicative stage (149)

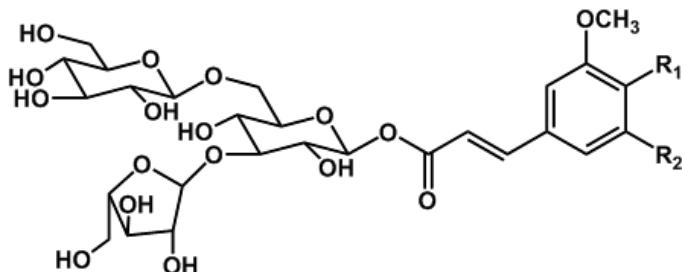
84. R<sub>1</sub> = OH, R<sub>2</sub> = H85. R<sub>1</sub> = OCH<sub>3</sub>, R<sub>2</sub> = OCH<sub>3</sub>

Fig. 9. The chemical structures of cinnamic acid ester glycosides in soy.

## 2.6 Cinnamic acid ester glycosides

### 2.6.1 Chemical characteristics of cinnamic acid ester glycosides

Two cinnamic acid ester glycosides were isolated by Hosny et al from soybean molasses, which is a by-product of aqueous alcohol soy protein concentrate production (70). They were identified as 1-O-(E)-feruloyl[ $\alpha$ -L-arabinofuranosyl-(1 $\rightarrow$ 3)][ $\beta$ -D-glucopyranosyl(1 $\rightarrow$ 6)] $\beta$ -D-glucopyranose (84) and 1-O-(E)-3,4,5-trimethoxycinnamoyl[ $\alpha$ -L-arabinofuranosyl(1 $\rightarrow$ 3)][glucopyranosyl(1 $\rightarrow$ 6)] $\beta$ -D-glucopyranose (85) (Figure 9).

## 3. Constituents formed during processing

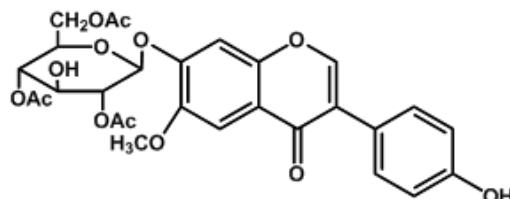
Food processing may dramatically change the compositions and relative contents of the constituents in soy products and artificial compounds may also occur (159). The processing of foods can improve nutrition, quality, and safety; though processing could also lead to the formation of anti-nutritional and toxic compounds. These multi-faceted consequences of food processing result from molecular interactions among nutrients and with other food ingredients, both natural and added (160).

Some components isolated from soy products such as soy sauce and fermented products do not occur naturally in the soybean. And the types of these compounds vary among different soy products based upon processing methods. These compounds may be formed by 1) soybean processing or fermentation; 2) compounds from other ingredients of soy products; 3) compounds from reactions of components in soybean and/or other ingredients during processing. Even though these compounds are not found naturally in soybean, they do exist widely in various soy products and may contribute to important beneficial or detrimental biological effects associated with soy consumption. Unfortunately, the key role of these compounds is largely ignored.

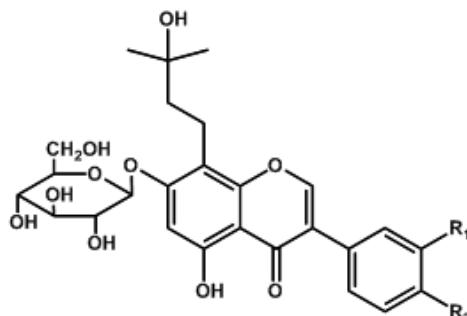
In Japan, soybeans molasses and soy fermented with *Bacillus subtilis* (*natto*) are very popular daily foodstuffs (161). Six isoflavones which differ from that isolated from soy were generated by this process. They are glycinein 7-O- $\beta$ -D-(2'',4'',6''-O-triacetyl)glucopyranoside (86), 8-( $\gamma$ -hydroxy- $\gamma,\gamma$ -dimethylpropyl)genistin (87), 5-hydroxy-8-( $\gamma$ -hydroxy- $\gamma,\gamma$ -dimethylpropyl)-3',4'-dimethoxyisoflavone-7-O- $\beta$ -D-glucopyranoside (88), 6''-O-succinylaidzin (89), 6''-O-succinylgenistin (90), and 6''-O-succinylglycitin (91) (161, 162) (Figure 10).

Another group of well-known compounds formed during food processing in soy is The Maillard reaction products (163, 164). The Maillard reaction is the heat-induced reaction of amino groups of amino acids, peptides, and proteins with carbonyl groups of reducing sugars such as glucose, that results in the concurrent formation of so-called Maillard browning products and acrylamide (165). In the past, extensive work has been done on Maillard reaction products in food products including several excellent reviews (160, 164-167). Five Maillard reaction products, fructose-lysine (92), fructose-alanine (93), fructose-valine (94), fructose-leucine (95) and fructose-isoleucine (96) (Figure 11) were isolated from soy sauce and miso (168, 169).

Other groups of compounds may also be found in different soy products. Guided by platelet aggregation analysis, two anti-platelet alkaloids, 1-methyl-1,2,3,4-tetrahydro- $\beta$ -carboline (97) and 1-methyl- $\beta$ -carboline (98), were obtained from soy sauce. These two compounds inhibited the maximal aggregation response induced by epinephrine, platelet-activating

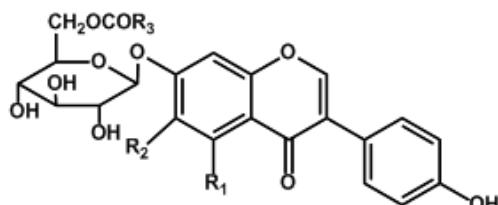


86. glycictein 7-O- $\beta$ -D-(2'',4'',6''-O-triacetyl)glucopyranoside



87. R<sub>1</sub>= H, R<sub>2</sub>= OH 8-( $\gamma$ -hydroxy- $\gamma$ , $\gamma$ -dimethylpropyl)genistin

88. R<sub>1</sub>= R<sub>2</sub>= OCH<sub>3</sub> 5-hydroxy-8-( $\gamma$ -hydroxy- $\gamma$ , $\gamma$ -dimethylpropyl)-3',4'-dimethoxysflavone-7-O- $\beta$ -D-glucopyranoside



89. R<sub>1</sub>= R<sub>2</sub>= H, R<sub>3</sub>= CH<sub>2</sub>CH<sub>2</sub>COOH 6''-O-succinyl daidzin

90. R<sub>1</sub>= OH, R<sub>2</sub>=H, R<sub>3</sub>= CH<sub>2</sub>CH<sub>2</sub>COOH 6''-O-succinyl genistin

91. R<sub>1</sub>= H, R<sub>2</sub>= OCH<sub>3</sub>, R<sub>3</sub>= CH<sub>2</sub>CH<sub>2</sub>COOH 6''-O-succinyl glycitin

Fig. 10. The chemical structures of new isoflavones generated during food processing in soy products.

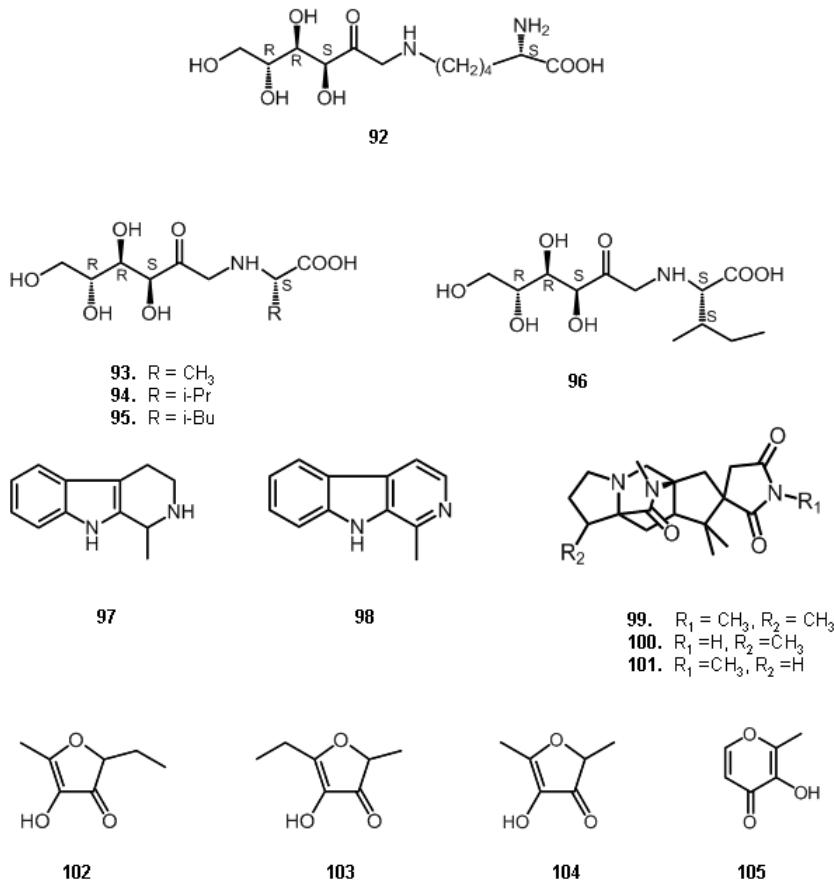


Fig. 11. The chemical structures of other components generated during food processing in soy products.

factor, collagen, adenosine 5'-diphosphate, and thrombin, respectively (170). Compound **97** had much greater inhibitory effect than that of **98** on platelet aggregation. Three other alkaloids, asperparalines A (**99**), B (**100**) and C (**101**) were found from okara (the insoluble residue of whole soybean) fermented with *Aspergillus japonicus* JV-23 (Figure 11) (171). Since soybean does not contain alkaloids, these compounds were likely brought into the soy products from other sources.

A series of aromatic compounds were isolated from soy sauce and miso (a traditional Japanese seasoning produced by fermented soybeans) (172-174). Their structures were identified as 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone (HEMF; **102**), 4-hydroxy-5-ethyl-2-methyl-3(2H)-furanone (**103**), 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF; **104**) and 3-hydroxy-2-methyl-4H-pyran-4-one (maltol; **105**) (Figure 11). They are considered to form the base of the sweet aroma of miso. All of these compounds were most likely generated during soy processing and/or fermentation.

Processing may also significantly elevate or reduce certain naturally occurring soybean components. For instance, a remarkable increase in folate content was found after fermentation, 5.2-fold and 1.7-fold higher than that of the boiled soybeans and soybean seeds, respectively (175). Thus, we must be aware that the composition of soybean products may differ substantially from the native soybean.

#### 4. Conclusion

The soy bean has been used as a human food source of high quality protein and other nutrients for hundreds of years and is currently a major source of protein in commercial food products for the beef, pork and chicken industry. This chapter summarizes our current knowledge about chemical structures and health effects of the major phytochemicals in soy. Clearly, the observed health effects from soy or soy-based foods (other than those attributed to nutrients like protein) are not solely from the actions of certain individual or types of compounds, but rather are due to the mixed effects of different compounds. Additive, synergistic, and/or antagonistic effects of different soy components combine to provide a final effect of soy foods and these effects may also be altered by phytochemicals from other non-soy foods in the meal. In order to fully understand the mechanism of health effects of soy, it is important to identify the genuine bioactive compounds in soy.

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# Sarcodon Mushrooms: Biologically Active Metabolites

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## 1. Introduction

Thelephorales Corn ex. Oberw. belong to the Agaricomycetes (Basidiomycota, Fungi) and comprise more than 177 species (Kirk *et al.*, 2008) that are classified into two families: the *Bankeraceae* Donk (mostly with colourless basidiospores) and the *Thelephoraceae* Chevall. (mostly with brown to yellowish basidiospores) (Yorou & Agerer, 2007). Bankeraceae are characterized by a typical fenugreek odour, with pileate and stipitate fruit bodies, hydnoid to spinose hymenophores with brown and lobed as well as colourless and evenly ornamented basidiospores. Bankeraceae comprise 5 distinct genera: *Bankera* Coker & Beers ex Pouzar, *Hydnellum* P. Karsten, *Phelodon* P. Karsten, *Sarcodon* Quél. ex P. Karst and *Boletopsis* Fayod.

The name *Sarcodon* was proposed by Quélet in 1878, but as any binomial was formed, the name was established as a genus only later by Karsten (Banker, 1913).

The name, derived from the ancient greek stems *sarco*= flesh and *odon*=tooth, is due to the presence of the spines that look like teeth on the hymenophore. For this reason *Sarcodon* species are colloquial called “tooth fungi” (Fig. 1).

The genus comprises more than 72 species, most of which are not edible, due to the bitter taste. Among these species only few have been phytochemically and biologically studied: *S. aspratus* (syn *S. imbricatus*), *S. cyrneus*, *S. glaucopus*, *S. leucopus*, *S. laevigatum* (syn. *S. laevigatus*) and *S. scabrosus*.

Phytochemical studies were aimed to study either the composition of different extracts or to isolate new metabolites and to evaluate biological activity of extracts and isolated compounds.

## 2. Phytochemical studies on *Sarcodon* spp

### 2.1 *Sarcodon aspratus* syn *S. imbricatus*

*Sarcodon imbricatus* (L.) P. Karst. is the current name used for *S. aspratus* (Berk.) even though several synonyms are used for this mushroom (see Index Fungorum: [www.indexfungorum.org](http://www.indexfungorum.org)). It is commonly known as the *shingled hedgehog* or *scaly hedgehog*. The mushroom has a large, brownish cap with large brown scales and may reach 30 cm in diameter. On the underside it sports greyish brittle teeth instead of gills, and has white flesh.



Fig. 1. *Sarcodon imbricatus* (L.: Fr.) Karsten hymenophore

The fungus can be bitter, although this is less apparent in younger specimens. Submerging the mushrooms in boiling water will remove this. It can be pickled or dried and used as flavouring. In Bulgaria it is collected, dried and finely ground to be used as an aromatic mushroom flour. It is reported as edible, but of poor quality in the United States by some sources but as deliciously edible by others. In China *Sarcodon aspratus*, also known as “*black tiger's paw*”, is a popular natural edible mushroom. It is used for lowering cholesterol level, muscles relaxation and blood circulation. Its special musky aroma enhances the taste in meat's dishes, especially hams (S.K. Kim, 2006), and particularly brings out the sweet flavour when making a clear soup out of it.

Several papers have been published regarding the use of *S. aspratus* for manufacturing foods and beverages (e.g. Hwang & Na, 2009a, 2009b; B.C. Kim *et al.*, 2008, 2009; Jang, 2008; Wang & Wang, 2008; J.K. Kim, 2007).

### 2.1.1 Phytochemistry of *Sarcodon imbricatus*

In view of its use as a food, the phytochemical composition of *S. imbricatus* has been studied.

Proximate constituents (moisture, fat, crude protein, ash and carbohydrates) of this mushroom have been reported (Barros *et al.*, 2007a). On the basis of the proximate analysis, it can be calculated that a portion of 100 g of this mushroom provides, on average, 24 kcal (100 kJ). The analysis of fatty acid composition allowed to determine 15 fatty acids.

Unsaturated fatty acids (MUFA, monounsaturated fatty acids, 46%, PUFA, polyunsaturated fatty acids, 36%) were predominant over SFA (saturated fatty acids, 18%). Methanolic extract of *S. imbricatus* showed the presence (0.5%) of total polyphenols, expressed as g of gallic acid /100 g dry weight that justify the radical scavenging activity (DPPH) (Marcotullio *et al.*, 2008). On the other hand the amounts of ascorbic acid,  $\beta$ -carotene and lycopene found in the mushroom were very low (Barros *et al.*, 2007b). Sterol fraction was



Fig. 2. *Sarcodon imbricatus* (L.: Fr.) Karsten

studied by several research groups. In addition to ergosterol, several other ergostane and cholestanol compounds have been isolated (Huang et al., 2002; Ueno et al., 1999; Marcotullio et al., 2008). Particularly interesting is the presence in the methanolic extract of *S. imbricatus* of ergosterol peroxide (or 3- $\beta$ -hydroxy-5 $\alpha$ ,8 $\alpha$ -epidioxyergosta-6,22-diene) that shows a plethora of biological activities, such as antileukemic and anticancer (Kobori et al., 2006), apoptotic-inducing (Takei et al., 2005), and anti-inflammatory (Kobori et al., 2007). It is interesting to note that processing and cooking practices determine a lowering of nutrients concentrations and of antioxidant activity (Barros et al., 2007c).

Other interesting metabolites isolated and identified from *S. aspratus* are polysaccharides. Han and coworkers isolated a water soluble polysaccharide (HCP) with molecular mass of  $6.7 \times 10^5$  Da (Han et al., 2011). HCP resulted to be a linear glucan with a backbone structure of (1 $\rightarrow$ 6)-linked- $\alpha$ -glucopyranosyl residues. Another polysaccharide (HBP) with a molecular weight of  $4.3 \times 10^5$  Da was isolated by the same author in 2010. Even in this case the backbone structure was constituted by (1 $\rightarrow$ 6)-linked- $\alpha$ -glucopyranosyl residues, which occasionally branched at O-3 position (Han et al., 2010).

Other constituents isolated from *S. aspratus* were ceramide compounds (Yaoita et al., 2002).

## 2.2 *Sarcodon cyrneus* Maas Gest

*Sarcodon cyrneus*, according Myco Bank ([www.mycobank.org](http://www.mycobank.org)), has a "pileus up to 65 mm across, planoconvex to somewhat depressed in centre, finely tomentose at margin, becoming felted farther back, or tomentum collapsed to form smooth and shiny pellicle. Stipe 15-30 x 6-15 mm, broader when fused, equal or somewhat enlarged below, with abruptly pointed base, straight to curved, tomentose, in places glabrescent, pale grey-brown, darkening with age and becoming more or less concolorous with, pileus, at extreme base with yellowish grey mycelium. Spines up to 3 mm long, 0.1-0.3 mm broad, long decurrent, often almost reaching base of stipe, crowded, subulate, first whitish, becoming purplish brown. Context pallid (whitish suffused with pinkish when fresh, according to the collector), not greenish in base of stipe" (Fig. 3). The taste is bitter and the mushroom is not edible.



Fig. 3. *Sarcodon cyrneus* Maas Geest.

#### 2.2.1 Phytochemistry of *Sarcodon cyrneus*

In the literature only two papers regarding the secondary metabolites isolated from *S. cyrneus* have been reported (Marcotullio *et al.*, 2006b; Marcotullio *et al.*, 2007) and they deals with the structural identification of five cyathane diterpenes. These compounds were named cyrneine A-D (1-4) (Fig. 4) and glaucopine C (7) (Fig. 6).

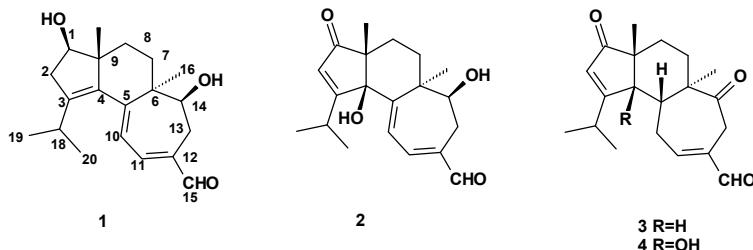


Fig. 4. Structures of cyrneines

#### 2.3 *Sarcodon glaucopus* Maas Geest. & Nannf.

According MycoBank ([www.mycobank.org](http://www.mycobank.org)) the mushroom has a “pileus up to 110 mm across, plano-convex to somewhat depressed; at first tomentose, then matted, forming a cuticle which breaks up into scales near margin, into areoles in centre; scales adhering to appressed, yellow-brown with vinaceous shade or pale to dark purplish brown, occasionally locally violet-grey (giving a peculiar leaden grey impression), at times very dark brown in centre, contrasting with dingy yellowish ground colour, not infrequently covered with minute yellowish dots of excreted matter when dried. Stipe 27-75 x 10-40 mm, cylindrical, tapering below or somewhat broadened below, tomentose, fibrillose, covered with adnate fibrillose squamules or partly matted, dingy whitish, soon pinkish brown to purplish brown above, grey-green below, with pointed, whitish base. Spines up to 5 mm long, 0.1-0.2 mm broad, whitish, finally purplish brown” (Fig. 5). The taste is bitter and the mushroom is not edible.



Fig. 5. *Sarcodon glaucopus* Maas Geest. & Nannf.

### 2.3.1 Phytochemistry of *Sarcodon galucopus*

No phytochemical studies regarding proximate composition have been reported for *S. glaucopus*. In the literature there are only two studies on the secondary metabolites of *S. glaucopus*, and they deal with the isolation and structure elucidation of three cyathane diterpenes named glaucopine A (5), glaucopine B (6) (Curini *et al.*, 2005) and glaucopine C (7) (Marcotullio *et al.*, 2006a) (Fig. 6).

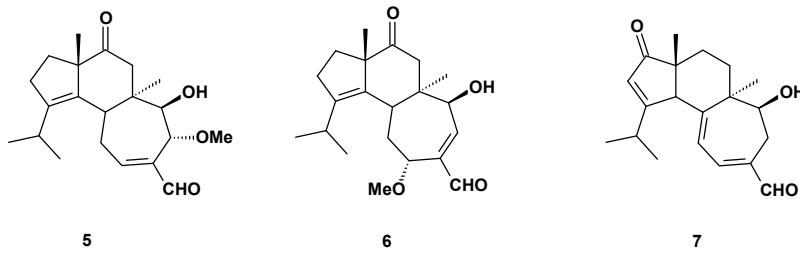


Fig. 6. Structures of glaucopines

### 2.4 *Sarcodon laevigatus* (Sw.) P. Karst.

Index Fungorum ([www.indexfungorum.org](http://www.indexfungorum.org)) reports for *S. laevigatus* (syn. *S. leucopus*, often the term *S. laevigatum* is used instead of *S. laevigatus*). A description of *Sarcodon leucopus* (Pers.) Maas Geest. & Nannf. has been reported in MycoBank: "Pileus up to ca. 200 mm across, planoconvex or slightly depressed, without concentric or radiate markings, at first finely felted; felt collapsed to form smooth, more or less shiny, innate-scaly cuticle, the latter subsequently radiately rimose near margin, breaking up into areoles in centre, here with scales somewhat more pronounced and tips sometimes slightly raised; pale purplish brown

on yellowish drab ground colour or a rich purplish brown to dark brown. Stipe 40-80 x 20-60 mm, cylindrical to ventricose, finely tomentose, later with smooth or innate-scaly cuticle, concolorous with pileus or paler, whitish below, after some time with green spots (always?). Spines up to c. 15 x 1 mm, whitish, finally purplish brown. Context up to 40 mm thick near centre of pileus, whitish, suffused with purplish brown to violet tints, after some time pale greenish. Odour commonly experienced as disagreeable. Taste bitter after some time".

#### 2.4.1 Phytochemistry of *Sarcodon laevigatus* or/and *S. leucopus*

In the literature no references to *S. laevigatus* have been reported, while it is possible to find two papers referring to *S. laevigatum* and to *S. laevigetum*. The first one reports the isolation and the structure identification of three p-terphenyls (**8-10**), of which **8** (named sarcodon) was a new compound (Ma *et al.*, 2006). The second report is a Patent and deals with the preparation of compound **9** (named B1-V) by extraction from *S. laevigetum* (Ma, 2009).

On the other hand, Tringali and coworkers (Tringali *et al.*, 1987) reported the isolation and structure identification of several p-terphenyls from *Sarcodon leucopus*.

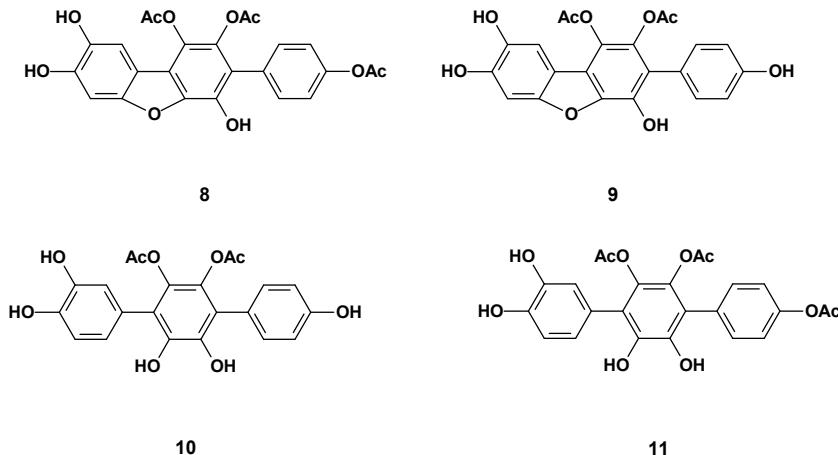
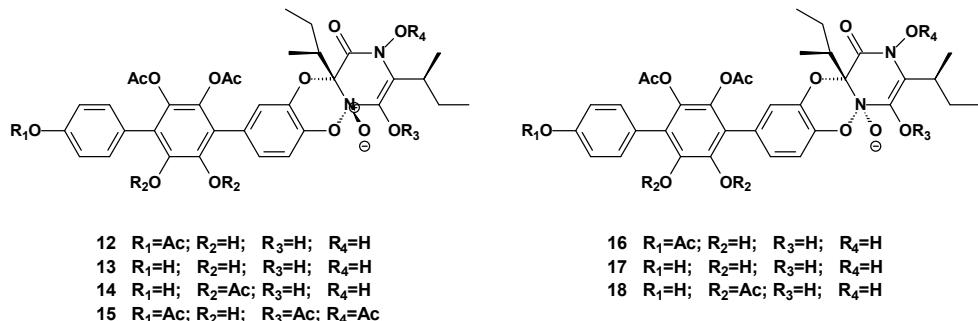
The first two isolated compounds were **10** and **11**. (Fig. 8)



Fig. 7. *Sarcodon leucopus* (Pers.) Maas Geest. & Nannf.

It is evident from these reports how the correct identification of the species is important for preventing mistakes in the identification of new secondary metabolites.

Later, the same research group isolated other nitrogen-containing p-terphenyl derivatives that they named **sarcodonins**. The first to be isolated was **12** in 2000 (Geraci *et al.*, 2000) and other six related compounds (**13-18**) were isolated in 2004 (Cali *et al.*, 2004) (Fig. 9).

Fig. 8. Structures of *p*-terphenyls isolated from *S. laevigatum*Fig. 9. Structures of sarcodonins isolated from *S. leucopus*

Compounds **13-15** were named sarcodonin  $\alpha$ ,  $\beta$ , and  $\gamma$ , respectively, while compounds **16-18** *epi*-sarcodonin, *epi*-sarcodonin  $\alpha$  and  $\beta$ , respectively. Cali *et al.*, (2004) from the same mushroom isolated oxidated sarcodonins and named them sarcoviolin (**19**) and *epi*-sarcoviolin (**20**) (Fig. 10).

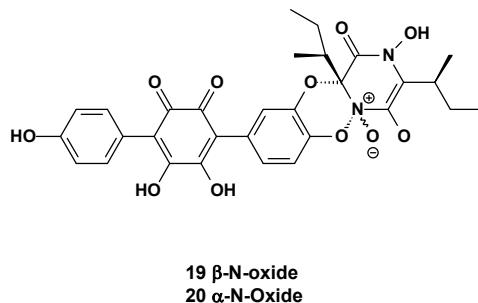


Fig. 10. Structures of sarcoviolins

Recently the structures of sarcodonins and sarcoviolins have been revised by synthesis, and the new structure for sarcodonin **12** is reported in Fig. 11 (Lin *et al.*, 2011).

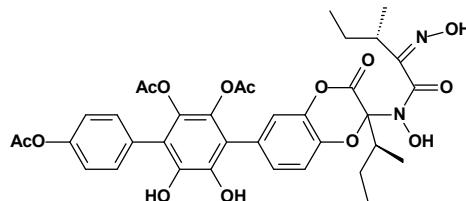


Fig. 11. Revised structure proposed for sarcodonin **12**

It is very interesting to notice that screening the literature with the term “sarcodonin” different structures with the same name have been reported, as we will see checking the compounds isolated from *S. scabrosus*.

## 2.5 *Sarcodon scabrosus* (Fr.) P. Karst.

Myco Bank describes *S. scabrosus* “with pileus up to 120 mm across (fresh), plano-convex, more or less deeply depressed in the centre, coarsely scaly, the scales erect in the centre, decumbent farther outwards, adnate and woolly near the margin, brown in various shades (brick-colour, fulvous, ferruginous, bay) on a fairly pale yellow-brown ground, in some specimens passing into a delicate lilac at the margin, with age becoming very dark purplish brown, and the scale-tips even blackish, somewhat shiny when dried. Stipe 20–120 x 10–60 mm (fresh), tapering downwards, usually with pointed base, felted to subfibrillose, pinkish brown to brick-colour, becoming concolorous with the pileus, the lower part or the base grey-green, when young covered with white mycelium. Spines up to 5 mm long (dry), slender (up to 0.3 mm), decurrent, crowded, subulate, long remaining yellowish brown, finally purplish brown. Context dingy whitish in the pileus and the top of the stipe, vinescent, brownish-marbled with age, grey-green in the base of the stipe. Greenish mycelium from the base of the stipe staining dingy pinkish brown to reddish brown in KOH solution. Odour of water-melon (*Citrullus vulgaris*) when cut fresh” (Fig. 12).

Due to its bitter taste it is not edible.

### 2.5.1 Phytochemistry of *Sarcodon scabrosus*

An analysis of the literature revealed that *Sarcodon scabrosus* (syn *Hydnnum scabrosus*), together with *S. imbricatus*, is one of the most studied species among *Sarcodon*. Two classes of secondary metabolites have been isolated from this species, and both are represented by cyathane diterpenes: scabronines and sarcodonins. The use of the same name for different classes of secondary metabolites clearly shows how the use of common names can give rise to mistakes.

The first report about the isolation of cyathane diterpenes from *S. scabrosus* was by Shibata *et al.* (1989) and it is about sarcodonin A (**21**) and G (**22**) (Fig. 13).



Fig. 12. *Sarcodon scabrosus* (Fr.) P. Karst.

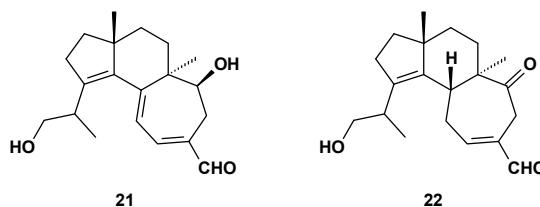


Fig. 13. Sarcodonins A and G

Generally sarcodonins and neosarcodonins isolated from *S. scabrosus* are characterized by the presence of a hydroxyl-methyl group in C-19 (Kryczkowski *et al.*, 2008).

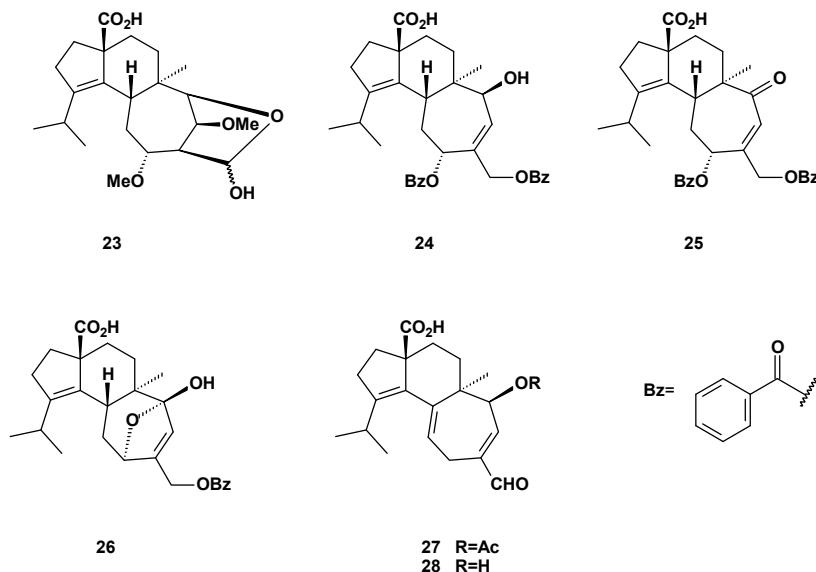


Fig. 14. Isolated scabronines

Scabronines are characterized by the oxidation of C-17 to carboxylic group. The first scabronine that was isolated was scabronine A (**23**) (Ohta *et al.*, 1998a) and later scabronines B-F (**24-28**) were isolated (Kita *et al.*, 1998) (Fig. 14).

Among different cyathane diterpenes, the most interesting for its biological properties is scabronine G (**29**) (Fig. 15), that has been isolated in 1998 (Ohta *et al.*, 1998b). In 2004, Ma and coworkers reported the isolation of another *scabronine G* (**30**), but this compound is structurally related to sarcodonins (Ma *et al.*, 2004).

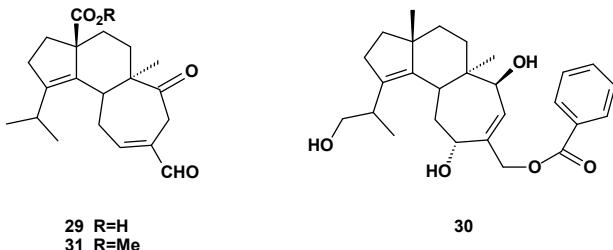


Fig. 15. Structures of the two scabronines G

Ma *et al.* isolated other scabronines (H-J), but they still have sarcodonins structures (Ma *et al.*, 2004; Ma *et al.*, 2008).

From *S. scabrosus* in addition to the already mentioned sarcodonins A (**21**) and G (**22**) in 2002 other three cyathane diterpenoids have been isolated (Hirota *et al.*, 2002). Three of them were named neosarcodonins A-C (**31-33**). The same group in 2004 isolated neosarcodonin O (**34**) and three acyl derivatives of sarcodonin A (**35-37**) (Kamo *et al.*, 2004) (Fig. 16).

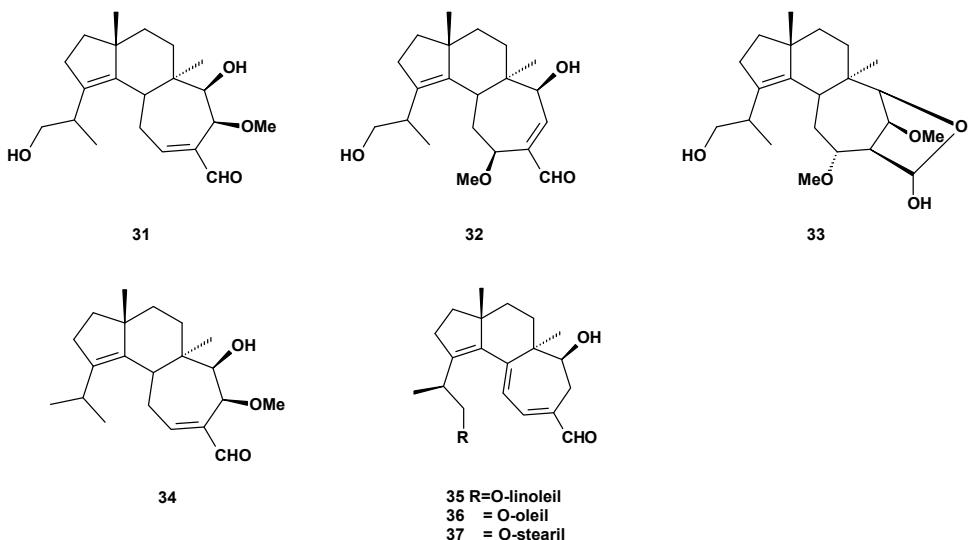


Fig. 16. Neosarcodonins isolated from *S. scabrosus*

### 3. Biological activities of *Sarcodon* metabolites

#### 3.1 Biological activities of p-terphenyl derivatives

Terphenyls are aromatic hydrocarbons consisting of a chain of three benzene rings. From a structural point of view three different isomers are possible, in which the terminal rings are *ortho*-, *meta*-, or *para*-substituents of the central ring. Most of the natural terphenyls are *p*-terphenyl derivatives. In recent years, some terphenyls have been reported to exhibit significant biological activity, *e.g.*, potent immunosuppressant, neuroprotective, antithrombotic, anticoagulant, specific 5-lipoxygenase inhibitory, and cytotoxic activities (Liu, 2007).

##### 3.1.1 Antibiotic properties

Compounds **10** and **11** (Tringali *et al.*, 1987) were tested against several Gram-positive and Gram-negative bacteria in an agar dilution test and the results showed that among Gram-positive the most susceptible microorganism was *Proteus mirabilis* and among Gram-negative *Staphylococcus aureus* (MIC 75 and 50 µg/ml respectively).

##### 3.1.2 Antitumoral activity

Sarcodonin (**12**) was tested against two different tumor cell lines and it resulted moderately active toward KB (ED<sub>50</sub> 10.0 µg/ml) and P-388 (ED<sub>50</sub> 27.0 µg/ml) (Geraci *et al.*, 2000). The higher cytotoxic activity of **10** with respect to **12**, coupled with mild antibacterial activity already described (Tringali *et al.*, 1987) suggest that **10** could play a role in the chemical defense of the mushroom.

Compounds **12**, **13** (sarcodonin  $\alpha$ ), **15** (sarcodonin  $\gamma$ ), and **17** (episarcodonin) and the mixture of **19** and **20** (sarcoviolins) were tested in the three-cell line panel High Throughput PreScreen (one-dose primary anticancer assay) carried out at National Cancer Institute (Bethesda, USA). Fully aromatic terphenyls proved to be cytotoxic at a concentration of 5  $\times 10^{-5}$  M against NCI-H460 (Lung), MCF7 (Breast), and SF-268 (CNS) cell lines. In particular, **12**, **15**, and **16** show the highest cytotoxicity towards SF-268 cells, with 96, 93, and 95% of cells killed, respectively. Sarcoviolins (**19** and **20**) significantly reduced the growth of all cell lines at 10<sup>-4</sup> M (MCF7 totally blocked).

#### 3.2 Biological activities of cyathane diterpenoids

In 1965 Brodie discovered a new bird's nest fungus of the genus *Cyathus* that was named *C. helenae* (Brodie, 1966) and he showed that the metabolites of the mushroom (cyathine complex) possessed antibiotic activity (Allbutt *et al.*, 1971). From this first report, in these 40 years, a great number of cyathane diterpenoids have been isolated, structurally identified and tested for their biological activities.

##### 3.2.1 Antibiotic activity

In 1998 Shibata (Shibata *et al.*, 1998) and coworkers isolated from *S. scabrosus* sarcodonins L and M and sarcodonin A (**21**) and G (**22**). Sarcodonin L and M resulted to be identical to scabronines C (**25**) and B (**24**), respectively (Kita *et al.*, 1998). All these compounds were tested against *B. subtilis* and *S. aureus* and the results are reported in Table 1.

Compound	<i>B. subtilis</i>			<i>S. aureus</i>		
	1.0%*	0.2%	0.05%	1.0%	0.2%	0.05%
<b>21</b>	6.5**			7.0		
<b>22</b>	6.5			7.5	6.5	
<b>24</b>	14.0	11.0	8.0	22.0	15.0	9.0
<b>25</b>	9.5	7.5	7.0	18.0	12.0	7.5

\*Disc were soaked in 35 µl of each test sample (w/v%); \*\*The diameter of the inhibitory zone was measured in mm.

Table 1. Antibiotic activity of sarcodonins A, G, L and M

### 3.2.2 Anti-inflammatory activity

Anti-inflammatory activity has been tested for sarcodonin A (**21**), sarcodonin G (**22**), neosarcodonin A-C (**31-33**) (Hirota *et al.*, 2002), neosarcodonin O (**34**), acyl derivatives of sarcodonin A (**35-37**) (Kamo *et al.*, 2004) and glaucopines A-C (**5-7**) (Curini *et al.*, 2005; Marcotullio *et al.*, 2006a). The results are reported in Table 2. The topical anti-inflammatory activity of compounds was evaluated observing the reduction of oedema induced by TPA, for sarcodonins and neosarcodonins, and Croton oil, for glaucopines, in mouse ear. Indomethacin was used as positive control in all tests.

Compound	Dose	Animals	% Oedema Reduction
<b>5</b>	1.0 <sup>a</sup>	10	62
<b>6</b>	1.0 <sup>a</sup>	10	55
<b>7</b>	1.0 <sup>a</sup>	6	39
<b>Indomethacin</b>	0.3 <sup>a</sup>	10	66
<b>21</b>	0.63 <sup>b</sup>	5	75
<b>22</b>	0.63 <sup>b</sup>	5	84
<b>31</b>	0.57 <sup>b</sup>	5	49
<b>32</b>	0.57 <sup>b</sup>	5	64
<b>33</b>	0.53 <sup>b</sup>	5	87
<b>34</b>	0.63 <sup>b</sup>	5	36
<b>35</b>	0.63 <sup>b</sup>	5	61
<b>36</b>	0.63 <sup>b</sup>	5	46
<b>37</b>	0.63 <sup>b</sup>	5	72
<b>Indomethacin</b>	0.56 <sup>b</sup>	5	16

<sup>a</sup> Dose= µMoles/cm<sup>2</sup>; <sup>b</sup> Dose= µmol

Table 2. Anti-inflammatory activity of sarcodonins, neosarcodonins and glaucopines

### 3.2.3 Antiproliferative and antitumoral activities

The first report about the antitumor activity of a cyathane diterpene was about sarcodonin G isolated from the dichloromethane extract of *Sarcolon scabrosus* Karst, on HeLa cells in vitro. Sarcodonin G (**22**), isolated from the mushroom *Sarcolon scabrosus* and already reported to have anti-inflammatory activity, inhibited proliferation of HeLa cells (Dong *et al.*, 2009).

### 3.2.4 Stimulation of NGF (Nerve Growth Factor)

*Sarcodon scabrosus* and *S. cyrneus* metabolites have been studied for their unique activity to act as neuroprotective agents being able to stimulate the production of neurotrophic factors *in vitro*. Scabronines A-G (23-29) have been tested for their activity to induce NGF secretion from 1321N1 human astrocytoma cells and induce neuritogenesis in PC12 cells (rat pheochromocytoma cells) (Ohta *et al.*, 1998a, 1998b; Kita *et al.*, 1998; Oshima, Y. *et al.*, 1999). Scabronines increased the expression of mRNA for NGF, and the secretion of NGF from 1321N1 cells in a concentration-dependent mechanism (Obara *et al.*, 1999). Among different scabronines, scabronine G methyl ester (SG-ME) (31) resulted to be the most active and the mechanism of action was deeply studied. SG-ME activates PKC- $\zeta$ , induces the translocation NF- $\kappa$ B to nucleus and enhances its transcriptional activity (Fig. 17) (Obara *et al.*, 2001).

Cyrneine A-D (1-4) and glaucopine C (7) were tested to evaluate their activity to induce NGF production from 1321N1 cells, but they resulted much less active than scabronine G (Fig. 18) (Marcotullio *et al.*, 2007). Nevertheless, cyrneine A and B showed an interesting activity, being able to induce directly differentiation on PC-12 cells (Marcotullio *et al.*, 2006b).

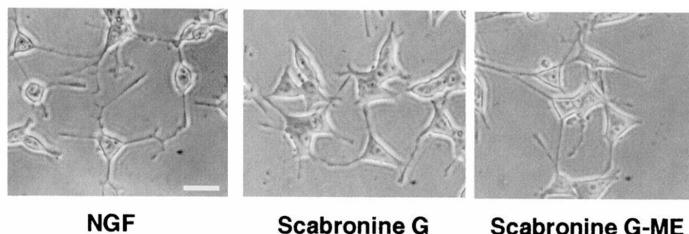


Fig. 17. Morphological changes of PC-12 cells by the 1321N1 cell culture medium conditioned by scabronines. NGF used as control.

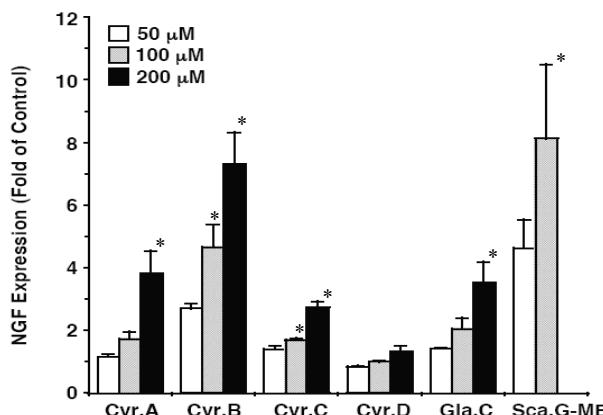


Fig. 18. Evaluation of NGF synthesis from 1321N1 induced by cyrneines and glaucopine C. SG-ME used as control.

As a transcriptional regulation is required for neurite extension, and the activity of three major transcription factors (activator protein-1 (AP-1), nuclear factor- $\kappa$ B, and CREB) was determined. Cyrneines A and B enhanced activation of AP-1 and NF- $\kappa$ B. Moreover, treatment with cyrnejine A led to actin translocation and subsequently, to accumulation of F-actin at the tip of neurites. Rac1 activity was increased by cyrnejine A and expression of a dominant-negative Rac1 mutant significantly inhibited the cyrnejine A-induced extension of neurites. These results suggest that cyrnejine A induces neurite outgrowth in a Rac1-dependent mechanism (Obara *et al.*, 2007).

#### 4. Conclusions

The analysis of the literature about *Sarcodon* genus showed that these mushrooms produces interesting biologically active secondary metabolites such as cyathane e triphenyl derivatives. It is interesting to note that the use of trivial names instead of systematic ones generated some confusion (e.g. the term sarcodonins has been used to name both terphenyls and cyathanes). Furthermore, it was evidenced that sometimes there are problems in the use of different synonyms for the same species. Nevertheless, the interesting biological properties of *Sarcodon* metabolites and the nutritional value of some spp make this genus worthy of further investigations.

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# By-Products from Plant Foods are Sources of Dietary Fibre and Antioxidants

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## 1. Introduction

Fruit and vegetables have been recognized as important sources for a wide array of non digestible components and phytochemicals that individually, or in combination, may act synergistically to contribute to the nutritional and health benefits of these food commodities.

World Health Organization (WHO) and worldwide health authorities such as United States Department of Agriculture (USDA) promote a high consumption and variety of fruit and vegetables. In addition, the source of fibre in a healthy dietary pattern such as Mediterranean Diet has been described as an important qualitative difference on health. Fruit and vegetable related dietary fibre transports a significant amount of polyphenols and carotenoids linked to the fibre matrix through the human gut. Despite these effects and recommendations, the intake of plant foods remains low and, consequently, both dietary fibre and antioxidant compounds are usually deficient in most diets around the world.

On the other hand, the food industry processing plant foods produces large amounts of waste and residues (leaves, stems, wastewaters, etc.) that are good sources of dietary fibre and phytochemicals. Several of them contain more dietary fibre than their respective edible portion.

A variety of plant food byproducts are rich in fibre and polyphenolic compounds meeting the criteria of antioxidant dietary fibre. A broad spectrum of these will be summarized in the present work. In this chapter information on nutritional and phytochemical composition will be also included. Special attention nutritional claims criteria with reference to European Regulation has been given to quality ingredients for functional foods and/or dietary supplements. The application of byproducts in the food industry results in added value to both, the industry and the consumer. The industry benefits from economic incomes and the consumer from the excellent nutritional value of these materials with potential health claims.

## 2. A healthy diet: Mediterranean dietary pattern based on dietary fibre and bioactive compounds

Mediterranean Diet was declared by UNESCO as an Intangible Cultural Heritage, offering important benefits for the health, quality of life and well-being of the communities. The

Mediterranean Diet offers a nutritional model enriched by diverse cultures which over centuries has essentially maintained the same food structure and the same proportions: olive oil, fresh fruits and vegetables, grains and derivatives, fish and to a lesser extent, nuts, dairy products and meat. There is also a moderate consumption of wine, coffee and tea during meals while respecting religious rules and beliefs. Mediterranean Diet is not just a diet (mixture of food), it means a lifestyle because it also implies the way to prepare food and how foods are consumed around the same table. To eat in the Mediterranean Dietary Pattern is a moment of social meeting that complies with several functions: social, cultural and nutritional. The recognition of dietary qualities, and the positive impact on the health of the people who follow this dietary pattern, indicates the model of healthy diet we must try to encourage (UNESCO, 2010).

The definition of the Mediterranean Diet is mainly based on consumption of foods. However, despite the robust inverse association between Mediterranean Diet and mortality found in observational and epidemiological studies, it is not clear which food constituents of this dietary pattern contribute most to its health effects.

With regards to the intake of nutrients and / or food constituents, it appears that both dietary patterns are similar in terms of energy, energy profile and dietary fibre. As far as micronutrients are concerned, clear significant differences in the intake of vitamins and minerals in Northern and Southern European countries have not been reported (Elmadfa et al, 2004). However, the intake of monounsaturated fatty acids is higher in the Mediterranean area in opposition to the intake of saturated fatty acids whose values are higher in countries of Northern and Central Europe where there is a higher prevalence of chronic diseases. These facts are related to food consumption patterns that show a remarkable difference. In countries with Mediterranean diet, consumption of fruit and vegetables is significantly higher, being very important not only the quantity of these foods but also the variety of them. Moreover, there are also differences in relation to fat consumption. Mediterranean recorded high availability of olive oil and unprocessed red meat, while Central and Northern European preferably consumed fat from meat products (Naska et al., 2006). These data are in agreement with the fatty acid profile of food consumed in both dietary patterns.

On the other hand, plant foods in Mediterranean Diet, of which there is a considerable amount and variety, seems to have a significant impact on the nutritional assessment of the Mediterranean Diet, as mentioned later, defines some health indicators of this dietary pattern. Plant foods, especially fruit and vegetables contribute a large proportion of the overall dietary intake of dietary fibre and bioactive compounds and a small proportion of the overall dietary intake of energy. Therefore, the role of bioactive compounds or phytochemicals as a key factor in the health effects of the Mediterranean dietary pattern is an attractive hypothesis. The composition and the physicochemical structure of dietary fibre and phytochemicals in fruit and vegetables have specific characteristics which lend these food groups significant nutrition and health related properties. Indeed, the potential health benefits of fruit and vegetables are mainly attributed to the effects of dietary fibre and antioxidants.

It is argued that an increasing intake of 400 to 800 g/person/day of fruits and vegetables is a public health strategy of considerable importance for individuals and communities worldwide. For this reason the World Health Organization (WHO) recommends a daily intake of more than 400 g per person daily, and health authorities worldwide promote high

consumption of fruit and vegetables. Currently, the Food Standards Agency recommends the consumption of five portions of fruit and vegetables daily. There is no guidance on the specific type of fruit and vegetables to consume other than a suggestion of a variety; this is because to date, there is a lack of convincing evidence for their identification. It is therefore of priority to identify fruit and vegetables which would significantly contribute to a reduction in cardiovascular diseases and other chronic non-communicable diseases. This way we could make public health recommendations and provide scientific information to the food industry about the selection of ingredients for functional foods.

Although inconclusive, evidence has alluded to synergistic effects of combinations of polyphenols, which may be more protective against cardiovascular diseases than isolated polyphenols, and that a combination of fruits, in the context of a balanced diet and healthy lifestyle, would help to protect against these pathologies (Chong et al., 2010) Many of the putative chemoprotective phytochemicals in fruits and vegetables are coloured. Therefore, a good strategy may be guidelines based on selecting one daily serving of fruits and vegetables from each of seven colour classes, so that a variety of phytochemicals is consumed. In this context, a complementary definition of the Mediterranean Diet was recently proposed, based on the following dietary indicators: 1) monounsaturated/saturated lipid ratio; 2) intake of dietary fibre defined as food indigestible fraction; 3) intake of antioxidant capacity of the whole diet; 4) intake of phytosterols. These indicators were selected based on the scientific evidence to support the beneficial health effects and because they are differential features in Mediterranean Diet (Saura-Calixto & Goñi, 2009).

There is general consensus among scientists as to the significant role of the monounsaturated to saturated fat ratio in disease aetiology. This ratio is predictive of total mortality and is a common feature in Mediterranean countries, where is much higher than in other parts of the world including northern Europe and North America (Naska et al, 2006).

As it is indicated above, dietary fibre intake is quantitatively similar in Mediterranean and non-Mediterranean European countries (around 20 g per capita). However, there are qualitative differences arising from the fact that a large proportion of the dietary fibre intake in Mediterranean countries comes from fresh fruit and vegetables, while in Northern European countries it comes more from cereals. Consequently, the composition and properties of the dietary fibre in the Mediterranean Diet has specific characteristics related to the type of food, nutrient and phytochemicals contents and healthy properties.

There is a lack of comprehensive data on the antioxidant capacity of whole diets. This parameter is derived from the accumulative and synergistic antioxidant power of a wide variety of sources. Total dietary antioxidant capacity is probably higher in Mediterranean Diet than in other dietary patterns because of the amount and variety of plant foods rich in antioxidant phytochemicals.

Finally, the intake of phytosterols has been established as a factor in the lower cardiovascular disease death rates in Mediterranean countries and it is a specific essential dietary indicator.

The intake of bioactive compounds in recognized healthy diets such as the Mediterranean Diet may serve as a benchmark until scientific knowledge in this field is sufficiently

advanced to establish daily allowances. Traditional Mediterranean foods are rich in dietary fibre and bioactive compounds and the Mediterranean Diet is a specific type of healthy diet.

### **3. Fruit and vegetables are source of dietary fibre and antioxidant phytochemicals**

Plant foods, particularly fruit and vegetables, have been consistently identified in epidemiological research as the key components of dietary patterns that reduce risk for the development of chronic and degenerative diseases, including atherosclerotic cardiovascular diseases, insulin resistance and type II diabetes and many cancers (Hu et al., 2000; Kant et al., 2004; Mokdad et al., 2000). One of the predominant mechanisms of their protective action is due to their antioxidant activity and the capacity to scavenge free radicals. There has been increasing interest in the nutritional properties of fruit and vegetables as sources of dietary fibre and other health-promoting phytochemical compounds (Knekt et al., 2002; Kris-Etherton et al., 2004; Mennen et al., 2004; Most, 2004).

Fruit and vegetables are generally high in water, low in fat and, in addition to vitamins and minerals, contain significant amounts of dietary fibre and phytochemicals - mainly polyphenols and carotenoids - with significant biological properties, including antioxidant activity.

The composition and the physicochemical structure of dietary fibre and phytochemicals in fruit and vegetables are specific characteristics which lend significant nutrition- and health-related properties to this food group. Indeed, the potential health benefits of fruit and vegetables are mainly attributed to the effects of dietary fibre and antioxidants.

#### **3.1 Phytochemicals**

Phytochemicals or phytonutrients are bioactive substances that can be found in foods derived from plants and are not essentials for life. The human body is not able to produce them. Phenolic compounds are widely distributed throughout the plant kingdom and range from simple molecules such as phenolic acids to complex polymerised compounds (i.e. polyphenols) (Rice-Evans et al., 1996). Recently, some of their characteristics, mainly their antioxidant capacity, have given rise to research related to their protective properties on health and the mechanisms of action involved. The health benefits of antioxidant of natural origin are associated with their role in the prevention of several disorders called oxidative stress pathologies (Herrera et al., 2009). These are related to the damaging effect of oxygen free radicals, or more generally reactive oxygen species, products of normal metabolism that become harmful when they cannot be neutralized by the cellular antioxidant defense systems. In this condition of oxidative stress an uncontrolled oxidizing process may occur that damages biological molecules, disturbs cellular functions, and can potentially lead to the development of one or more diseases (Valko et al., 2007).

*Dietary phytochemicals* are defined as bioactive, non-nutrient plant compounds that are associated with reduced risk of chronic diseases (Liu, 2004). Prospective cohort studies consistently suggest that when consumed in whole foods, these phytochemicals may contribute to important protection against chronic diseases, such as cardiovascular diseases and certain cancer (Okarter & Liu, 2010). The additive and synergistic effects of these bioactive phytochemicals found in plant foods may be responsible for the health benefits

associated with the diet; additionally, the phytochemicals present in the different groups of plant foods in the diet complement each other when they are consumed together (Adom et al., 2005; Liu, 2004; Okarter & Liu, 2010).

Plant foods phenolic compounds may provide benefits to human subjects via several mechanisms (Nijveldt et al., 2001). The best described and most well known mechanism is through their antioxidant properties and modulation of biological oxidative stress to prevent damage to cellular lipids, protein and DNA. Directly, they may scavenge superoxide and other reactive oxygen species such as hydroxyl and peroxy radicals. Overall, phenolic compounds have multiple paths for benefiting human health, most notably, through their actions as antioxidants and modifying cellular events. Their specific actions are likely to be dependent on the composition and time course of metabolites appearing in plasma (Crozier et al., 2009; Hollman et al., 1997; Manach et al., 2005). The intake of these antioxidants can lead to sustained reduction of the kind of oxidative damage to lipids, proteins and DNA that is associated with the development of chronic diseases (Evans & Halliwell 2001).

Notwithstanding the need for more research, the collected data suggest that the consumption of phenolic-rich fruits increases the antioxidant capacity of blood, and when they are consumed with high fat and carbohydrate "pro-oxidant" foods, they may counterbalance their negative effects. Given the content and availability of fat and carbohydrate in the Western diet, regular consumption of phenolic-rich foods, appears to be a prudent strategy to maintain oxidative balance and health (Burton-Freeman, 2010). Vitamins (C and E), polyphenolic compounds and carotenoids are the main groups of antioxidants present in fruit and vegetables. Vitamins are single molecules, but polyphenols and carotenoids are made up of hundreds of compounds with a wide range of structures and molecular masses.

Dietary fibre and antioxidants are generally addressed separately as groups of food constituents in both chemical and nutritional studies. However, it is a little known fact that a substantial proportion of the antioxidant polyphenols and carotenoids contained in fruit and vegetables are linked to dietary fibre (Saura-Calixto et al., 2007), and some of the postulated benefits of the fibre intake can be attributed to these associated compounds.

Most biological properties of polyphenols depend on their bioavailability; the latter is largely influenced by chemical and physical properties and plant-derived conjugation. While a small proportion of some dietary polyphenols can be absorbed through the small intestine, the majority are either not absorbed, or are excreted and become fermentable substrates for bacterial microflora in the colon along with the non digestible food fraction (Williamson & Manach, 2005). Polyphenols bound to dietary fibre can account for a substantial part of total polyphenols in foods. These polyphenols are not bioavailable in the human upper intestine and reach the colon, where they become fermentable substrates for bacterial microflora, along with the other dietary fibre components. The fermentation of polyphenols in the colon improves antioxidant status and yields different metabolites with potential systemic effects.

The majority of studies on determining the bioavailability of phenolic compounds are conducted by the analysis of blood phenolic metabolites a short time after ingestion. However, it should be noted that among the many polyphenols present in food, most of

them do not reach the bloodstream in the early hours of food digestion. Nevertheless, they do the transit until the large intestine where they can be metabolized through other pathways involving the enzymatic activities of the colonic microbiota. Therefore, it is expected that the protective effects of polyphenols are broader than those listed, as will be indicated later.

### 3.2 Dietary fibre

Dietary fibre is a major constituent of plant foods and its importance in nutrition and health is widely recognized. Numerous clinical and epidemiological studies have addressed the role of dietary fibre in intestinal health, prevention of cardiovascular disease, cancer, obesity, and diabetes (Buttriss & Stokes, 2008). The recommended daily intake of dietary fibre is 25–35 g/person (Buttriss & Stokes, 2008).

	Male	Female	All
Italy	Not applicable	22.92 (6.94)	22.92 (6.94)
Spain	24.93 (9.12)	22.02 (7.29)	22.92 (8.01)
United Kingdom	29.12 (8.34)	23.35 (7.08)	25.52 (8.08)
Netherlands	20.00 (7.50)	20.39 (7.06)	20.27 (7.20)
Germany	26.77 (7.61)	22.55 (5.49)	23.63 (6.38)
Sweden	23.83 (7.51)	21.55 (6.41)	22.53 (7.00)
Denmark	21.33 (7.78)	19.08 (6.47)	20.06 (7.16)
Total	22.98 (8.26)	21.52 (6.90)	21.97 (7.38)

Table 1. Fibre intake (g per day) among EPIC cohort (Bingham et al., 2003). *Data are mean (SD). EPIC: European Prospective Investigation into Cancer and Nutrition. Cohort numbers: 134 012 males; 300197 females; 4340209 in total.*

Just as there are significant differences in the consumption of fruit and vegetables among European countries we also note differences in dietary fibre intake. The source of fibre in a healthy dietary pattern is an important qualitative difference. **Table 1** summarizes the daily intake of dietary fibre among European countries (Bingham et al., 2003).

The intake of dietary fibre is low and quantitatively similar in Mediterranean and non-Mediterranean European countries. These data were consistent with other published works (Elmadfa et al., 2005). Dietary fibre intake in Mediterranean countries comes from fresh fruit and vegetables, while in Northern European countries it comes more from cereals. Accordingly, the incidence of chronic diseases is higher in northern European countries.

Currently there is not a harmonized definition of dietary fibre at the European Community level. The term dietary fibre was originally defined as the portion of food which is derived from cellular walls of plants which are digested very poorly by human beings (Trowell, 1976). The recognition that other food components could have effects similar to those originating from plant cell walls led to a redefinition of dietary fibre to include all undigestible food components. The indigestible fraction of food has been defined as the part of plant foods that is not digested nor absorbed in the small intestine, reaching the colon where it is a substrate for the fermentative microbiota. As such, it comprises not only dietary fibre (traditional concept), but also other compounds of proven resistance to the action of

digestive enzymes such as a fraction of dietary starch (resistant starch), protein, certain polyphenols, and other associated compounds (Saura-Calixto et al., 2000, 2007). This definition, basically physiological in nature, has been accepted by the majority of scientists working in the field. In this line, a method to quantify the food indigestible fraction in plant foods was presented. In this method, the digestible portion of the food is removed by using digestive enzymes and mimicking the digestive process in the small intestine. Then, a majority of indigestible components are isolated (Saura-Calixto et al., 2000). The value of dietary fibre includes resistant starch in this analytical method. However, resistant oligosaccharides and inulin are not included, and therefore need to be measured separately and subsequently added to the total food indigestible fraction estimate. The concept of indigestible fraction as dietary fibre is more reliable when applied to epidemiological and nutritional studies (Saura-Calixto & Goñi, 2004).

On the other hand, a number of physiological effects in human beings, e.g. decreased intestinal transit time, increased stools bulk, reduction of blood total and/or LDL cholesterol levels, and reducing post-prandial blood glucose and/or insulin levels, are often associated to the intake of dietary fibre. However, these effects vary depending on fibre component. Therefore, each fibre may have specific effects, which suggests not including physiological properties in the definition.

### **3.3 Dietary fibre as a carrier of bioactive compounds**

Fruit and vegetables possess a higher soluble/insoluble fibre ratio than cereals, what is considered as an indicator of nutritional quality. A part of the postulated benefits of the Mediterranean dietary pattern might then be attributable to the intake of food undigestible components (Saura-Calixto et al., 2000). It is important to note at this point that the use of food dietary fibre data in nutrition may be subject to some limitations arising from the concept of dietary fibre itself and from the methodology used to determine dietary fibre in foods.

Dietary fibre does not constitute a defined chemical group but a combination of chemically heterogeneous substances. Moreover, dietary fibre, especially from fruits and vegetables, is a carrier of bioactive compounds. Dietary fibre of fruit and vegetables transports a significant amount of polyphenols and carotenoids linked to the fibre matrix through the human gut (Saura-Calixto et al., 2006, 2007). Therefore, associated phytochemicals can make a significant contribution to the health benefits attributed to the dietary fibre of fruit and vegetables. Thus, phytochemicals may be considered dietary fibre constituents in view of the similarity of their properties in terms of resistance to digestive enzymes and colonic fermentability (Saura-Calixto et al., 2006, 2007).

Physiological and physicochemical effects of dietary fibres depend on the relative amount of individual non-digestible components. Therefore, when a dietary fibre contains associated compounds with antioxidant activity, this property is conferred to the total dietary fibre complex and may be considered as antioxidant dietary fibre (Saura-Calixto et al., 1998).

In the Spanish diet, considered as Mediterranean pattern diet, fruit and vegetables provide a daily intake of 700-1000 mg of polyphenols/person/diet, a major fraction of this (600 mg/person/day) associated with dietary fibre (Saura-Calixto et al., 2007). These issues constitute an important qualitative difference in relation to other dietary patterns.

#### 4. By-Products from plant foods as sources of dietary fibre and antioxidants

Fruit and vegetables have been recognized as important sources for a wide array of non digestible components and phytochemicals that individually, or in combination, may act synergistically to contribute to the nutritional and health benefits of these food commodities. Despite the consumption recommendations, the intake of fruit and vegetables remains low and, consequently, both dietary fibre and antioxidant compounds are usually deficient in most diets around the world.

Nowadays dietary fibre and bioactive compounds are widely used as functional ingredients in processed foods. The market in this field is competitive and the development of new types of quality ingredients is a challenge for the food industry. In this regard, it is interesting to consider not only the nutritional quality of the ingredient, but also its distribution, cost and other additional benefits, since the use of these ingredients would give added value to the production of these materials.

	Byproducts	Edible part	Reference
Agave	40% (rind and pith)	60%	Iníguez-Covarrubias et al., 2001
Apple	11% (pulp and seed core)	89%	Ayala-Zavala et al., 2010
Artichoke	Around 60% (outer bracts, receptacles and stems)	40%	Llorach et al., 2002
Asparagus	Up to 40-50% (spear)	50-60%	Rodríguez et al., 2006
Banana	Up to 30% (peel)	70%	Schieber et al., 2001
Cactus pear cladodes	20% (spines, glochids and peel)	80%	Bensadon et al., 2010
Cactus pear fruit	45% (spines, glochids, peel and unusable pulp)	65%	Bensadon et al., 2010
Carrot	30-40% (pomace)	60-70%	Schieber et al., 2001
<i>Cyphomandra betacea</i>	15-35% (skin, pulp and seeds)	65-85%	Ordóñez et al., 2010
Guava	10-15% (peel and seeds)	85-90%	Schieber et al., 2001
Mandarin	16% (peels)	84%	Ayala-Zavala et al., 2010
Mango	13.5% (seeds), 11% (peels) and 17.9% (unusable pulp)	58%	Ayala-Zavala et al., 2010
Orange	66% (peel)	44%	Li et al., 2006
Papaya	6.5% (seeds), 8.5% (peels) and 32.1% (unusable pulp)	53%	Ayala-Zavala et al., 2010
Passion fruit	>75% (rind and seeds)	25%	Schieber et al., 2001
Pineapple	9.1% (core), 13.5% (peels), 14.9% (top) and 14.5% (pulp)	48%	Ayala-Zavala et al., 2010
Potato	15-40% (peel)	60-85%	Schieber et al., 2001
Tomato	3-7% (peel and seeds)	93-97%	Schieber et al., 2001
Tiger nuts ("Chufa")	Up to 60% (solid and liquid wastes)	40%	Sánchez-Zapata et al., 2009

Table 2. Amount of byproducts generated from fruit and vegetable processing industry.

There is a trend to find new sources of functional ingredients such as plant food byproducts that have traditionally been undervalued (Rodríguez et al., 2006). The term "byproduct" suggests that plant food wastes might be usable and have their own market (Sánchez-Zapata et al., 2009). The plant food processing industry produces large amounts of wastes and residues, estimated to be around 800 000 tons per year of fresh fruit and vegetable matter globally, without considering the wastage during processing (Ayala-Zavala et al., 2010). In India, fruit and vegetable wastes constitute about 5.6 million tons annually (Arvanitoyannis & Varzakas et al., 2008). These byproducts might reach around 60% of harvested plants. These residues are very perishable products that are difficult to manage because of environmental problems in the industries (Arvanitoyannis & Varzakas et al., 2008).

Dietary fibre does not constitute a defined chemical group but a combination of chemically heterogeneous substances. The health significance of foods fibres has led to the development of a large and potential market for fibre-rich products and ingredients (Rodríguez et al., 2006). In general, agricultural and industries residues are important sources of dietary fibre.

**Table 2** shows the percentage of byproducts generated from fruit and vegetables processing industries. These byproducts are made up mainly of skins, seeds, stems, leaves, wastewaters and unusable pulp which are normally discarded (Ajila et al., 2007). The amount of byproducts might represent more than 40% of total plant food in cases such as artichoke, asparagus, cactus pear fruit, mango, orange, papaya, pineapple, red chicory and tiger nuts.

**Table 3** displays the total dietary fibre content of byproducts from plant foods widely consumed. It is remarkable the important amount of fibre fraction present in byproducts ranging from 27 up to 80%, comprising both soluble and insoluble fibre compounds.

Dietary fibre of fruit and vegetables transports a significant amount of polyphenols and carotenoids linked to the fibre matrix through the human gut (Saura-Calixto et al., 2006, 2007). Therefore, associated phytochemicals can make a significant contribution to the health benefits attributed to the dietary fibre of fruit and vegetables. Moreover, phytochemicals may be considered dietary fibre constituents in view of the similarity of their properties in terms of resistance to digestive enzymes and colonic fermentability (Saura-Calixto et al., 2006, 2007).

An interesting approach to providing an added value to byproducts is their use as sources of dietary fibre and also as natural antioxidant compounds. Particularly, plant food residues are a good source of phytochemicals such as polyphenols.

The phenolic content of a wide variety of plant food byproducts are displayed in Tables 4 and 5. It can be noted that peel byproducts from grape, mango, pomegranate, apple, bambangan, cactus pear and cladodes as well as seeds of avocado, longan and mango, are remarkably the highest in polyphenol concentration. Likewise, general byproducts from asparagus, artichoke, blueberry, cranberry, buckwheat and grape seed are rich in polyphenols including proanthocyanidins. Grape antioxidant dietary fibre is the most concentrated source of polyphenols with a concentration of 19740 mg/100 g dry weight (Pérez-Jiménez et al., 2008).

Byproducts	Dietary fiber (g/100 g dry weight)	Reference
Apple	44.0	McKee & Latner, 2000
Brewer's dried grain	60.0	McKee & Latner, 2000
Cabbage outer leaves	40.5	McKee & Latner, 2000
Carob	53.0	Bravo, 1994
Carrot	48.0	McKee & Latner, 2000
Cauliflower	65.0	McKee & Latner, 2000
Chia	56.5	Vázquez-Ovando et al., 2009
Pepper	80.4	McKee & Latner, 2000
Cocoa hulls	60.5	Lecumberri et al., 2006
Coconut	63.2	Raghavendra et al., 2006
Coffee silverskin	69.2	Napolitano et al., 2007
Date	71.0	McKee & Latner, 2000
Grape	77.9	McKee & Latner, 2000
Grape antioxidant dietary fibre	73.5	Pérez-Jiménez et al., 2008
Grapefruit	58.6	Larrauri et al., 1997a
Guava	48.6	Sánchez-Zapata et al., 2009
Jack vean	55.9	Vázquez-Ovando et al., 2009
Kiwi	25.8	McKee & Latner, 2000
Lime	64.3	Jongaroontaprangsee et al., 2007
Tangerine	52.9	Rincon et al., 2005
Mango	74.0	Larrauri et al., 1996
Oat bran	8.2	McKee & Latner, 2000
Olive	80.0	McKee & Latner, 2000
Orange	57-71	Vázquez-Ovando et al., 2009
Passion fruit	63.3	Pérez-Navarrete, 2003, as cited in Sánchez-Zapata et al., 2009
Peach	36.0	McKee & Latner, 2000
Pear	43.9	McKee & Latner, 2000
Peas	82.3	McKee & Latner, 2000
Pineapple	70.6	Larrauri et al., 1997b
Rice bran	27.4	McKee & Latner, 2000
Tiger nuts	59.7	Sánchez-Zapata et al., 2009

Table 3. Total dietary fibre content of byproducts from plant foods.

	Peel/skin	Pulp	General byproducts	Reference
Asparagus			284-371	Rodríguez et al., 2005
Blanched artichoke			360-440	Llorach et al., 2002
Cauliflower			110-180	Llorach et al., 2003
Chicory			77-82	Llorach et al., 2004
Grape	2890			Saura-Calixto et al., 1998
Grapefruit	155	135		Gorinstein et al., 2001
Guava	58.70			Jimenez-Escrig et al., 2001
Hazelnut	577*		127-241*	Shahidi et al., 2007
Lemon	190.0	164.0		Gorinstein et al., 2001
Lettuce			14-156	Llorach et al., 2004
Mango	7000			Larrauri et al., 1996
Orange	179.0	154.0		Gorinstein et al., 2001
Peach	133.7	41.5		Chang et al., 2000
Pomegranate	24990	2440		Li et al., 2005
Raw artichoke			300-320	Llorach et al., 2002
Tomato cherry	10.4-40	9.20-27.0		George et al., 2004

Table 4. Phenolic content (mg/100 g fresh weight) measured in solvent extracts of plant byproducts. \*mg/g of extract.

A wide array of phenolic compounds has been described in plant food by-products. Almond hull extracts contain hydroxybenzoic and cinnamic acid derivatives, with minor presence of flavan-3-ols, including the presence of epicatechin and glycosylated flavonols (Rubilar et al., 2007). Five phenolic acids (gallic acid, caffeic acid, p-coumaric acid, ferulic acid, and sinapic acid) were identified in hazelnut byproducts (both free and esterified forms) (Shahidi et al., 2007).

Caffeic acid derivatives are the main phenolic compounds in artichoke heads, with a wide range of caffeoquinic acid derivatives with chlorogenic acid (5-Ocaffeoquinic acid) as the most important of these derivatives. Other phenolics such as the flavonoids apigenin and luteolin (both glucosides and rutinosides) as well as different cyanidin caffeoyleglucoside derivatives have been identified (Llorach et al., 2002). The analysis of cauliflower byproduct extracts revealed the presence of both flavonoids and hydroxycinnamic acids (caffeic acid and sinapic acid). Different combinations of flavonols such as kaempferol and quercetin with sinapic acid and glucose were the main phenolic compounds present (Llorach et al., 2003).

Analyses of lettuce byproducts revealed the occurrence of both hydroxycinnamic acids and flavonoids. The flavonoid profile of lettuce byproducts was composed of flavones (luteolin derivatives) and flavonols (quercetin derivatives), whereas the chicory byproducts were composed only of kaempferol derivatives (Llorach et al., 2004).

	Peel/ skin	Pulp	Seed	General byproducts	Reference
Apple	3300	11800			Schieber et al., 2003; Wolfe & Liu et al., 2003
Asparagus				284-371	Rodríguez et al., 2005
Avocado			8820		Soong & Barlow, 2004
Bambangan peel powder	9830				Hassan et al., 2011
Banana	928	232			Someya et al., 2002
Blueberry				459*	Khanal et al., 2009
Buckwheat bran				406	Zduńczyk et al., 2006
Buckwheat hulls				448	Zduńczyk et al., 2006
Cactus pear	2760	3180	1610		Bensadón et al., 2010; Ramírez-Moreno et al., 2011
Cladodes	3710				Bensadón et al., 2010
Cranberry				448*	Khanal et al., 2009
<i>Cyphomandra betacea</i>				72	Ordóñez et al., 2010
Grape antioxidant dietary fibre				19740	Pérez-Jiménez et al., 2008
Grape pomace				20-200	González-Paramás et al., 2004
Grape seed				120-710	González-Paramás et al., 2004
Jackfruit		90	2770		Soong & Barlow, 2004
Longan		160	6260		Soong & Barlow, 2004
Mango	5467- 9020	240	11700		Ajila et al., 2010a; Soong & Barlow, 2004
Oat bran with hulls				155	Zduńczyk et al., 2006

Table 5. Phenolic content (mg/100 g dry weight) in different plant foods byproducts. \*mg proanthocyanidins/100 g dry weight.

The prominent phenolic compounds identified in peels of raw and ripe mango fruits were protocatechuic acid, gentisic acid and gallic acid. Gallic acid, syringic acid, mangiferin, ellagic acid, gentisyl-protocatechuic acid, quercetin were the phenolic compounds identified in both raw and ripe peels, while raw peel showed the presence of glycosylated iriflophenone and maclurin derivatives also (Ajila et al., 2010b). The main flavonoids found in citrus species are hesperidin, narirutin, naringin and eriocitrin. Peel and other solid residues of lemon waste mainly contained hesperidin and eriocitrin, while the latter was predominant in liquid residues (Schieber et al., 2001). Otherwise, the polyphenol profile of solvent extracts of various byproducts (barks, kernels, peels, and old and young leaves) from Brazilian mango crops showed the occurrence of xanthone C-glycosides, gallotannins, and benzophenones (Barreto et al., 2008).

Apple pomace is a good source of polyphenols which are predominantly localized in the peels and are present in the juice to a minor extent (Schieber et al., 2001). The phenolic compounds quantified in the apple skin were: the proanthocyanidins (procyanidin B1 and

B2), the flavan-3-ols (epicatechin and catechin), the flavonols (quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, quercetin-3-O-glucoside, quercetin-3-O-rutinoside), the dihydrochalcone (phloretin-2-O-glucoside), the anthocyanin (cyanidin-3-O-galactoside), and the phenolic acid (chlorogenic acid) (Huber & Rupasinghe, 2009). Also, banana bracts are an abundant source of anthocyanins such as delphinidin, cyanidin, pelargonidin, peonidin, petunidin and malvidin (Schieber et al., 2001).

In the composition of extracts of white and red grape pomace several of these compounds were also detected but basically consisted of glycosylated flavonols (quercetin, kaempferol) (Rubilar et al., 2007). Phenolic compounds present in grape pomace antioxidant dietary fibre have been widely described (Tourino et al., 2008), comprising: hydroxybenzoic and phenolic acids and their derivatives, monomeric flavonoids (catechins and flavonols aglycones) and flavonoids such as oligomeric proanthocyanidins, flavonols, flavones, and flavanones; anthocyanin, anthocyanidins delphinidin, cyanidin, petulin, peonidin, petunidin, pelargonidin and malvidin) and their mono and acetyl glucoside derivatives were also identified as well as polymeric proanthocyanidins (types A and B).

One recognized characteristic of a healthy diet includes components that counteract oxidative stress, which is involved in the etiopathogeny and progression of chronic diseases, contributing to the process of aging (Herrera et al., 2009). Dietary fruit and vegetables provide a significant amount of compounds (vitamins, carotenoids and polyphenols) that act as physiological antioxidants. Polyphenols are the major dietary antioxidants.

Since part of the total content of antioxidant phytochemicals is linked to dietary fibre as noted above, an appreciable proportion of the total antioxidant capacity in fruit and vegetables is associated with dietary fibre. We address mainly polyphenols associated with dietary fibre in fruit and vegetables because of the important biological properties derived from them and the significant phenolic content in these foods. Table 6 summarizes the antioxidant or radical scavenging activity of plant food byproducts. Those from bambangan, cactus pear, cladodes, hazelnut and, especially grape antioxidant dietary fibre, possess a potent antioxidant capacity.

## 5. Application of plant food byproducts as functional ingredients

The definition of a *functional food* by the International Life of Science Institute (ILSI 1999) is "a food product can be functional if it has satisfactorily been proven that it produces a beneficial effect on one or more physiological functions, besides its conventional nutritional effects, being this relevant for improving human health and/or reducing the risk of suffering certain diseases" (Roberfroid, 2000). Dietary fibre holds all the characteristics required to be considered as an important ingredient in the formulation of functional foods due to its proven beneficial effects (Rodríguez et al., 2006).

The Regulation on Nutrition and Health Claims (European Comission 2007, EU Regulation (EC) No 1924/2006) allows claims to be made with respect to the fibre content of food (Table 7) as *source of fibre* if its levels exceed 3 g per 100 g or *high in fibre* for 6 g per 100 g (Buttriss & Stokes, 2008). The claims for dietary fibre are as follows (Table 7)

	Extract	Byproducts	Method	Reference
Bambangan peel powder	Methanol, acetone and water	44.50 µg/mL IC <sub>50</sub>	DDPH	Hassan et al., 2011
Blanched artichoke	Methanol/water	0.18-0.27g TEAC/100 g FW	ABTS	Llorach et al., 2002
Buckwheat bran	Methanol and water	24.24 µmol TEAC/g DW	ABTS	Zduńczyk et al., 2006
Buckwheat hulls	Methanol and water	26.15 µmol TEAC/g DW	ABTS	Zduńczyk et al., 2006
Cactus pear	Acidic methanol, acetone and water	66.33 µmol TEAC/g DW	ABTS	Bensadón et al., 2010; Ramírez-Moreno et al., 2011
Cauliflower	Ethanol and water	0.86-3.20 g TEAC/1 kg FW	ABTS	Llorach et al., 2003
Chicory	Methanol or water	0.6-0.8 mg TEAC/g FW	ABTS	Llorach et al., 2004
Cladodes	Acidic methanol, acetone and water	57.55 µmol TEAC/g DW	ABTS	Bensadón et al., 2010
Coffee silverskin	Methanol and water	1-4 mmol TEAC/g FW	ABTS	Napolitano et al., 2007
<i>Cyphomandra betacea</i>	Acidic ethanol and water	12.24 µmol TEAC/g DW	ABTS	Ordóñez et al., 2010
Grape antioxidant dietary fibre	Acidic methanol, acetone, water and butanol.	375.5 µmol TEAC/g DW	ABTS	Pérez-Jiménez et al., 2008
Hazelnut	Ethanol, water and methanol	117-148 µmol TEAC/g of extract	ABTS	Shahidi et al., 2007
Lettuce	Methanol or water	0.4-1.3 mg TEAC/g FW	ABTS	Llorach et al., 2004
Oat bran with hulls	Methanol and water	7.96 µmol TEAC/g DW	ABTS	Zduńczyk et al., 2006
Raw artichoke	Methanol/water	0.14-0.25 g TEAC/100 g FW	ABTS	Llorach et al., 2002

Table 6. Antioxidant activity or radical scavenging activity of plant food byproducts. TEAC: trolox equivalent antioxidant capacity. DW: dry weight. FW: fresh weight.

Claim	<i>Source of fibre</i>	<i>Increased in fibre</i>	<i>High in fibre</i>
Requirement	Either >3 g/100 g or >1.5 g fibre/100 kcal	>25% more than a similar food which no claim is made	Either >6 g/100 g or >3 g fibre/100 kcal

Table 7. Nutrient claims for dietary fibre based on AOAC (American Organization of Analytical Chemists) analysis (EU Regulation (EC) No 1924/2006).

Byproducts obtained when processing cereal, algae, fruit and vegetables can be added as functional ingredients, providing advantageous dietary fibre and bioactive compounds. These byproducts serve as non-caloric bulking agents, enhance water and oil retention, and improve emulsion and oxidative stability. The literature reports addition of fibre to food products such as baked goods, beverages, confectionary, dairy, meat and pasta (Elleuch et al., 2011).

Addition of byproducts in bakery products are muffin butter supplemented with peach dietary fibre (Grigelmo-Miguel et al., 1999), and cake dough enhanced with prickly pear cladode fibre (Ayadi et al., 2009) at levels up to 5%. Incorporation of cauliflower by-products into ready-to-eat snacks enhanced nutritional and textural characteristics, increasing dietary fibre levels in the finished product by over 100% (Stojceska et al., 2008).

Fibres can also be introduced into meat products. Addition of 1.5% of orange fibre or 3% of carrot fibre to dry fermented sausages does not affect its organoleptic characteristics (Eim et al., 2008; Garcia et al., 2002). Citrus fibre with associated antioxidant bioactive compounds when added to meat products inhibits lipid oxidation and decrease residual nitrite levels (Fernández-Ginés et al., 2003). Pork burgers elaborated with tiger nut fibre had higher nutritional value in terms of fibre content and better cooking characteristics such as higher cooking yield, fat and moisture retention (Sánchez-Zapata et al., 2010). Grape pomace antioxidant dietary fibre when added to minced fish (Sánchez-Alonso et al., 2007) and chicken hamburgers (Sáyago-Ayerdi et al., 2009) improves oxidative stability and thus prolong shelf life. By-product fibre addition to burgers is a promising and convenient application considering dietary fibre of burgers can be significantly increased without changes in sensory acceptance.

The addition of unripe banana flour to spaghetti increased the dietary fibre and the content of phenolic compounds. Consequently, spaghetti had a slow and low rate for enzymatic hydrolysis and an increased antioxidant capacity (Ovando-Martínez et al., 2009). Mango peel powder was incorporated into macaroni (7.5%) to increase dietary fibre from 8.6 to 17.8%, polyphenols from 0.46 to 1.80 mg/g and carotenoid content from 5 to 84 µg/g of macaroni, resulting in an enhanced nutritional quality without affecting its cooking, textural and sensory properties (Ajila et al., 2010a).

## 6. Conclusion

Several fruit and vegetable byproducts from food processing industries meet the criteria of antioxidant dietary fibre definition. They are certain to be an excellent source of dietary fibre and natural antioxidants if used as high-quality ingredients in functional foods or dietary supplements. Furthermore, compliance with the nutritional claims criteria recognized in the

European Regulation would be met. The applications of plant food byproducts will definitely bring about added value to both, the industry and the consumer. The industry benefits from economic incomes and the consumer from the excellent nutritional value of these materials with potential health claims.

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# Oral Bioavailability and Disposition of Phytochemicals

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## 1. Introduction

Higher plants produce a vast variety of secondary metabolites known as phytochemicals (PCs) which appear to protect the plant against a variety of stresses such as UV irradiation, pathogenic attacks, and perhaps even consumption by herbivores. Many of these non-nutrient PCs have been shown to exert a wide range of biological effects, and epidemiological and nutritional studies have identified a protective role for PCs in the prevention of cancer, diabetes, cardiovascular and neurodegenerative diseases (Kris-Etherton et al., 2002; Aruoma et al., 2003; Surh, 2003; Balunas & Kinghorn, 2005; Duthie, 2007; Espin et al., 2007; Russo, 2007; Dembinska-Kiec et al., 2008; Hooper et al., 2008; Khan et al., 2008). Unlike some cytotoxic chemicals derived from natural products (e.g., etoposide, daunorubicin and paclitaxel), PCs are found in high concentrations in fruits, vegetables, nuts, wine and tea, and intake can be up to several hundred milligrams per day (Manach et al., 2005). PCs which have been associated with health benefits, include glucosinolates, organic isothiocyanates, dibenzocyclooctadienes, sulphur-containing compounds of alliaceae, terpenoids (carotenoids, monoterpenes, and phytosterols), flavonoids and polyphenols (e.g., anthocyanins, flavones, flavan-3-ols, isoflavones, stilbenoids, and ellagic acid) (Balunas & Kinghorn, 2005; Espin et al., 2007). Bioavailability and tissue distribution of these PCs in humans are key factors that need to be clearly established in association with their biological effects. Recently various drug metabolizing enzymes and drug transporters such as the ATP binding cassette (ABC) and the solute carrier (SLC) transporters have been cloned and functional analyses suggest that they play significant roles in the absorption and disposition of most drugs and PCs (Zhang et al., 1998; Zhang & Benet, 2001; Borst & Elferink, 2002; Faber et al., 2003; Sarkadi et al., 2006; Shitara et al., 2006; Hu et al., 2003; Zhou et al., 2004; Morris & Zhang, 2006; Zhang et al., 2007; Shukla et al., 2008; Zhang et al., 2009). They are present in all tissues and play pivotal roles in the defense of the body against amphipathic carcinogens and toxins. Many drug metabolizing enzymes and transporters are under tight transcriptional regulation by nuclear receptors, suggesting their functions are subject to environmental and dietary influences (Borst & Elferink, 2002; Petri et al., 2003; Lancon et al., 2007; Giacomini et al., 2010). In addition, PCs may modulate the expression and function of drug metabolizing enzymes and drug transporters which govern xenobiotic bioavailability (Wang & Morris, 2007; Kim et al., 2009; Shukla et al., 2009). This review will highlight the various barriers to dietary phytochemicals, approaches for assessing these interactions, and their implications in pharmacokinetics and potential clinical applications.

## 2. Absorption from gastrointestinal (GI) tract

To achieve their beneficial effects, other than on the GI tract itself, these PC molecules must be delivered to target tissues and organs after overcoming several absorption barriers in the GI tract (Figure 1). Firstly, they must dissolve in the fluids of the GI tract and survive the different pH environment ranges from extreme low in the stomach to slightly basic in some segments of the small intestine. They may also be subjected to degradation and metabolism by intestinal enzymes, such as the glycosidases, esterases, oxidases and hydrolases, originating both from the host and the myriad of microbiota that inhabit the GI tract (Sousa et al., 2008). Actually the large intestine accommodates most of the GI microbiota and the rate and extent of metabolism by bacteria will be influenced by the amount of the PC that reaches the distal gut. Many plant flavonoids exist in the O-glycoside form within the plant and undergo rapid GI hydrolysis to remove the glucose conjugates and form their respective aglycons (Crespy et al., 2002; Walle et al., 2005). The latter are more lipophilic and thus are more efficiently absorbed across the GI wall than the parent glycoside, providing they are not subject to intestinal transporter-mediated absorption. However, interactions with intestinal ABC and SLC transporters may cause unpredictable absorption kinetics of many PCs which cannot be simply predicted from their physicochemical properties. Transporter-related absorption phenomena, such as the limited and nonlinear intestinal permeability and absorption of PCs, may lead to extensive variability in their oral bioavailability, resulting in low plasma concentrations and lack of pharmacological effect on the one hand, or elevated concentrations and toxicity on the other.

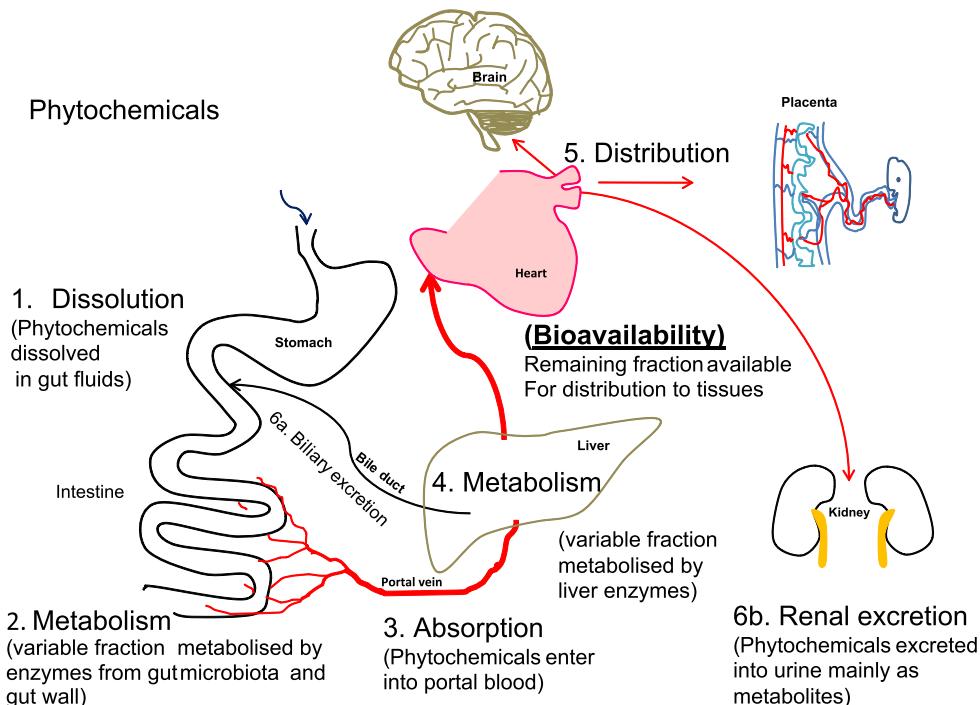


Fig. 1. Absorption and disposition of phytochemicals in humans.

The human family of ABC transporters contains 49 members with 7 subfamilies including several important xenobiotic transporters, such as P-glycoprotein (P-gp, ABCB1), multidrug resistance protein 1-9 (MRP 1-9, ABCC1-6 and ABCC10-12, respectively) and breast cancer resistance protein (BCRP, ABCG2) (Borst & Elferink, 2002). They actively transport chemically diverse substrates including amino acids, lipids, inorganic ions, peptides, saccharides, metals, xenobiotics, and proteins out of cells. In most examples of primary active transport that have been observed, transport of the substrates against their concentration and chemical potential gradients was driven by the hydrolysis of ATP (Higgins, 1992). To date, important SLC transporters involved in xenobiotic absorption and disposition mainly include organic cation transporter (OCT) and organic anion transporter (OAT); and organic anion transporting polypeptide (OATP) families.

Many ABC and SLC transporters have been identified in the GI tract including OATPs, P-gp, MRPs and BCRP on the apical membrane and OCT1 and MRP 3, 4, 5 on the basolateral (blood) side (Figure 2). PCs can act as substrates for ABC transporters (Table 1), which can severely limit their bioavailability. The expression of BCRP transcripts in human jejunum are higher than that of P-gp (Taipaleensuu et al., 2001; Maliepaard et al., 2001), suggesting that BCRP may play an important role in limiting the intestinal absorption of its substrates. Knocking out mouse Bcrp led to significant increases in the oral bioavailability of daidzen (3.7-fold) and genistein (1.8-fold) compared to wild type mice (Enokizono et al., 2007). Several flavonoids such as quercetin, kaempferol, and diverse anthocyanins and anthocyanidins commonly found in grapes and berries, malvidin, petunidin, malvidin-3-galactoside, malvidin-3,5-diglucoside, cyanidin-3-galactoside, peonidin-3-glucoside and cyanidin-3-glucoside have also been identified as BCRP substrates (Dreiseitel et al., 2009; An et al., 2010). Thus BCRP may limit the absorption of these PCs, but to date, human pharmacokinetic data are not available to confirm this.

Accumulating evidence, mainly from *in vitro* studies has indicated that many flavonoid aglycones and anthocyanins and anthocyanidins are P-gp and BCRP inhibitors, including genistein, biochanin A, quercetin, morin, phloretin, silymarin (a mixture of silibinins,

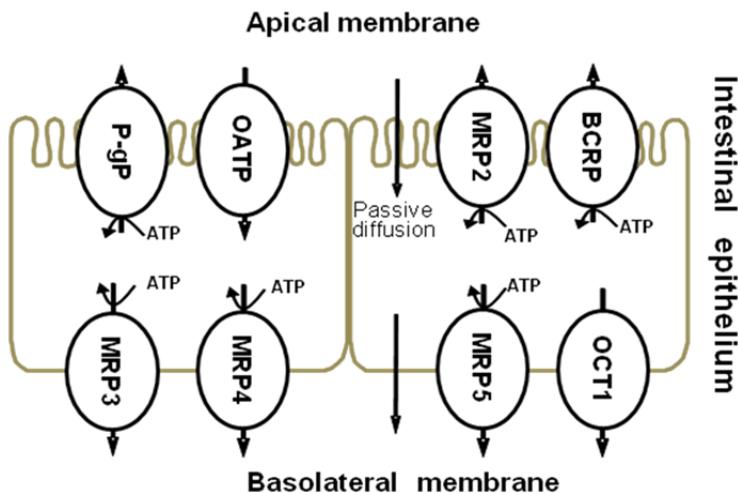


Fig. 2. Localization of ABC and SLC transporters in human small intestine.

Name	Symbol	Tissue location	PC substrates	PC inhibitors	References
P-gp	ABCB1 MDR1	Liver, intestine, brain	Quercetin, EGCG, Biochanin A	Genistein, naringenin, hesperetin, acacetin, apigenin, chrysin.	(Jodoin et al., 2002; Wang et al., 2002; Zhang & Morris, 2003; de Castro et al., 2007; Taur & Rodriguez-Proteau, 2008)
MRP1	ABCC1	All major tissues	(-)-Epicatechin-3-gallate	Quercetin, Naringenin, Kaempferol, Apigenin, Genistein Schisandrin A and B, Schisandrol A and B	(Versantvoort et al., 1993; Versantvoort et al., 1994; Versantvoort et al., 1996)
MRP2	ABCC2,	Liver, kidney, intestine, brain	(-)-Epicatechin-3-gallate, 4-O-methyl-EGCG	Myricetin, robinetin	(Borst et al., 1999; Zhou et al., 2008)
MRP3	ABCC3	Small intestine, pancreas, colon, placenta, adrenal gland	Baicalein-7-glucuronide,	?	(Borst et al., 1999; Zhou et al., 2008)
MRP4	ABCC4	Kidney	?	Quercetin, silymarin	(Borst et al., 1999; Zhou et al., 2008b; Wu, 2005)
MRP5	ABCC5	Most tissues	?	Quercetin, silymarin	(Borst et al., 1999; Zhou et al., 2008b; Wu, 2005)
BCRP	ABCG2	Placenta, liver, the small intestine, colon, lung, kidney	Quercetin, genistein, resveratrol, malvidin, petunidin, malvidin-3-galactoside, malvidin-3,5-diglucoside, cyanidin-3-galactoside, peonidin-3-glucoside and cyanidin-3-glucoside	Genistein, naringenin, hesperetin, acacetin, apigenin, chrysin, diosmetin, luteolin, galangin, kaempferide, kaempferol, cyanidin, peonidin, cyanidin-3,5-diglucoside, malvidin, pelargonidin, delphinidin, petunidin, delphinidin-3-glucoside, cyanidin-3-rutinoside, malvidin-3-glucoside, pelargonidin-3,5-diglucoside, malvidin-3-galactoside, cannabinoids	(Litman et al., 2001; Holland et al., 2007; de Wolf et al., 2008; Dreiseitel et al., 2009)

Table 1. An overview of tissue distribution, substrates and inhibitors of P-gp, MRP<sub>s</sub> and BCRP.

isosilyolin A and B, silychristin A and B, and silydianin), chrysins, hesperetin, naringenin, and the green tea polyphenols, epicatechin gallate, catechin gallate and epigallocatechin gallate (Table 1) (Castro & Altenberg, 1997; Jodoin et al., 2002; Wang et al., 2002; Zhang & Morris, 2003; de Castro et al., 2007; Taur & Rodriguez-Proteau, 2008; Dreiseitel et al., 2009). It has been suggested that such inhibitory PCs could be used to reverse the ABC transporter-based constraints on the GI absorption of other substrate PCs and that this may represent a useful strategy for improving their bioavailability. For example, the oral bioavailability of biochanin A was increased approximately 2-fold when coadministered with the BCRP inhibitors quercetin and epigallocatechin-3-gallate (EGCG) in rats (Moon & Morris, 2007). However this effect may at least partially be due to inhibition of metabolizing enzymes, as there is evidence that both quercetin and the green tea polyphenols may inhibit both Phase 1 and 2 metabolism (Cermak & Wolffram, 2006). Kaempferol has also been reported to increase quercetin permeability across MDCKII-Bcrp monolayers by inhibition of Bcrp-mediated quercetin efflux (An et al., 2010). Recently it has also been suggested that intestinal ABC transporters may function as barriers to absorption of PCs (e.g., resveratrol, green tea catechins and flavonoids) by cooperating with intestinal Phase 2 metabolizing enzymes (Zhang et al., 2004; Ebert et al., 2005; Zhang et al., 2007; Juan et al., 2010), implying a joint role in limiting oral absorption of PCs. In an *in situ* intestine perfusion model in Mrp2-deficient rats, Bcrp was shown to limit net intestinal absorption of quercetin by pumping quercetin glucuronides back into the lumen (Sesink et al., 2005).

Several uptake transporters, such as organic anion transporting polypeptide (OATP) and organic cation transporter (OCT) from the SLC transporter superfamily are also functionally expressed on human intestine tissues (Figure 2) (Hagenbuch & Meier, 2003; Giacomini et al., 2010), and have recently been associated with the oral absorption of some PCs. For example, quercetin was absorbed by passive diffusion and a pH-dependent mechanism mediated by OATP in a Caco-2 cell monolayer model (Nait Chabane et al., 2009). As quercetin is also a substrate for the efflux transporters P-gp and BCRP, a balance between these counteracting transporters may allow a more precise control of the cellular accumulation of such substrate compounds, but the actual biological implication of this fine-tuning mechanism remains unclear at the moment. This transport process may be further complicated in that many PCs present in plants are linked to sugar moieties, which may have an impact on their oral absorption. For example, there is *in vitro* and *in silico* evidence that the human glucose transporter 1 (SLC2A1) and rat glucose transporter 4 (slc2a4) transports quercetin (Strobel et al., 2005; Cunningham et al., 2006). In addition, the pig but not human sodium-dependent glucose transporter-1 (SGLT1) appeared to be involved in the intestinal uptake of quercetin glucosides (Cermak et al., 2004; Kottra & Daniel, 2007).

### 3. Metabolism (enterocytes & hepatocytes)

Possibly of greater importance as a defensive barrier against these invading foreign molecules is the battery of both Phase 1 and Phase 2 enzymes present in the enterocytes (Figure 3). The Phase 1 reactions include oxidation, reduction and hydrolysis, which primarily serve to increase the hydrophilicity of the molecule, and expose or add a functional group (such as a hydroxyl group) to facilitate Phase 2 conjugation reactions. Oxidation is the most predominant reaction involved in the Phase 1 metabolism of xenobiotics, and is principally carried out by a family of closely related isozymes known as

the cytochrome P450-dependent mixed-function oxidases (CYPs). In humans, CYP1A, CYP2C, CYP2D and CYP3A are responsible for metabolising the bulk of xenobiotics that enter the body via the oral route (Lewis & Ito, 2008). CYP3A4/5 with its broad substrate specificity is particularly important in xenobiotic metabolism, making up 70 and 30% of total CYPs in the intestines and liver, respectively (Zhang & Benet, 2001).

The parent PCs (or their Phase 1 metabolites) that contain suitable functional groups (e.g., a hydroxyl group) often undergo conjugation reactions with endogenous compounds to yield more polar and water soluble compounds. The latter are usually ideal substrates for active transport out of the cell, and eventually excretion from the body. The principal conjugation reaction is the formation of  $\beta$ -glucuronides catalysed by a family of enzymes known as the uridine diphosphoglucuronosyl transferases (UGTs), but conjugation with a sulpho moiety ( $\text{SO}_3^-$ ) or glutathione also occurs, catalysed by various sulphotransferases (SULTs) and glutathione-S-transferases (GSTs), respectively. Less polar conjugates may also be formed by methylation, catalysed by catechol-O-methyl transferase (COMT). These Phase 2 conjugation reactions are particularly important for PCs such as epi-gallocatechin-3-gallate (EGCG), which is the most abundant catechin in green tea. EGCG has numerous hydroxyl groups and undergoes extensive Phase 2 metabolism, including glucuronidation, sulphation, and methylation (Lambert et al., 2007; Yang et al., 2007). Several recent studies using liquid chromatography-tandem mass spectrometry (LC-MS/MS) have demonstrated

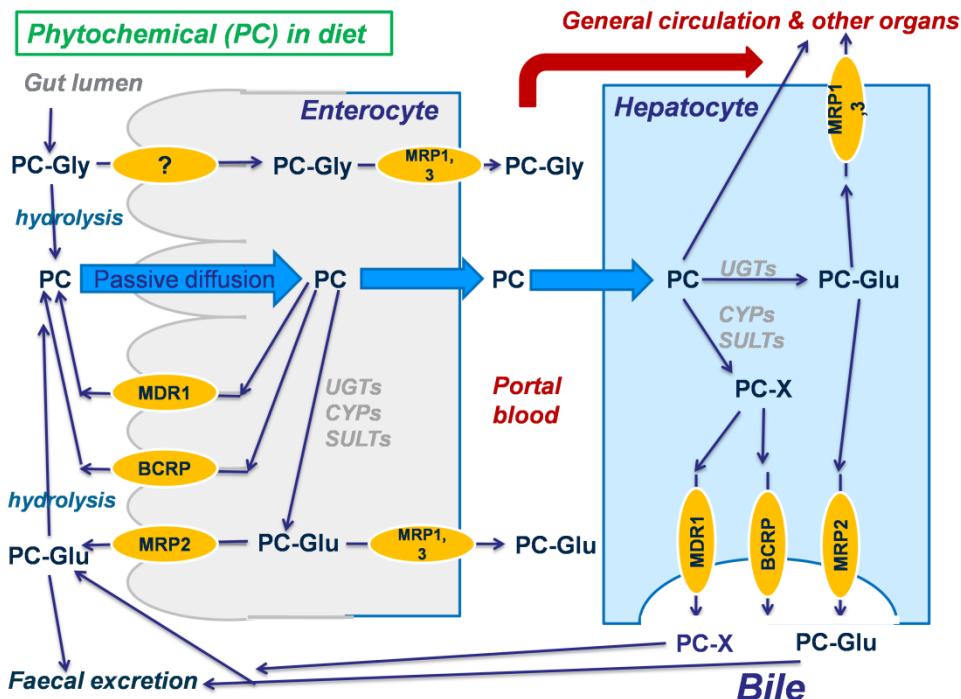


Fig. 3. Metabolism and disposition of phytochemicals (PC) and their metabolites in enterocytes and hepatocytes. PC-Gly, glycoside phytochemical; PC-Glu, glucuronide conjugate; PC-X, phytochemical metabolite

that after ingestion of some flavonoids, Phase 2 conjugates of the aglycon such as glucuronides, sulphates and methylated metabolites predominate in the blood circulation, rather than the original plant glycoside or aglycon (Janisch et al., 2004; Zhang et al., 2007). The extent to which these metabolites contribute to the overall beneficial effects of PCs in the body is largely unknown, and needs further investigation. Most evidence for drugs has indicated that their Phase 2 conjugates have little pharmacological activity but exceptions certainly exist, such as morphine-6-glucuronide and ezetimibe-glucuronide which appear to have greater pharmacological activity than their parent compound (Lotsch & Geisslinger, 2001; Kosoglou et al., 2005). In studies with green tea polyphenols, the metabolites mostly had reduced biological activity, but in some systems the metabolites had the equivalent or greater activity than the parent PC (Lambert et al., 2007). Other *in vitro* studies have shown that phloridzin-glucuronide is significantly more potent at protecting human SH-SY5Y neuroblastoma cells from hydrogen peroxide-mediated cell death than the parent molecule phloridzin (Stevenson et al., 2008), and that quercetin-3-glucuronide was significantly more potent than quercetin in a model of inflammation using human neutrophils (Suri et al., 2008). There is also evidence that the position of the glucuronide conjugate on the flavonoid can influence its biological activity (Day et al., 2000; O'Leary et al., 2003). Certainly these conjugated metabolic products are ideal substrates for various active transmembrane transport processes, in particular the excretory processes of the liver and kidney.

Although the Phase 1 and 2 metabolic enzymes are found in the epithelial cells of the gut wall, by far the greatest concentrations are found in the liver (Figure 3), where they form a major barrier to the further distribution of the parent PC to other organs, such as of the heart, kidney, lungs and brain. The liver's location and the portal venous blood supply from the intestines make it well suited for the protection of the body from possible toxic xenobiotics contained in our diet. During this first passage through the liver, many PCs will undergo substantial extraction and metabolism (known as first-pass metabolism). The resulting metabolic products are then exported back out of the liver into the blood stream and carried to the kidney where they may be excreted in the urine. Alternatively, metabolites such as glucuronide conjugates may be exported in the bile and released into the gut lumen. Thereafter, the metabolite conjugate may be excreted in the faeces, or alternatively it may be further metabolised by gut microbial enzymes, such as  $\beta$ -glucuronidase, which has the ability to cleave off the glucuronide and reform the less-polar aglycon, which may then be reabsorbed. This cycle is known as enterohepatic recirculation and may result in a longer exposure of the body to the PC. Evidence for such enterohepatic recirculation has been obtained for the flavonoid baicalein 7-O-glucuronide with a rat model (Xing et al., 2005), but whether a similar process occurs for some PCs in humans is not known.

#### 4. Distribution

Presumably, if the PC overcomes the defence mechanisms of the gut and the liver, it will enter the systemic circulation and be distributed in the bloodstream to the other major organs of the body and possible site(s) of action. In pharmacology, the term bioavailability is used to indicate the relative amount of the ingested parent xenobiotic that reaches the main cardiovascular circulation. Bioavailability is usually measured by taking peripheral blood samples over a period of time after ingestion and analysing for xenobiotic concentration. It is assumed that this blood concentration is an acceptable index for the concentration or

exposure at the site of action. Most PCs can pass with ease through the pores of the capillaries of organs such as the heart and lungs, but not some pharmacological sanctuaries, such as the brain, testis, fetus and stem cells. The tissue distribution of xenobiotics is significantly influenced by ABC transporters as the latter contribute to the maintenance of these pharmacological sanctuaries (Borst & Elferink, 2002; Huls et al., 2009; Mruk et al., 2011). For many PCs present in the bloodstream either as parent compound or metabolite, active efflux by ABC transporters may represent a major rate-limiting factor in their distribution or access to these sanctuaries. Phytochemical interactions with these efflux transporters could result in either: a further decrease in substrate PC distribution to the site, if the transporters were stimulated or induced; or accumulation of substrate, if the transporters were inhibited. The brain capillaries are surrounded with a protective cellular sheath of glial cells (the so-called blood brain barrier, BBB) resulting in permeability characteristics more closely resembling those of tightly bound tissue cell walls (Pardridge, 1993). To gain access to the brain, a PC must be highly lipid-soluble, or subject to uptake transport processes. MDR1, BCRP, MRP1, MRP3, MRP4 and MRP5 genes were shown by qRT-PCR to be expressed in the BBB (Dauchy et al., 2009). P-gp, BCRP and MRP1 have been located at the apical membrane of brain endothelial cells (Figure 4) (Kubota et al., 2006; Dauchy et al., 2009), and there is evidence that P-gp limits the penetration of various drugs across the BBB (Linnet & Ejsing, 2008). However the effects of several potent PC inhibitors of P-gp on passage across the BBB appeared to be minor (Tsai et al., 2001). Similarly, the uptake of the BCRP substrates, mitoxantrone and dehydroepiandrosterone sulfate, into the brain did not vary significantly between wild type and Bcrp knockout mice (Lee et al., 2005). In contrast, significant increases in the brain concentrations of various phytoestrogens including genistein (9.2-fold), daidzen (5.6-fold) and coumestrol (3.9-fold) were reported in Bcrp knockout mice compared to wild type (Enokizono et al., 2007). Similar results were also observed in the testis, suggesting that Bcrp may play a protective role reducing the accumulation of such compounds in both the brain and the testis. Likewise in a rat

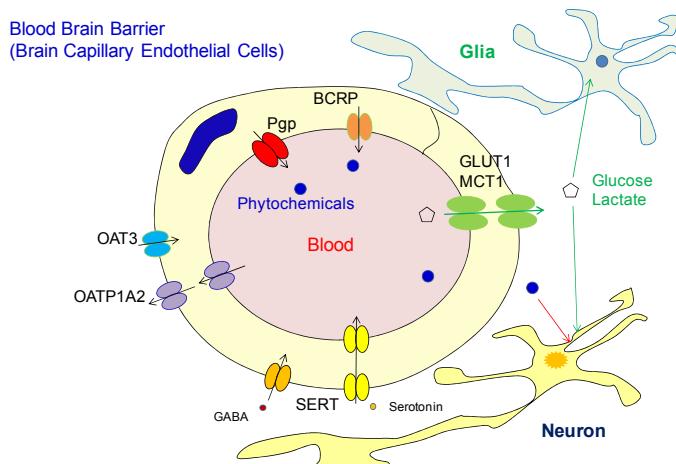


Fig. 4. Localization of ABC and SLC transporters in human BBB. GLUT1, glucose transporter 1; MCT1, monocarboxylate transporter 1; SERT, serotonin transporter

hemisphere perfusion study, the brain accumulation of quercetin was dramatically increased by pretreatment with GF120918 (a P-gp and BCRP inhibitor), but not with PSC833 (a P-gp inhibitor) (Yousdim et al., 2004). A recent study using the *ex vivo* rat BBB model indicated that curcumin inhibited BCRP activity at nanomolar concentrations and significantly increased the penetration of sulfasalazine across the BBB (Shukla et al., 2009). However, due to the low oral bioavailability of curcumin, such concentrations are rarely achieved with low to sub nanomolar concentrations typically being observed in plasma, even after oral dosing at 8 g/day for 18 months (Dhillon et al., 2008). BCRP is most abundantly expressed in the apical membrane of placental syncytiotrophoblasts, suggesting that it may play a role in protecting the fetus by impeding xenobiotic penetration across the placental barrier (Jonker et al., 2000; Evseenko et al., 2006). It has been shown that genistein accumulates in *Bcrp* knockout mice fetuses when genistein was included in the diet of pregnant mice during gestation (Enokizono et al., 2007), implying the involvement of *Bcrp* in genistein efflux in mouse placenta. However, a recent study shows that genistein at low, environmentally relevant concentration (10 ng/mL) can transfer across the human placenta at term at a extent similar to antipyrine (a well-known passive diffusion marker) in a placenta perfusion system (Balakrishnan et al., 2010), which suggests human BCRP may play a minor role in limiting the fetal exposure to genistein. These discrepancies may be due to species differences or differences between *in vivo* and *ex vivo* experimental systems. Since fetal exposure to genistein may have adverse consequences with regard to the development of the fetus (North & Golding, 2000), the regulatory role of BCRP in genistein transfer in term placenta needs further studies. As many flavanoids present in diet are potent modulators of BCRP and dietary flavonoids are in greater use, their influence on fetal exposure to various PC substrates of BCRP may also require further studies.

## 5. Excretion

The ABC and SLC transporters are abundantly expressed in the liver and kidney and regulate the excretion of many compounds, including PCs and their metabolites. In the liver, OAT2, OCT1 and OATPs are located in the sinusoid membrane to extract xenobiotics from blood; while BCRP, P-gp and MRP2 are found in the hepatocyte canalicular membrane effluxing compounds into the bile (Hooiveld & van Montfoorta, 2000). The inhibition of these transporters in hepatocytes can increase the concentrations of PC substrates in the bloodstream and/or decrease their biliary excretion and prolong their stay in the body. For example, biliary excretion of glucuronide and sulfate conjugates of silymarin flavonolignans was reduced by approximately 96 and 78%, respectively, in *Mrp2*-deficient Wistar rats, compared to wildtype (Miranda et al., 2008). Since biliary excretion of glucuronide and sulfate conjugates is the major route for silymarin's elimination in humans and rodents (Miranda et al., 2008), the pharmacokinetics of silymarin may be susceptible to MRP2 inhibition/induction or to pathological conditions where MRP2 may be deficient, such as in cholestatic liver disease or in Dubin-Johnson Syndrome (Keitel et al., 2000; Borst & Elferink, 2002). In contrast, MRP1 and 3 are found at the sinusoid membrane effluxing substrates back into the bloodstream, but are expressed at low levels under normal conditions (Ros et al., 2003). However in situations where MRP2 may be deficient, MRP3 may be upregulated, apparently to compensate for the diminished ability to excrete organic acids into bile (Keitel et al., 2000). Chronic administration of PCs however, may result in the upregulation of transporters via activation of the pregnane X receptor (PXR) or the aryl hydrocarbon

receptor (AhR) in hepatocytes, lessening the effects of inhibition (Lim & Lim, 2006; Zhang et al., 2007; Zhang et al., 2009).

In the kidneys, P-gp, MRP2, MRP4 and BCRP are expressed in the apical membranes and OCT2, OATs and OATP4C1 in the basolateral side of the cells lining the proximal tubules, transporting xenobiotics out of the blood into the urine (Ichikawa et al., 1991; van Aubel et al., 2002; Leslie et al., 2005; Huls et al., 2008; Giacomini et al., 2010). MRP2, 4 and BCRP efflux anionic or conjugated compounds (e.g., Phase 2 metabolites of some flavanoids), while more hydrophobic compounds are extruded by P-gp. As many PCs are excreted as conjugated metabolites by the kidney, there exists the possibility of competitive inhibition of these transport processes with drugs whose major route of elimination is by the kidney. It would appear cautionary to monitor plasma concentrations for drugs with a narrow therapeutic index in patients who are also consuming large amounts of PCs, perhaps as herbal medications or health supplements.

## 6. Modulation of bioavailability by PCs

There is accumulating evidence that PCs are able to modulate the activity of some ABC and SLC transporters by numerous mechanisms, resulting in significant changes in the oral bioavailability of substrate xenobiotics. For example, 1-hour pretreatment of mice with oral curcumin (400 mg/kg) resulted in a 13-fold increase in the bioavailability (as measured by the area under the plasma concentration-time curve (AUC)) of the Bcrp substrate sulfasalazine (anti-inflammatory drug) (Shukla et al., 2009). Although inhibition of sulfasalazine's metabolism could not be ruled out, it was believed that direct competitive inhibition of Bcrp was the major mechanism involved. In contrast, more prolonged treatment of rats with a lower dose of oral curcumin (60 mg/kg for 4 days) produced a down regulation in P-gp concentrations (> 50% reduction) in the gut, leading to a 1.6-fold increase in the AUC of the  $\beta$ -blocking drug, celiprolol (Zhang et al., 2007). The latter does not undergo metabolism and is a P-gp substrate, and thus the reduction in P-gp in the gut was deemed responsible for the increased bioavailability of celiprolol. It was also intriguing to note that the effect of curcumin was tissue specific, resulting in a greater than 2-fold P-gp increase in the liver but no effect in the kidney. Most studies of PC-transporter interactions have been undertaken using *in vitro* systems (Table 2) or *in vivo* animal studies and the difficulty in extrapolating to humans is apparent. Recently Molnar et al. (2006) have demonstrated that a wide range of lipophilic PCs (diterpenes, triterpenes and carotenoids) were able to inhibit human P-gp *in vitro* at the low  $\mu\text{g}/\text{ml}$  range, whereas other combinations had positive synergistic activity (Molnar et al., 2006). Using purified PCs on P-gp over-expressing cells *in vitro*, Patel et al. (2004) showed that quercetin, hypericin and kaempferol were able to increase the cellular uptake of ritonavir by 5- to 8-fold (Patel et al., 2004). It is also interesting to note that *in vitro* assays or short-term exposure to these polyphenols *in vivo* appears to inhibit the action of efflux pumps and increase substrate bioavailability, whilst chronic exposure in healthy volunteers actually increases the expression of P-gp. For example, extracts from St John's Wort (SJW) have been shown to up-regulate the expression of intestinal P-gp (Durr et al., 2000; Hennessy et al., 2002), that may subsequently reduce the bioavailability of substrates, such as the antiviral drugs indinavir and saquinavir that are used in the treatment of acquired immune deficiency syndrome (AIDS) (Perloff et al., 2001). Treatment with SJW is able to reduce plasma concentrations of

these drugs by up to 57% in healthy human volunteers, potentially leading to sub-therapeutic levels (Piscitelli et al., 2000). Hyperforin has been identified as the most likely candidate causing this inducing effect by binding and subsequent activation of PXR leading to increased expression not only of P-gp but also various Phase 1 and 2 metabolizing enzymes (Moore et al., 2000). In fact, the induction of CYPs and UGTs is probably the more important mechanism causing the majority of the many clinically significant drug interactions with SJW that have been reported (Hennessy et al., 2002).

Quercetin has been identified as a P-gp substrate and inhibitor, and has been reported to cause a 55 % increase in oral bioavailability of fexofenadine in healthy volunteers (Kim et al., 2009). Fexofenadine is non-sedating antihistamine which is a P-gp substrate that undergoes negligible metabolism in humans. Interestingly the oral bioavailability of fexofenadine is significantly decreased when taken with grapefruit juice (GFJ) or one of its major PC components, naringin (Bailey et al., 2007). The mechanism responsible is thought to be

	P-gp	BCRP	MRP1	MRP2	MRP4	MRP5	References
<i>Phytochemicals</i>							
<b>Apigenin</b>	?	I	I	I	?	?	(Versantvoort et al., 1994; Imai et al., 2004)
<b>Biochanin A</b>	I <sup>ca</sup>	I <sup>ca</sup>	I <sup>ca</sup>	?	?	?	(Versantvoort et al., 1993; Zhang & Morris, 2003; Zhang et al., 2004)
<b>Curcumin</b>	I	I	I	?	?	I	(Anuchapreeda et al., 2002; Chearwae et al., 2006; Shukla et al., 2009; Li et al., 2010)
<b>Cyanidin</b>	I	I	?	?	?	?	(Dreiseitel et al., 2009)
<b>Daidzein</b>	?	I <sup>ca</sup>	I	?	?	?	(Versantvoort et al., 1994; Imai et al., 2004)
<b>EGCG</b>	x	?	S	S	?	?	(Jodoi et al., 2002; Hong et al., 2003)
<b>Epicatechin</b>	?	?	S	S	?	?	(Hong et al., 2003)
<b>Genistein</b>	I <sup>ca</sup>	S <sup>ca</sup> , I <sup>ca</sup>	I	S	?	?	(Imai et al., 2004)
<b>Narigenin</b>	S, I	I	I	S	?	I	(Imai et al., 2004)
<b>Naringin</b>	I <sup>ca</sup>	I	?	?	?	?	(Imai et al., 2004)
<b>Puerarin</b>	?	?	?	?	?	?	(Imai et al., 2004)
<b>Quercetin</b>	S, I	I	I	S, I	I	I	(Wu et al., 2005)
<b>Resveratrol</b>	I	I	I	?	I	x	(Nabekura et al., 2005; Wu et al., 2005; Breedveld et al., 2007)
<b>Silymarin</b>	I <sup>ca</sup>	I	I	?	I	I	(Zhang & Morris, 2003; Cooray et al., 2004; Wu et al., 2005)

Table 2. Phytochemical substrates and inhibitors of P-gp, MRPs and BCRP. I, inhibitor; S, Substrate; Ca, Experiments carried only in cancer cells; x, not a substrate or inhibitor; ? Not determined.

inhibition of OATP1A2 which is involved in intestinal uptake of fexofenadine, and naringin (and hesperidin, also found in GFJ) have been identified as potent inhibitors of this transporter. This is an uncharacteristic interaction with GFJ as most reported interactions have involved markedly increased drug bioavailability, often resulting in significant toxicity (Bailey et al., 1991). Further studies have indicated that the main mechanism was inhibition of intestinal CYP3A4 by the furanocoumarins in GFJ, including bergamottin and 6',7'-hydroxybergamottin (Dahan & Altman, 2004). However these may not be the sole contributors, as other PCs found in GFJ, such as quercetin and kaempferol have also been shown to cause inhibition of the CYPs *in vitro* (Zhou et al., 2003; Dahan & Altman, 2004; Rodeiro et al., 2008). These studies serve to illustrate the potential complexity of the interactions between multiple PCs acting by different mechanisms dependent on the period of exposure to modulate uptake and efflux transporters and metabolizing enzymes in both enterocytes and hepatocytes.

## 7. Improving the bioavailability of PCs

Although many PCs have postulated health benefits, most appear to suffer from poor oral bioavailability which makes their utility as such agents rather tenuous. The biological activity of many of these PCs have been amply demonstrated *in vitro* but the effects *in vivo* are much more limited, probably due to the sub-micromolar concentrations achieved in plasma after ingestion (Baur & Sinclair, 2006; Moon & Morris, 2007; Zhang et al., 2007) (Anand et al., 2007). Apart from modulating ABC transporter function, PCs can also act as substrates for these efflux pumps, which can severely limit their bioavailability. For example, quercetin has very limited bioavailability through gut epithelia and regardless of the amount consumed orally, plasma concentrations of quercetin rarely exceed 1 µM. Although not specifically proven, this is likely to be at least in part due to BCRP expression in gut epithelia (see (Murakami & Takano, 2008) for review). Curcumin has well demonstrated antitumour activity *in vitro* and is currently in clinical trials for the treatment of various cancers but with limited success (Dhillon et al., 2008; Hatcher et al., 2008). However, the poor absorption and rapid first-pass metabolism resulting in poor oral bioavailability and low systemic concentrations, continue to be a major problem with curcumin's use in the clinic and appear to be responsible for the disconnect between curcumin's *in vitro* vs *in vivo* biological activity (Anand et al., 2007). To overcome this, the development of liposomal and nanoparticle formulations of curcumin has been investigated with reports of significant increases in its bioavailability (Bisht et al., 2007; Chen et al., 2009; Cui et al., 2009). Such strategies are also being employed to increase the oral bioavailability of resveratrol to improve its therapeutic potential (Santos et al., 2011). However, P-gp substrates (e.g., paclitaxel), delivered to P-gp overexpressing cells by nanoparticles, are susceptible to efflux by P-gp (Chavanpatil et al., 2006). It has also been shown that conjugation of negatively charged nanoparticles to glutathione led to nanoparticle efflux from the cell via MRP-transporters (Zhang et al., 2007; Holpuch et al., 2011). Some excipients are also potent ABC transporter inhibitors (Yamagata et al., 2007) and may be used in novel formulations to further improve PC bioavailability and targeted tissue distribution.

## 8. Conclusions

In conclusion, it is now apparent that the physicochemical properties of phytochemicals are not the only factors determining their oral bioavailability, and that there is a complex

interplay between these properties and the processes of metabolism and active transport in absorption, distribution and excretion, which determine the extent of exposure to their bioactive site(s) in the body. There is still much to be learned about the application of phytochemicals to the improvement of human health as these compounds have multiple complex effects on the body apart from their interactions with drug metabolizing enzymes and transporters. Despite limited data from human studies, the sheer amount and diversity of available phytochemicals continues to encourage researchers to look for new candidates with therapeutic potential and to improve their bioavailability and consequently their efficacy by developing new delivery systems.

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## **Part 2**

### **Anticancer Properties**



# Anticancer and Antimicrobial Potential of Plant-Derived Natural Products

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## 1. Introduction

Plant use in treating diseases is as old as civilization (Fabricant & Farnsworth, 2001) and traditional medicines are still a major part of habitual treatments of different maladies (Alviano & Alviano, 2009). In recent times and due to historical, cultural, and other reasons, folk medicine has taken an important place especially in developing countries where limited health services are available. However, the absence of scientific evaluation of medicinal plants to validate their use may cause serious adverse effects (Souza et al., 2004).

Plants are considered as one of the main sources of biologically active materials. Recent records reported that medicinal herbs are used by 80% of the people living in rural areas as primary healthcare system (Sakarkar & Deshmukh, 2011). It has been estimated that about 50% of the prescription products in Europe and USA are originating from natural products including plants or their derivatives (Cordell, 2002; Newman et al., 2003). Out of the 250,000 - 500,000 plant species on earth, only 1-10 % have been studied chemically and pharmacologically for their potential medicinal value (Verpoote, 2000). In the Middle East region 700 species of the identified plants are known for their medicinal values (Azaizeh et al., 2006).

In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous (Raskin et al., 2002). Compared with chemical synthesis, plant derived natural products represent an attractive source of biologically active agents since they are natural and available at affordable prices (Ghosh et al., 2008). Also plants derived agents may have different mechanisms than conventional drugs, and could be of clinical importance in health care improvement (Eloff et al., 1998). Plant materials might be bioactive secondary metabolites that have the potential to treat different afflictions. Examples of these compounds include phenols, phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides and glucosinolates (Mukherjee et al., 2001; Quiroga et al., 2001). Such plant derived natural products are the main focus of many scientists to develop new medication for different diseases like cancer and microbial infection.

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells (Karp, 1999). The high mortality rate among cancer patients is an indication of the

limited efficiency of the current therapies including radiation, chemotherapy and surgery (Xu et al., 2009). Cancer development is a multi-step process including induction of genetic instability, abnormal expression of genes, abnormal signal transduction, angiogenesis, metastasis, and immune evasion (Boik, 2001). For many years, scientists were searching for miracle cures for cancer using chemically synthesized or natural pure compounds. In the last few decades, research has been focused on the use of natural products such as crude plant extracts or a combination of different phytochemicals for cancer therapy; this trend is based upon: first, the synergistic effect of the different plant metabolites in the crude extract, second, is the multiple points of intervention of such extracts (Neergheen, 2009). This is one of the many faces of using plants in the quest of controlling different diseases. Another face is the use of such plant products in controlling microbial resistance spread. As a result of the uncontrolled use of many antibiotics, their efficiency is being threatened by the emergence of microbial resistance to existing chemotherapeutic agents (Cowan, 1999; Pareke & Chanda, 2007). Bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), pencillin-resistant *Streptococcus pneumoniae* (PRSP), and Vancomycin- resistant enterococci (VRE) in addition to the development of multidrug-resistant (MDR) bacterial strains (Alanis, 2005) are just few examples that made the search for new and novel bioactive substances among the first priorities in the search for antitumor, antibacterial, and antifungal substances (Ficker et al., 2005).

Realizing all the aforementioned, it is clear that there is a pressing need to participate in the search for new and novel bioactive agents that would help in providing new avenues in fighting diseases and reducing suffering. This chapter will provide information about selected plants that have a potential to provide new anticancer and/or antimicrobial agents.

## 2. The history of traditional medicine

For thousands of years and in different parts of the world, medicinal plants have been used to treat different diseases (Palombo, 2009). Fossil records documented the use of medicinal plants by humans before 60,000 years (Fabricant & Farnsworth, 2001). Nowadays plants continue to be the major source of medicine in rural regions of developing countries (Chitme et al., 2003) and it has been estimated that about 80% of peoples in developing countries are still using medicinal plants for their health care ( Kim, 2005).

The Eastern region of the Mediterranean has been characterized by high inventory of medicinal herbs used by local traditional healers to treat different ailments (Azaizeh et al., 2006). Research on medicinal plant treasures is based on present day and historical systems of traditional and local medicine (Al-Qura'n, 2009). During the Ottoman Empire and following the Byzantine traditions, hospitals were run by physician who used pharmacists to gather medicinal plants and prepare remedies originating from classical Greek and folk medicinal practice (Littlewood et al., 2002).

A comprehensive study on practitioners and herbalist in Palestine revealed that approximately 129 plant species are still prescribed to treat different diseases including liver, digestive tract, respiratory system, skin, cancer and other diseases (Azaizeh et al., 2003). On the other hand, the high diversity of plant species in Jordan encouraged many to study the distribution and use of medicinal plants in this country. More than 100 herbalists were interviewed in an extensive survey for Jordanian medicinal plants indicated the

presence of 150 herbal plants used in folkloric medicine (Abu-Irmaileh & Afifi, 2003). Seventy nine plant species are still in use in traditional medicine in the Showbak region (south of Jordan) while forty six are part of the popular medicine in the Ajloun Heights region (north of Jordan) and some of the plants are used in both regions ( Aburjai et al., 2007; Al-Qura'n, 2009). Most of the practitioners were not licensed and have no scientific information about medicinal plants (Abu-Irmaileh & Afifi, 2003; Azaizeh et al., 2003).

The diversity of plants in the Mediterranean region is declining, were recent estimates reported less than 200-250 plant species are used to treat different ailments in the Arab traditional medicine (Said et al., 2002; Abu-Irmaileh & Afifi, 2003; Saad et al., 2005), compared to more than 700 species which were identified for their medicinal uses in previous decades (Azaizeh et al., 2006). The high rate of plant extinction on the earth necessitates an increase in the efforts to study plant natural products for their potential to provide treatment for different afflictions.

### **3. Cancer biology**

DNA damage causes conversion of normal cell into a cancer cell. Cancer cells lack the ability to communicate with their neighboring cells. The first cancer cell starts to divide producing daughter cells, which in turn divide to produce more and more cancer cells. As cancer cells divide, they develop malignant characteristics including metastasis, immune system evasion, and induction of blood vessels formation (angiogenesis). Continuous cell division of cancer cells lead to the formation of tumors. In solid tumors, blood vessels become structurally and functionally abnormal; this abnormality leads to heterogeneous blood flow which creates chronically hypoxic and acidic regions in the core of the solid tumor (Brown & Wilson, 2004). These hypoxic regions lead to the activation of angiogenesis and cell survival genes in addition to other genes that induce drug resistant (Chen et al., 2003). Furthermore, the low pH microenvironment of cancer cells in the tumor core may prevent the active uptake of some anticancer drugs (Mahoney et al., 2003).

The two traditional therapies (chemotherapy and radiation) are not greatly efficient in treating hypoxic cancer cells (Tannock et al., 1998). The killing effect of ionizing radiation depends on the presence of oxygen which is absent or very low in the tumor core and the poor vascularization minimizes the delivery of chemotherapeutic agents (Brown & Wilson, 2004). This makes the poorly vascularized regions of tumors a major obstacle to effective treatment and opens the door to other therapies that may use different mechanisms to targets highly resistant cancer cells.

### **4. Oncogenes and tumor suppressor genes**

Two sets of genes are controlling cancer development. Oncogenes are the first set of genes and are involved in different cell activities including cell division. However, overexpression of these genes transforms a normal cell into a cancer cell. On the other hand, the second set of genes (tumor suppressor genes) inhibits cancer cell formation by different mechanisms. Tumor suppressor genes are underexpressed in cancer cells while, oncogenes are overexpressed. Table 1 summarizes the main oncogenes and tumor suppressor genes and their role in cancer development. Oncogenes and their products represent good targets for cancer therapy. Other targets include enzymes involved in cell division like topoisomerases

that unwind the DNA during replication. The diversity of plant derived natural products can provide therapeutic products attacking different targets in cancer cells.

Oncogenes	Functions of their proteins
<i>Bcl-2</i>	Inhibits apoptosis and protect cancer cell from free radicals.
<i>c-myc</i>	Initiate cell division and inhibits differentiation.
<i>HER-2/neu( c-erb-2)</i>	Facilitates signal transduction, expressed in 33% of breast cancers.
<i>MDM2</i>	Protect cancer cells from apoptosis by binding and inhibiting <i>p53</i> (tumor suppressor gene that induces apoptosis in damaged cells).
<i>ras</i>	Facilitate tumor invasion by stimulation of collagenase production and inhibits apoptosis by increasing <i>MDM2</i> expression.
<i>fos and jun</i>	Promote uncontrolled proliferation by their participation in cell cycle initiation.
Tumor suppressor genes	Functions of their proteins
<i>Bax</i>	An inducer of apoptosis
<i>Cx32, Cx 43 and other connexin producing genes</i>	Inhibits carcinogenesis by restoring communication between cells through gap junctions.
<i>p53</i>	Initiates DNA repair and induce apoptosis in cells that cannot be repaired.

Reference: Boik, 2001

Table 1. The main oncogenes and tumor suppressor genes.

## 5. The use of plants in cancer therapy

Cancer is one of the major causes of death and the number of cancer patients is in continuous rise. Every year 2-3 % of deaths recorded world wide arise from different types of cancer (Madhuri & Pandey, 2009). The available treatment methods include surgery, chemotherapy, and radiation (Tannock et al., 1998). The increasing costs of conventional treatments (chemotherapy and radiation) and the lack of effective drugs to cure solid tumors encouraged people in different countries to depend more on folk medicine which is rooted in medicinal plants use (Wood-Sheldon et al., 1997). Such plants have an almost unlimited capacity to produce substances that attract researchers in the quest for new and novel therapeutics (Reed & Pellecchia, 2005). Although some plant products are used in cancer therapy, plant derived anticancer agents represent only one-fourth of the total treatments options. Since 1961, nine compounds originating from plants have been approved for use in cancer therapy in the United States. These agents are vinblastine, vincristine, navelbine, etoposide, teniposide, taxol, taxotere, topotecan, and irinotecan (Lee, 1999). In an extensive study, the anticancer properties of 187 plant species were evaluated. Among them, only 15 species have been used to treat cancer clinically (Kintzios, 2006). It was observed that different plants contain different bioactive compounds and these vary with area, climate and mode of agricultural practice if they are not present in wild environment.

Herbivory, pathogens and competition are the driving forces that induce plant species to develop chemical defense compounds. These plant origin compounds are good models for elucidation of their functional roles in medication and treatment of different afflictions (Wood-Sheldon et al., 1997). For example, the lignin in the roots of *Anthriscus sylvestris* showed an insecticidal activity (Kozawa et al., 1982). Poisonous plants exposed to frequent grazing by animals are commonly rich in alkaloids which have many biological activities including anticancer potential (Kintzios, 2006). However, the growth regulatory properties of some plant metabolites allow them to act as chemotherapeutical agents. Flavonoids from *Scutellaria baicalensis* act on cyclin-dependent kinases to inhibit cancer cell proliferation (Dai & Grant, 2003; Chang et al., 2004).

Thirteen distinct groups of plant-derived natural products with antitumor properties were documented (Kintzios, 2006). Among them, alkaloids (Facchini, 2001), phenylpropanoids (Dixon & Paiva, 1995) and terpenoids (Trapp & Croteau, 2001) are well known for their antitumor potentials.

An integrated part of cancer cell development is the resistance to programmed cell death (apoptosis). Re-establishment of apoptosis in cancer cells is a target mechanism for anticancer agents (Joshi et al., 1999). Some plant-derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal property for successful anticancer agents (Hirano et al., 1995). Identifying the mode of action of anticancer agents of plant origin provide helpful information for their future use. Thus it is important to screen the apoptotic potential of plants either in their crude extract form or as pure compounds (Tarapadar et al., 2001). Due to their multiple intervention strategies, crude plant extracts have been proposed to prevent, arrest, or reverse the cellular and molecular processes of carcinogenesis (Neergheen et al., 2009).

Since the distribution of bioactive compounds differs according to the plant used, different solvents were used to extract these compounds from different plants. The methanol extract of *Scutellaria orientalis* showed potent anti-leukemic activity against HL-60 cell line (Ozmen et al., 2010). The water extract of *Rheum officinale* exhibited significant antiproliferative activity by inducing apoptosis in MCF-7 and A549 cell lines (Li et al., 2009). A potent antiproliferative activity was also reported for the hexane extract of *Casearia sylvestris* stem bark against different cancer cell lines (Mesquita et al., 2009) and the butanol extract of *Pfuffia paniculata* demonstrated high cytotoxic activity against MCF-7 cell line (Nagamine et al., 2009). Additionally, the *Physalis minima* chloroform extract induced apoptosis in human lung adenocarcinoma cell line (Leong et al., 2009). Out of 76 Jordanian plant species, the ethanolic extracts of *Inula graveolens*, *Salvia dominica*, *Conyza canadiensis* and *Achillea santolina* showed potent antiproliferative activity against MCF-7 cell line (Abu-Dahab & Afifi, 2007). The aqueous methanol of *Ononis hirta* and *Inula viscosa* showed high ability to selectively target MCF-7 cancer cells and induced apoptosis (Talib & Mahasneh, 2010a).

A substantial progress has been made in the treatment of cancer since the early years of anticancer drug research. An example of successful anticancer plant products are the vinca alkaloids which were isolated from *Catharanthus roseus*. The first identified vinca alkaloids were Vincristine and Vinblastine (Duflos et al., 2002). The antitumor activities of these compounds involve binding to tubulin and disruption of mitotic spindle assembly (Nobili et al., 2009). Myelosuppression and neurotoxicity were reported as side effects of vinca

alkaloids (Yun-San et al., 2008). Another example of plant products in cancer therapy is taxol which was extracted from the bark of *Taxus brevifolia* (Wani et al., 1971). Its cytotoxic activity is mediated by stabilizing microtubules rather than destabilizing them (Horwitz et al., 1993). Since taxol drugs have low solubility, they are administered together with solvents which cause some adverse effects like hypersensitivity and neuropathies (Onetto et al., 1993). Other anticancer plant products mediate their activities by inhibiting the enzyme DNA topoisomerase. Plants under this category include camptothecins extracted from the Chinese tree *Camptotheca acuminata* (Wall et al., 1966) and Podophyllotoxins extracted from the roots of the Indian plant *Podophyllum peltatum* (Nobili et al., 2009).

In spite of the success of previously mentioned anticancer plant products, the development of multi-drug resistance in cancer chemotherapy still one of the major problems (Xu et al., 2009). To avoid this problem, researchers focused on other targets including starving cancer cells by targeting the process of angiogenesis (formation of new blood vessels) which represent an attractive target since tumors depend on angiogenesis for survival and metastasis (Griggs et al., 2001). Some plant derived agents showed promising anti-angiogenic activity such as stilbene combretasatin- A4 which was isolated from *Combretum caffrum* (Young & Chaplin, 2004). Another strategy to target multi-drug resistance cancers is to use a combination of chemotherapy with other strategies including anaerobic bacteria which experimentally showed promising results in targeting solid tumors (Dang et al., 2001). Many plants exhibited promising anticancer activities (Table 2). Such plants may provide compounds that can change the list of drugs available for cancer treatment in the future.

Family	Species	Compounds
Anacardiaceae	<i>Rhus succedanea</i>	Flavones, aldehydes
Annonaceae	<i>Annona chrimola</i> , <i>A. muricata</i> , <i>A. senegalensis</i> ,	Annonaceous acetogenins
	<i>A. reticulata</i> , <i>A. squamosa</i> , <i>A. bullata</i>	(lactones)
	<i>Goniothalamus gardneri</i> , <i>G. amuyon</i> and <i>G. giganteus</i>	lactones
	<i>Polyathia barnesii</i>	Clerodane diterpenes
	<i>Xylopia aromatica</i>	Annonaceous acetogenins
Apiaceae	<i>Anthriscus sylvestris</i>	Lignans
	<i>Seseli mairei</i>	Polyacetylenes
Apocynaceae	<i>Alstonia scholaris</i> R. BR.	Extract, indole alkaloids
	<i>Ervatamia divaricata</i> , <i>E. microphylla</i> , <i>E. heyneana</i>	Alkaloids
	<i>Plumeria rubra</i>	Alkaloids (iridoids), lignans
Araliaceae	<i>Dendropanax arboreus</i>	Oxylipins (linoleic acid derivatives)
	<i>Panax ginseng</i> , <i>P. quinquefolius</i> , <i>P. vietnamensis</i>	Saponins, polysaccharides, polyacetylenic alcohols

Aristolochiaceae	<i>Aristolochia elegans, A. versicolor</i>	Sesquiterpene lactones
Asclepiadaceae	<i>Calotropis procera, C. gigantea</i>	Glycosides
Asteraceae	<i>Neurolaena lobata</i>	Sesquiterpene lactones
Bignoniaceae	<i>Kigelia pinnata</i>	Dichloromethane extracts
Burseraceae	<i>Bursera simaruba, B. permollis, B. morelensis, B. microphylla, B. klugii, B. schlechtendalii</i>	Lignans
Caesalpiniaceae	<i>Caesalpinia sappan</i>	Ethyl acetate extracts
Campanulaceae	<i>Platycodon grandiflorum</i>	Polysaccharides
Capparaceae	<i>Polanisia dodecandra</i>	Flavonols
Celastraceae	<i>Glyptopetalum sclerocarpum</i>	Terpenoids
	<i>Maytenus boaria, M. guangsiensis, M. ovatus, M. senegalensis, M. wallichiana</i> and <i>M. emarginata</i>	Triterpenes
Combretaceae	<i>Bucida buceras</i>	Flavanones, diterpenes
	<i>Terminalia arjuna</i>	Flavones, tannins
Compositae	<i>Eupatorium cannabinum</i>	Lactones
	<i>E. cuneifolium, E. rotundifolium, E. semiserratum, E. altissimum</i>	Flavones
	<i>Hellenium microcephalum, H. hoopesii</i>	Sesquiterpene lactones
	<i>Xanthium strumarium</i>	Alkaloids
	<i>Inula viscosa</i>	Flvonoids and alkaloids
	<i>Inula graveolens, Achillea santolina, Conyza canadiensis</i>	Ethanol extract
Coniferae	<i>J. virginiana, J. chinensis</i>	Podophyllotoxin (lignans)
Cucurbitaceae	<i>Cucurbita moschata, Mormodica charantia</i>	Proteins
Cupressaceae	<i>Chamaecyparis lawsoniana, Thujopsis dolabrata</i>	Alkaloids
Ericaceae	<i>Vaccinium macrocarpon, V. smallii</i>	Triterpenes, flavonol, glycosides
Eriocaulaceae	<i>Paepalanthus latipes</i>	Naphthoquinones
Euphorbiaceae	<i>Emblica officinalis</i>	Aqueous extracts, Norsesquiterpenoid, glycosides
	<i>Euphorbia amygdaloides, E. helioscopic, E. lathyris, E. mongolica, E. pubescens</i>	Jatrophane diterpenoids
	<i>Jatropha curcas, J. macrorhiza</i>	Jatrophane diterpenoids and triterpenoids
	<i>Mallotus philippinensis</i>	Rottlerin, phloroglucinol

		derivatives
	<i>Phyllanthus acuminatus, P. amarus, P. emblica, P. urinaria</i>	Glycosides
Fabaceae	<i>Ononis sicula, Ononis hirta</i>	Aqueous methanol extract
Flacourtiaceae	<i>Caesaria sylvestris</i>	Clerodane diterpenes
Guttiferae	<i>Garcinia hunburyi</i>	Processed extract
Hernandiaceae	<i>Hernandia sp.</i>	Lignans
Hyacinthaceae	<i>Scilla scilloides, S. peruviana</i>	Glycosides
Hypericaceae	<i>Hypericum perforatum, H. drummondii</i>	Polycyclic diones
Iridaceae	<i>Crocus sativus</i>	Carotenoids
Lamiaceae	<i>Hyptis martiusii, H. verticillata</i>	Diterpenes, lignans
	<i>Origanum vulgare, O. majorana</i>	Quinines, quinine glycosides
	<i>Rabdosia ternifolia, R. trichocarpa, R. macrophylla</i>	Diterpenoids, lactones
	<i>Salvia sclarea</i>	Lectins, Ursolic acid (carboxylic acids)
	<i>Salvia pinardi</i>	Aqueous methanol extract
	<i>Salvia przewalskii</i>	Quinones
	<i>Salvia dominica</i>	Ethanol extract
	<i>Scutellaria barbata, S. lateriflora, S. baicalensis</i>	Flavonoids, flavones
Lauraceae	<i>Cinnamomum camphora</i>	Cinnamaldehydes
Leguminosae	<i>Acacia catechu, A. victoriae, A. confuse, A. auriculiformis A. Cunn. and A. nilotica</i>	Proteins
	<i>Bauhinia racemosa</i>	Methanol extracts
	<i>Cassia acutifolia, C. angustifolia, C. torosa, C. leptophylla</i>	Anthraceranes, polysaccharides, piperidin (alkaloids)
	<i>C. pudibunda Glycyrrhiza glabra, G. uralensis, G. inflata</i>	Anthraquinones Glycyrrhetic acid, glycyrrhetic acid, flavonoids and
	<i>Tamarindus indica</i>	Triterpenoids, Polysaccharides
Liliaceae	<i>Colchicum autumnale, C. speciosum Crinum asiaticum var. toxicarium</i>	Alkaloids
Linaceae	<i>Linum usitatissimum</i>	Lignans
	<i>Viscum cruciatum</i>	Hexanoic acid extracts
Magnoliaceae	<i>M. officinalis, M. grandiflora, M. virginiana</i>	Neolignans
Malvaceae	<i>G. herbaceum G., indicum</i>	Cathechin (phenolics)

Meliaceae	<i>Azadirachta indica</i>	Limonoids (triterpenes)
	<i>Melia azedarach</i>	Limonoids (triterpenes)
Myrtaceae	<i>Eugenia jambos</i>	Hydrolyzable tannins
Ochnaceae	<i>Ouratea hexasperma, O. semiserrata</i>	Biflavonoids
Pinaceae	<i>Pseudolarix kaempferi</i>	Triterpene lactones, diterpenes
Polygalaceae	<i>Polygala vulgaris</i>	Xanthones
Polygonaceae	<i>Polygonum cuspidatum</i>	Flavonoids, anthraquinones
Ranunculaceae	<i>Nigella sativa</i>	Quinones, fatty acids
	<i>Pulsatilla koreana</i>	Lignans, saponins
Rosaceae	<i>Agrimonia pilosa</i>	Tannins, methanolic extracts
Rubiaceae	<i>Nauclea orientalis</i>	Angustine alkaloids
	<i>Psychotria. rubra, P. forsteriana</i>	Naphthoquinones, alkaloids
	<i>Rubia cordifolia, R. akane</i>	Cyclic hexapeptides Naphthoquinones, anthraquinones
Rutaceae	<i>Aegle marmelos Correa Fagara macrophylla</i>	Hydroalcoholic extract Alkaloids
	<i>Acronychia oblonga, A. porteri, A. pedunculata, A. Baueri</i>	Flavonols, alkaloids
	<i>Zieridium pseudobatusifolium</i>	Flavonols
Sapindaceae	<i>Koelreuteria henryi</i>	Anthraquinone, stilbene, and flavonoids
Simaroubaceae	<i>Brucea dysenterica, B. javanica</i>	Quassinoïd glycosides, Alkaloids
	<i>Eurycoma harmadiana</i>	Alkaloids, quassinoïds
	<i>Hannoia chlorantha, H. kleineana</i>	Quassinoïds, chaparrinones
Solanaceae	<i>Solanum pseudocapsicum</i>	Alkaloids
Scrophulariaceae	<i>Verbascum sinaiticum</i>	Aqueous methanol extract
Theaceae	<i>Camellia sinensis</i>	Polyphenols
Thymelaceae	<i>Stellera chasmaejasme</i>	Diterpenes
Tropaeolaceae	<i>Wikstroemia foetida, W. uvaursi, W. indica, Tropaeolum majus</i>	Polysaccharides, Aromatic plant hormones
Umbelliferaeae	<i>Angelica archangelica A. keiskei, A. sinensis, A. gigas, A. acutiloba, A. radix, A. japonika, A. edulis</i>	Pyranocoumarins, chalcones, polysaccharides
Urticaceae	<i>Ficus carica</i>	Lectins
Verbenaceae	<i>Vitex rotundifolia</i>	Flavonoids
Violaceae	<i>Viola odorata</i>	Nucleotides

**References:** (Kintzios, 2006), (Abu-Dahab and Afifi, 2007), (Talib and Mahasneh, 2010)

Table 2. Plants with potential anticancer activity

## 6. Plants as a source of antimicrobial agents

The discovery of antibiotics has decreased the spread and severity of a wide variety of inferior diseases. However, and as a result of their uncontrolled use, the efficiency of many antibiotics is being threatened by the emergence of microbial resistance to existing chemotherapeutic agents (Cowan, 1999). While bioactive natural compounds have been isolated mainly from cultivable microbial strains, an untapped biologically active metabolites of different resources including plants remains to be investigated (Quiroga et al., 2001) to alleviate or help responding to current health care situations; such situations include but not limited to unmet clinical needs, increasing cost of chemotherapy, mycobacterial reemergence, and the emergence of antibiotic resistant microbial strains such as MRSA (Alanis et al., 2005).

Microbial resistance occurs mainly in three general mechanisms: prevention of interaction of the drug with target; direct destruction or modification of the drug; and efflux of the drug from the cell (Alviano & Alviano, 2009). These mechanisms were used by different microorganisms and led to the emergence of many pathogenic bacterial strains (Alanis et al., 2005). With pathogenic fungi, the situation is not so bright also, where Amphotericin B was for many years the only treatment available for fungal infections. In late 1980s fluconazole and itraconazole was developed as additional therapeutic options (Ficker et al., 2002). Recently, azole derivatives are most widely used antifungal agents, although resistance for these drugs is emerging (Groll et al., 1998). All the available antifungal drugs used to date are not ideal in efficiency, safety, and antifungal spectrum (Di Domenico, 1998). Combination antifungal therapy was also used to increase the efficiency but there is a real demand for a next generation of safer and more powerful antifungal agents (Bartoli et al., 1998). Knowing that modifying known antimicrobial compounds is increasingly difficult created an urgent and very pressing need for isolation and identification of new bioactive chemicals from new sources including plants (Barker, 2006).

Plant derived natural products represent an attractive source of antimicrobial agents since they are natural, have manageable side effects and available at affordable prices (Ghosh et al., 2008). Also plants derived agents may have different mechanisms than conventional drugs, and could be of clinical importance in health care improvement (Ellof, 1998).

There are two main classes of plant derived agents. 1) phytoalexins which are low molecular weight compounds produced in response to microbial, herbivorous, or environmental stimuli (Van Etten et al., 1994). Phytoalexins include simple phenylpropanoid derivatives, flavonoids, isoflavonoids, terpenes and polyketides (Grayer & Harborne, 1994). 2) Phytoanticipins which are produced in plants before infection or from pre-existing compounds after infection (Van Etten et al., 1994). Phytoanticipins include: glycosides, glucosinolates and saponins that are normally stored in the vacuoles of plant cells (Osbourne, 1996). The antimicrobial potential of plant derived natural products is well documented. Schelz and colleagues reported the potential of menthol isolated from peppermint oil to eliminate the resistance plasmids of bacteria (Schelz et al., 2006). In another study carbazole alkaloids isolated from *Clausena anisata* stem bark showed high antibacterial and antifungal activities (Chakraborty et al., 2003).

Thousands of other phytochemicals having *in vitro* antimicrobial activities were also screened. Such screening programs are essential for validating the traditional use of

medicinal plants and for providing leads in the search for new antimicrobial agents (Alviano & Alviano, 2009). A number of studies of the bioactivity of plant extracts have been conducted and many of these studies showed promising results in developing new biologically active agents. The methanolic extract of clove *Caryophyllus aromaticus* showed antibacterial activity against many bacterial genera and the highest activity was against *Staphylococcus aureus* (Ushimaru et al., 2007). Association of clove extract and antibiotics showed synergistic antibacterial activity against antibiotic resistant *Pseudomonas aeruginosa* (Nascimento et al., 2000). Out of the 50 plants used in Indian traditional medicine, 72% showed antimicrobial activity including nine plants showed antifungal activity (Srivnivasan et al., 2001). When some Palestinian plants were tested for bioactivity, out of fifteen used in traditional medicine, only eight showed antibacterial activity against eight different bacterial strains (Essawi & Srour, 2000). Butanol extracts of *Rosa damascene*, *Narcissus tazetta*, and *Inula viscosa* exhibited potent antimicrobial activities against different microorganism including Methicillin-resistant *Staphylococcus aureus* and *Candida albicans* (Talib & Mahasneh, 2010b).

During the past decade there is an increase in the number of immuno-compromised patients. This is probably due to the alteration of the immune system caused by human immunodeficiency virus (HIV), cancer chemotherapy, and organ and bone marrow transplantation in addition to the use of immune suppressors to treat many diseases (Alexander & Perfect, 1997). The compromised immune system facilitates microbial infections including systemic mycosis. This leads to extensive use of Amphotericin B and azole derivatives as antifungal agents. Unfortunately, the wide range use of these antifungal agents leads to the emergence of drug resistant pathogenic fungi (Alexander & Perfect, 1997). *Candida albicans* is opportunistic yeast that can cause vaginal, oral, and lung infections in addition to systemic tissue damage in AIDS patients (Madigan & Martinko, 2006). This yeast was the target of many researchers to develop new antifungal agents. Out of twenty four medicinal plants used in traditional medicine in South Africa, two showed high potential to treat candidiasis (Motsei et al., 2003). Also some indigenous plants of Lebanon showed antimicrobial activity against *Candida albicans* and other tested microorganisms (Barbour et al., 2004).

Successive isolation of antimicrobial compounds from plants depends upon the type of solvent used in extraction procedure (Parekh & Chanda, 2007). Literature reported the use of different solvents to extract antimicrobial agents. The ethanol/methanol extracts of thirty four medicinal plants were more active than aqueous extracts against bacterial strains belonging to *Enterobacteriaceae* (Parekh & Chanda, 2007). Methanolic extract of *Terminalia chebula* showed higher antibacterial potential compared with aqueous extract (Ghosh et al., 2008). Mahasneh found that butanol extracts of several plants including: *Lotus halophilus*, *Pulicaria gnaphaloides*, *Capparis spinosa*, *Medicago laciniata*, *Limonium axillare* to exhibit superior antimicrobial activity compared with ethanol and aqueous extracts (Mahasneh, 2002). The ethanolic extract of 11 plant species from Argentina showed high antimicrobial activity against a list of microorganisms including methicillin, oxacillin, and gentamicin resistant *Staphylococcus* (Zampini et al., 2009). Of 16 plants studied, the methanolic and aqueous extracts of 10 Yemeni plants exhibited significant antimicrobial activity against three gram positive, two gram negative bacteria, and one fungus (Mothana et al., 2009).

Jordanian plants were the focus of many researchers for their antimicrobial activities. Butanol, ethanol, petroleum ether, and aqueous extracts were prepared from nine Jordanian plants. Butanol extract showed superior antimicrobial activity compared with other extracts (Mahasneh & El-Oqlah, 1999). Of 27 ethanol extracts prepared from indigenous Jordanian plants, six plants showed promising antimicrobial activity against different test microorganisms (Al-Bakri & Afifi, 2007). In addition to their broad antimicrobial activities some Jordanian plants like *Sonchus oleraceus* and *Laurus nobilis* exhibited high antiquorum sensing activities (Al-Hussaini & Mahasneh, 2009). Other Jordanian plants like *Crupina crupinastrum* and *Achillea biebersteinii* showed high antimicrobial activity against bacteria and fungi (Khalil et al., 2009). Additionally the methanolic extracts of two Jordanian plants *Artemisia herba-alba* and *Artemisia arborescens* showed high antibacterial activity against 32 isolates of *Mycoplasma* species (Al-Momani et al., 2007).

## 7. Conclusions

The potential isolation and use of new and novel bioactive products from plant origins is still very productive playground for the development of new drugs to improve health care in certain medical fields. It is essential to emphasize that extensive *in vitro* and *in vivo* tests must be conducted to assure the selection of active and nontoxic anticancer and antimicrobial phytochemicals.

## 8. References

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# Phytochemicals and Cancer Chemoprevention: Epigenetic Friends or Foe?

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## 1. Introduction

Cancer remains a major health problem and is responsible for one in eight deaths worldwide. Genome-wide association studies have identified hundreds of genetic variants associated with complex human diseases and traits, and have provided valuable insights into their genetic architecture. Despite the success of genome-wide association studies in identifying loci associated with cancer, a substantial proportion of the causality remains unexplained, leaving many questions how the remaining 'missing' heritability can be explained, although polygenic disease traits may account for some of these limitations (Maher, 2008; Manolio et al., 2009; Rakyan et al., 2011). Only a minority of cancers are caused by germline mutations, whereas the vast majority (90%) are linked to somatic mutations and environmental factors (Anand et al., 2008). Also, an estimated 55% increase in cancer incidence is expected by the year 2020 (Chaturvedi et al., 2011). A recent survey of the global incidence of cancer shows that the age-adjusted cancer incidence in the Western world is above 300 cases per 100,000 population, whereas that in Asian countries is less than 100 cases per 100,000. Observational studies have suggested that lifestyle risk factors such as tobacco, obesity, alcohol, sedentary lifestyle, high-fat diet, radiation, and infections are major contributors in cancer causes, which is further emphasized by the increase in cancer cases among immigrants from Asian to Western countries (Anand et al., 2008; Messina & Hilakivi-Clarke, 2009; Shu et al., 2009)). Reciprocally, a reasonable good fraction of cancer deaths may be prevented by modifying the diet composition (i.e. content of fiber, fruit, vegetable, fat/oil, protein, spices, cereals, etc.) and regular physical exercise (Anand et al., 2008; Bingham & Riboli, 2004; Boffetta et al., 2010; Tennant et al., 2010). Rather than the chemical conversion of food to energy and body matter of classic metabolism, food is now also a conditioning environment that shapes the activity of the (epi)genome and determines stress adaptative responses, metabolism, immune homeostasis and the physiology of the body.

The contribution of epigenetic changes (epimutations) in cancer is probably underestimated. Epigenetics encompasses several extra-genetic processes such as DNA methylation

(methylation of cytosines within CpG dinucleotides), histone tail modifications (including acetylation, phosphorylation, methylation, sumoylation, ribosylation and ubiquitination), noncoding RNA functions, regulation of polycomb group proteins and the epigenetic cofactor modifiers, all of which may alter gene expression but do not involve changes in the DNA sequence itself (Chi et al., 2010; Davalos & Esteller, 2010; Guil & Esteller, 2009; B. M. Lee & Mahadevan, 2009; Vanden Berghe et al., 2006b). Furthermore, many activities controlling chromatin dynamics require metabolites that shuttle between different cellular functions and pathways. One critical facet of histone and DNA modifying enzymes is that their activity also depends on intracellular levels of essential metabolites (acetyl-coA, Fe, ketoglutarate, NAD<sup>+</sup>, S-adenosylmethionine, see Figure 1) of which the concentrations are tightly linked to global cellular metabolism and energy levels (Bellet & Sassone-Corsi, 2010; Chang et al., 2010; Ladurner, 2009; Luo & Kuo, 2009; Wallace, 2010a; 2010b). Gene regulation is thus linked to the metabolic status of cells. To maintain uncontrolled cell proliferation of cancer cells, energy metabolism needs to be adjusted in order to fuel cell growth and invasion. In contrast to "healthy" cells which mainly generate energy from oxidative breakdown of pyruvate, cancer cell reprogram their glucose metabolism, limiting their energy metabolism largely to glycolysis. The fundamental difference in ratio of glycolysis to mitochondrial respiration between normal and cancerous cells is also known as the Warburg effect. As such, dynamic changes in energy levels and metabolite concentrations in the inflammatory tumor microenvironment can have significant epigenetic changes through variable activity of cofactor enzymes (Bonuccelli et al., 2010; Figueroa et al., 2010; Martinez-Outschoorn et al., 2011; Rathmell & Newgard, 2009; Teperino et al., 2010; Wellen et al., 2009).

The combinatorial nature of DNA methylation and histone modifications significantly extends the information potential of the genetic cancer code (Brower, 2011)(Figure 2). The most studied epigenetic lesion, which is DNA hypermethylation at the promoter region of many genes (Esteller, 2007; Mulero-Navarro & Esteller, 2008), is proved to be responsible for silencing of more than 600 cancer-related genes and this number is still rising. Besides effects on tumour suppressor genes, DNA methylation changes have also been detected in oncogenes as well as genes involved in the cell-cycle regulation, DNA repair, angiogenesis, metastasis and apoptosis (Herceg, 2007). Also oxidative stress (ROS, RNS) and inflammatory damage play an important role in epigenetic reprogramming of expression of cytokines, oncogenes and tumor suppressor genes, thereby setting up a ground for chronic inflammatory diseases and carcinogenesis (B. B. Aggarwal, 2009; B. B. Aggarwal & Gehlot, 2009; S. I. Grivennikov & Karin, 2010). On the other hand, global hypomethylation of the DNA is said to activate endoparasitic sequences and causes the global chromosome instability leading to various mutations and cancer progression (Esteller, 2008). Epigenetic defects in DNA methylation patterns at CpG sites (epimutations), abnormalities in histone modifications, chromatin remodelling and noncoding RNAs (microRNA, long noncoding RNA) or corrupt chromatin states of tumor suppressor genes or oncogenes recently emerged as major governing factors in tumor progression and cancer drug sensitivity (Backdahl et al., 2009; Davalos & Esteller, 2010; Esteller, 2007; 2008; Guil & Esteller, 2009; Hesson et al., 2010; Lai & Wade, 2011; Lujambio et al., 2008; Lujambio & Esteller, 2007; Vanden Berghe et al., 2006b). In addition, genetic mutations of epigenetic modifying ("writer") enzymes add another level of regulatory complexity (Chi et al., 2010; Dalgliesh et al., 2010; Delhommeau et al., 2009; Elsasser et al., 2011; Ko et al., 2010; Varela et al., 2011). Recent advances in genomic technologies have initiated large-scale studies to map cancer-associated epigenetic variation, specifically variation in DNA methylation and

chromatin states (Berdasco & Esteller, 2010; Birney et al., 2007; Brower, 2011; Ernst & Kellis, 2010; Ernst et al., 2011; Myers et al., 2011; Rakyan et al., 2011; Raney et al., 2011). Given the prevalence of reversible epigenetic abnormalities in different cancers, epigenetic therapy holds great promise for treatment. The future of specific and effective epigenetic drug design will rely on our ability in understanding epigenomic landscapes in normal and cancerous disease states (Hakim et al., 2011; Christopher A. Hamm & Costa, 2011; Gioacchino Natoli, 2010; G. Natoli et al., 2011; Tolhuis et al., 2011; B. van Steensel, 2011).

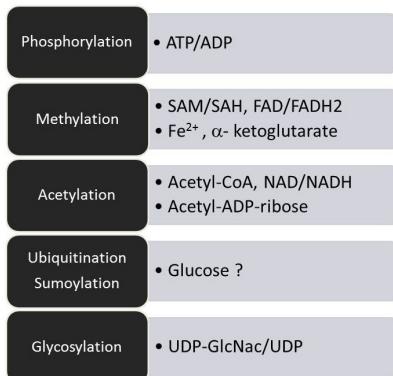


Fig. 1. Coupling of cancer metabolism, diet and epigenetics

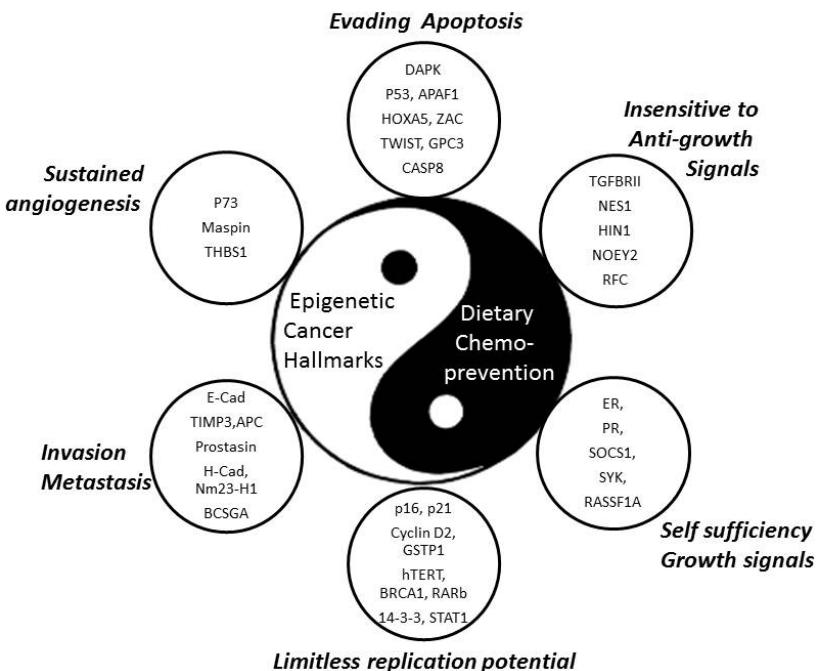


Fig. 2. Dietary reversal of epigenetic changes in cancer cells

Once critical facet of histone modifications is that they are elicited by specific enzymatic activities that depend on the intracellular levels of essential metabolites: these metabolites sense cellular metabolism, nutrients and energy levels in the cell.

Changes in DNA methylation have been recognized as one of the most common molecular alterations in human neoplasia and hypermethylation of gene-promoter regions is being revealed as one of the most frequent mechanisms of loss of gene function. This figure summarizes how changes in DNA-methylation (epimutations) contribute to the 6 hallmarks of a cancer cell i.e. limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, sustained angiogenesis and tissue invasion and metastasis. Since epigenetic changes (epimutations) are more easily reversible (when compared with genetic mutations), this has inspired various research efforts aiming to identify dietary phytochemicals (nutri-epigenomics) which can reverse epimutations and/or prevent cancer progression.

## 2. Dietary chemoprevention of cancer-inflammation

Cancer cells are distinguished by several distinct characteristics, such as self-sufficiency in growth signal, resistance to growth inhibition, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2011) (Figure 2). Tumor cells acquire these properties due to the dysregulation of multiple genes and associated cell signaling pathways, most of which are linked to inflammation. Immune cells also infiltrate in tumors, engage in an extensive and dynamic crosstalk with cancer cells (B. B. Aggarwal, 2009; S. I. Grivennikov et al., 2010a; Mantovani et al., 2008). Inflammation also affects immune surveillance and responses to therapy (D. Iliopoulos et al., 2011; M. Liu et al., 2010a; Rajasekhar et al., 2011; S. V. Sharma et al., 2010).

For that reason, rationally designed drugs that target a single gene product are unlikely to be of use in preventing or treating cancer. Moreover, targeted drugs can cause serious and even life-threatening side effects or therapy resistance (Hanahan & Weinberg, 2011). When a complex system starts to dysfunction, it is generally best to fix it early. Often, cancers have a long latency period –often 20 years or more-. By the time they are clinically detectable, the system has degenerated into a disorganized, chaotic mess at which point it may be beyond repair (Sporn, 2011). Therefore, there is an urgent need for safe and effective chemopreventive multifunctional drugs that act at entire networks in the body, rather than single targets (Deocaris et al., 2008). The basic idea of cancer chemoprevention is to arrest or reverse the progression of premalignant cells towards full malignancy using physiological mechanisms that do not kill healthy cells, but attenuate cancer-inflammation (B. B. Aggarwal & Gehlot, 2009; Anand et al., 2008; Jirtle & Skinner, 2007; Surh, 2003)(Figure 3).

The global demand for more affordable therapeutics and concerns about side effects of commonly used drugs has renewed interest in phytochemicals and traditional medicines which allow chronic use (Harvey, 2008; J. W. H. Li & Vederas, 2009; Singh, 2007). Studies on a wide spectrum of plant secondary metabolites extractable as natural products from fruits, vegetables, teas, spices, and traditional medicinal herbs have identified various bioactive plant phytochemicals that regulate multiple cancer-inflammation pathways and epigenetic cofactors, are cost effective, exhibit low toxicity, and are readily available (B. B. Aggarwal et al., 2011; Deorukhkar et al., 2007; Ki Won Lee et al., 2011; Yang & Dou, 2010). The recent

advances in genomics and metabolomics have enabled biologists to better investigate the potential use of immunomodulatory natural products for treatment or control of cancerous diseases. More recently, evidence is emerging that specific combinations of phytochemicals maybe far more effective in protecting against cancer than isolated compounds (Harvey, 2008; Kok et al., 2008). The cancer preventive or protective activities of the various immunomodulatory natural products lie in their effects on cellular defenses including detoxifying and antioxidant enzyme systems, and the induction of anti-inflammatory and antitumor or antimetastasis responses, often by targeting specific key transcription factors (i.e. like nuclear factor kappa B (NF $\kappa$ B), activator protein (AP-1), signal transducers and activators of transcription (STAT3), nuclear factor erythroid 2-related factor (NRF2), peroxisome proliferator-activated receptor-  $\gamma$  (PPAR  $\gamma$ ), estrogen receptor, liver X receptor (LXR), hypoxia inducible factor-1 (HIF-1)), epigenetic cofactors and microRNAs which are involved in tumor progression (Figure 4) (Meeran et al., 2010; Parasramka et al., 2011; Szarc vel Szcic et al., 2010). Typically, dysregulation of transcription factor activity is the result of numerous mechanisms, such as changes in gene expression, protein – protein interactions and post-translational modifications, leading to deregulation of gene products that are involved in both inflammation and carcinogenesis. Remarkably, “transient” inflammatory pathways can also trigger mitotic stable epigenetic switches from nontransformed to metastatic cancer cells via feedback signaling involving NF $\kappa$ B and Stat3 transcription factors, Lin28 and let-7 microRNAs and the cytokine IL6 in the tumor microenvironment (D. Iliopoulos et al., 2009; D. Iliopoulos et al., 2010). Furthermore, emerging data demonstrate the direct influence of certain anti-inflammatory dietary factors (for example polyphenols,

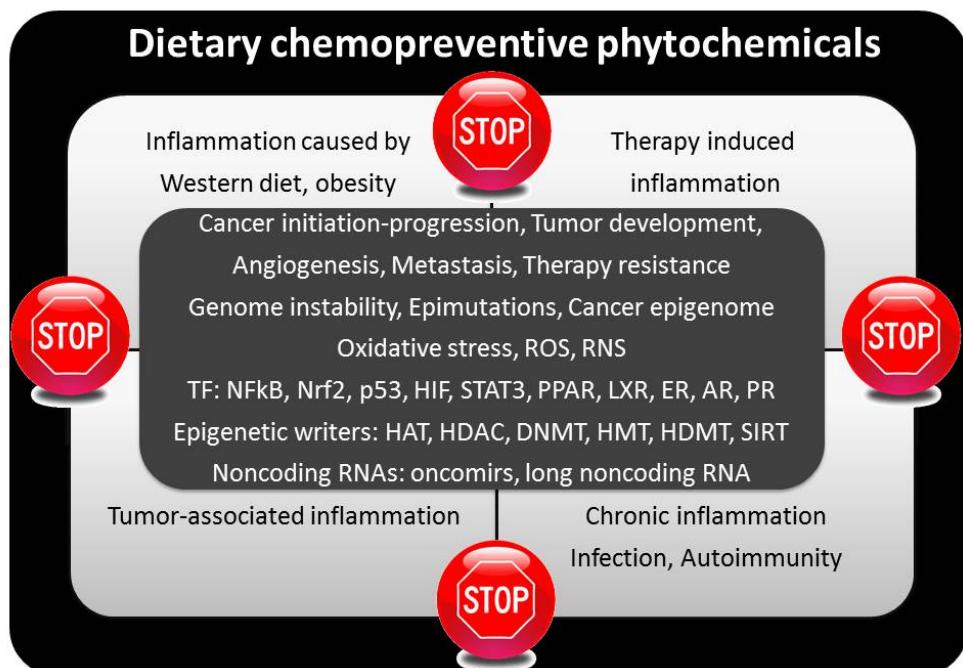


Fig. 3. Dietary chemoprevention of cancer-inflammation

isothiocyanates, epicatechins) and micronutrients (for example folic acid, selenium) on heritable gene expression, activity of the epigenetic machinery, DNA methylation or chromatin remodelling (Burdge & Lillycrop, 2010; Delage & Dashwood, 2008; Fang et al., 2007; Folmer et al., 2010; Hauser & Jung, 2008; Kirk et al., 2008; Kontogiorgis et al., 2010; Link et al., 2010; Suzuki & Miyata, 2006; Szarc vel Szic et al., 2010). Because epigenetic changes are reversible, developing drugs that control epigenetic regulation now attracts substantial research investment, including the development of functional foods or supplements as nutrition based epigenetic modulators for cancer chemoprevention (2008; Arasaradnam et al., 2008; Bingham & Riboli, 2004; Dashwood & Ho, 2007; Dashwood et al., 2006; Hurt & Farrar, 2008; Kawasaki et al., 2008; b; Parasramka et al., 2011; Szarc vel Szic et al., 2010)

The basic idea of cancer chemoprevention by dietary phytochemicals is to arrest or reverse the progression of premalignant cells towards full malignancy using physiological mechanisms by attenuating cancer-inflammation pathways. Chronic inflammation associated with infections or autoimmune disease precedes tumor development and can contribute to it through induction of oncogenic mutations, genomic instability, epimutations, changed expression of epigenetic "writer-reader-eraser" enzymes, oncomirs and long noncoding RNAs affecting early tumor promotion, and enhanced angiogenesis. Prolonged exposure to environmental irritants, Western diet or obesity can also result in low-grade chronic inflammation that precedes tumor development and contributes to it through the mechanisms mentioned above. Tumor-associated inflammation goes hand in hand with tumor development. This inflammatory response can enhance neoangiogenesis, promote tumor progression and metastatic spread, cause local immunosuppression, and further augment genomic instability. Cancer therapy can also trigger an inflammatory response by causing trauma, necrosis, and tissue injury that stimulate tumor re-emergence and resistance to therapy. However, in some cases, therapy-induced inflammation can enhance antigen presentation, leading to immune-mediated tumor eradication.

### **3. Chromatin states and methylomes in the epigenomic landscape**

In general, DNA is wrapped around nucleosomes, which are arranged as regularly spaced beads (146 bp DNA/nucleosome) along the DNA. Typically, nucleosomes consist of a histone octamer of histones (H)2A/B, H3 and H4. The DNA bridging two adjacent nucleosomes is normally bound by the linker histone H1 and is termed linker DNA. While the core histones are bound relatively tightly to DNA, chromatin is largely maintained by the dynamic association with its architectural proteins. Before most activators of a gene access their DNA-binding sites, a transition from a condensed heterochromatin ("solenoid-like fiber") to a decondensed euchromatin ("beads on a string") structure appears to take place. Conversely, the acquisition of a more condensed heterochromatin structure is often associated with gene silencing (Chi et al., 2010). This structural restriction of silenced chromatin on gene expression can be overcome by chromatin cofactor complexes, which remodel nucleosomes along the DNA or reversibly modify (acetylation, phosphorylation, ubiquitylation, glycosylation, sumoylation) histones on lysine, arginine, serine or threonine residues of amino-terminal histone tails. Since the discovery of histone-modifying enzymes, N-terminal histone tails protruding from nucleosomes were found to be 'velcro patches' for polycomb proteins, (de)acetylases (HDAC/HAT), (de)methylases (HMT/HDMT), ubiquitin

ligases, small ubiquitin-related modifier (SUMO) ligases, kinases, phosphatases, ribosylases, which together establish specific chromatin states involved in transcription (Chi et al., 2010; Ernst & Kellis, 2010; Ernst et al., 2011). Specific sets of histone modifications and/or variants are associated with genes that are actively transcribed or are repressed, a phenomenon defined as the "histone code" (Chi et al., 2010). Based on coexisting histone marks and genomewide ChIP-seq data available within the ENCODE consortium, principal component analysis allowed to reduce the complexity of the histone code into 9 different chromatin states with different functional regulatory features (Ernst & Kellis, 2010; Ernst et al., 2011).

To establish specificity of epigenetic marks, histone modifying complexes have to be recruited to relevant genomic locations by DNA-binding proteins, RNAs or protein-RNA complexes that bind to their specific DNA sites as a consequence of their own binding specificities and cellular concentrations (Brenner et al., 2005; Gupta et al., 2010; Hervouet et al., 2009; Perissi et al., 2010; Vire et al., 2006). It cannot come from the enzymatic activities per se as neither DNMTs, nor enzymes which modify histones, know which part of the genome needs to be tagged. Furthermore, there is now a large body of evidence showing that modifications of the histone tails provide signals ("binary switches") that are recognized by specific binding proteins, such as chromo-, bromo- or tudor-domains which in turn can influence gene expression and other chromatin functions (Fischle, 2008; Schreiber & Bernstein, 2002; Seet et al., 2006). The dynamic time-dependent combinations of histone modifications or threedimensional locus configuration further increase the complexity of information contained in chromatin (Bickmore et al., 2011; Chi et al., 2010; Fischle, 2008; G. Natoli, 2010; G. Natoli et al., 2011; Schreiber & Bernstein, 2002; B. van Steensel, 2011).

DNA methylation is the best known epigenetic mark (Bird, 2002; Esteller, 2007). It is catalyzed by two types of DNMTs: DNMT1 is a maintenance methyltransferase, whereas both DNMT3A and DNMT3B are *de novo* methyltransferases (P. A. Jones & Liang, 2009; Law & Jacobsen, 2010). The role of DNMT2 in DNA methylation is minor, its enzymology being largely directed to tRNA. DNA methylation is normally associated with gene inactivation and it usually occurs in CpG dinucleotides. Alternatively, DNA methylation of transcription factor binding sites which prevents binding of repressor proteins, may paradoxically induce gene activation. CpGs are normally methylated when scattered throughout the genome, but are mostly unmethylated when they are clustered as CpG islands at 5' ends of many genes. Hypermethylation of CpG-rich promoters triggers local histone code modifications resulting in a cellular camouflage mechanism that sequesters gene promoters away from transcription factors and results in stable silencing of gene expression. DNA methylation at CpG dinucleotides occurs upon transfer of S-adenosylmethionine (SAM) on cytosine by DNMTs. Whereas DNMT3A/B are responsible for DNA methylation during development (differentiation), DNMT1 is in charge of maintaining DNA methylation patterns in DNA replication during cell division. In mammalian cells, the fidelity of maintenance of methylation is 97–99.9% per mitosis, whereas *de novo* methylation is as high as 3–5% per mitosis, thus creating possibilities for epigenetic changes. DNA methylation also regulates genomic imprinting (Lees-Murdock & Walsh, 2008), X-chromosome inactivation (K. D. Robertson, 2005) and silencing of repetitive sequences (Miranda & Jones, 2007). Although in most cases DNA methylation is a stable epigenetic mark, reduced levels of methylation can also be observed during development. This net loss of methylation can either occur passively by replication in the absence of functional maintenance methylation pathways, or actively, by removing methylated cytosines. In plants active demethylation is achieved by

DNA glycosylase activity, probably in combination with the base excision repair pathway. In mammals, coupling of 5-methylcytosine deaminase and thymine DNA glycosylase activities maybe responsible for DNA demethylation. Alternatively, a role for the 5-hydroxymethylcytosine modification in mammalian DNA demethylation has also been proposed as an intermediate in an active DNA demethylation pathway involving DNA repair and 5-hydroxymethylcytosine-specific DNA glycosylase activity (Law & Jacobsen, 2010). Of particular interest, ROS and oxidative stress may affect DNA demethylation by DNA oxidation or TET-mediated DNA hydroxymethylation (Perillo et al., 2008; Luan Wang et al., 2011).

Although DNA methylation is the best-known epigenetic mark (P. A. Jones & Liang, 2009; Lande-Diner & Cedar, 2005; Scarano et al., 2005), DNA methylation does not act alone. It occurs in the context of nucleosome positioning, DNA sequence composition and histone modifications (Chodavarapu et al., 2010; B. M. Lee & Mahadevan, 2009; Vaissiere et al., 2008). For example, high resolution DNA methylation analysis has revealed 10-base periodicities (i.e one helical turn) in the DNA methylation status of nucleosome-bound DNA and found that nucleosomal DNA was more highly methylated than flanking DNA (Chodavarapu et al., 2010). These data revealed that nucleosome positioning influences DNA methylation patterning of promoters and intron-exon boundaries throughout the genome and that DNA methyltransferases preferentially target nucleosome-bound DNA. Whether nucleosome strings provide a combinatorial histone code is a matter of debate (Chi et al., 2010; Cosgrove & Wolberger, 2005; Fischle, 2008; Guil & Esteller, 2009; Jenuwein & Allis, 2001; B. M. Lee & Mahadevan, 2009; Margueron et al., 2005), but in any event, histone modifications influence gene activity and regulation. For example, acetylation of lysines is generally associated with transcriptional activation whereas lysine methylation can dictate either activation (e.g. H3K4, H3K36, H3K79) or suppression (e.g. H3K9, H3K27 or H4K20). Specific histone modifications have been shown to be associated with DNA hypermethylation of CpG islands, including deacetylation of histones H3 and H4, loss of H3K4me, and gain of H3K9me3 and H3K27me3 (R. S. Jones, 2007; A. G. Robertson et al., 2008). DNA methylation marks are recognized by DNA methyl-binding proteins (MBD) which can interact with corepressor-associated enzymes (i.e. HDACs, enhancer of zeste homologue (EZH)2, ...), thus further linking DNA methylation and chromatin regulation (Perissi et al., 2010; Perissi & Rosenfeld, 2005). Altogether, "histone code" may only become biologically meaningful at the level of domains which, upon integration of conformations of multiple nucleosomes, translates allosteric changes into specific gene (cluster) activities, in order to establish specific regulatory programs at the genome level (Chi et al., 2010; Fujioka et al., 2009; Nolis et al., 2009; Nunez et al., 2009; B. van Steensel, 2011). In analogy to allosteric control of enzymes, specific gene activity may be determined by the spatial organization (compartmentalization in discrete territories) and structural landscape (three-dimensional structure) of a gene locus, by altering the higher order structure of chromatin (*cis* mechanism) or by generating a binding platform for effector proteins (*trans* mechanisms) (Lieberman-Aiden et al., 2009; Metivier et al., 2006; G. Natoli, 2010; G. Natoli et al., 2011; Nolis et al., 2009; Nunez et al., 2009; Bas van Steensel, 2011).

There is good evidence that also noncoding RNAs regulate chromatin architecture (Guil & Esteller, 2009; Gupta et al., 2010; Mattick et al., 2009a; Mattick et al., 2009b; Taft et al., 2009a; Taft et al., 2009b; Tsai et al., 2010). The term noncoding RNA (ncRNA) is commonly employed for RNA that does not encode a protein. Although it has been generally assumed

that most genetic information is transacted by proteins, recent evidence suggests that the majority of the genomes of mammals and other complex organisms is in fact transcribed into ncRNAs, many of which are alternatively spliced and/or processed into smaller products. Besides tRNA and rRNA, these ncRNAs include long-noncodingRNAs (lncRNAs), microRNAs (miRNAs) and tinyRNAs (tiRNAs) as well as several other classes of, sometimes yet-to-be-discovered, small regulatory RNAs such as snoRNAs (Gupta et al., 2010; Mattick et al., 2009b; Taft et al., 2009a; Taft et al., 2009b). These RNAs (including those derived from introns) appear to comprise a hidden layer of internal signals that control various levels of gene expression in physiology and development, including chromatin architecture/epigenetic memory, transcription (enhancer function), RNA splicing, editing, translation and turnover (De Santa et al., 2010). RNA regulatory networks may determine most of our complex characteristics and play a significant role in disease (De Santa et al., 2010). For example, miRNAs can change expression levels of the epigenetic machinery (DNMT, HDAC, sirtuin (SIRT), polycomb (Pc) proteins, etc.) by posttranscriptional gene regulation involving base pairing with 3' untranslated (UTR) regions in their target mRNAs resulting in mRNA degradation or inhibition of translation (Denis et al., 2011; Guil & Esteller, 2009; Lujambio et al., 2008; Lujambio & Esteller, 2007; 2009; 'M.N. Ndlovu et al., 2011; Parasramka et al., 2011). Alternatively, long ncRNAs and tiRNAs can regulate gene expression and/or DNA methylation by promoter association (De Santa et al., 2010; Gupta et al., 2010; Taft et al., 2009a; Taft et al., 2009b; Tsai et al., 2010). DNA-methylation can thus also be RNA-directed (Denis et al., 2011; Guil & Esteller, 2009; Mahfouz; 'M.N. Ndlovu et al., 2011).

#### **4. Immunity, cancer-inflammation and the epigenomic landscape**

Pathologists have long recognized that some tumors are densely infiltrated by cell of both the innate and adaptive arms of the immune system and thereby mirror inflammatory conditions arising in non-neoplastic tissues (Hanahan & Weinberg, 2011). Originally, these immune responses were believed to eradicate tumors, which to some extent is true, although this pressure on the tumor triggers some escape programs to evade immune destruction. As such, solid tumors that do appear have somehow managed to avoid detection by the various arms of the immune system or have been able to limit the extent of immunological killing, thereby evading eradication. For example, cancer cells may paralyze infiltrating CTLs and NK cells, by secreting TGF $\beta$  or other immunosuppressive factors. As such, immuno-evasion can be considered as an emerging hallmark of carcinogenesis.

Furthermore, since 2000, various clues were reported that the tumor-associated inflammatory response had the unanticipated, paradoxical effect of enhancing tumorigenesis and progression (B. B. Aggarwal, 2009; B. B. Aggarwal & Gehlot, 2009; S.I. Grivennikov & Karin, 2010a& c; Ning Li et al., 2011; Naugler & Karin, 2008). Inflammation contributes to cancer progression by supplying bioactive molecules to its microenvironment, including growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion and metastasis, and signals that trigger activation of endothelial mesenchymal transition (S.I. Grivennikov et al., 2010b). The complexity of the inflammatory response requires that its many functional programs are controlled coordinately in some situations but independently in others (Medzhitov & Horng, 2009; Pasparakis, 2009). This is

achieved through multiple mechanisms that operate at different levels, including alterations in the composition of immune cells in tissues, changes in cell responsiveness to inflammatory stimuli, regulation of signaling pathways and epigenetic control of gene expression.

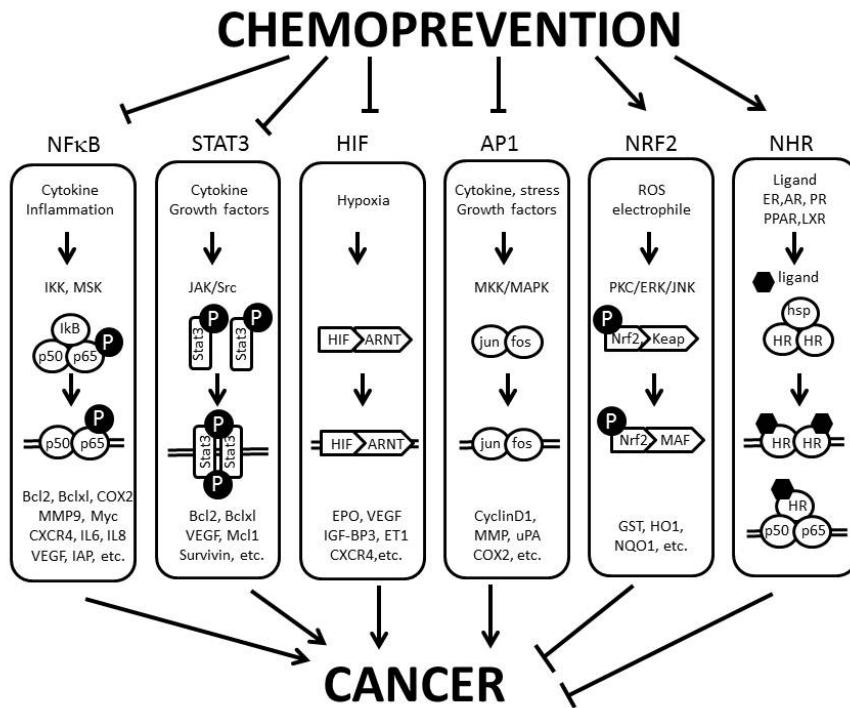
#### **4.1 Cell specific mechanisms in the tumor microenvironment**

Today, tumors are increasingly recognized as organs which can only be understood by studying the individual specialized cell types within it as well as the tumor microenvironment. Besides cancer cells, the parenchyma and stroma of tumors contain cancer stem cells, immune cells, endothelial cells, invasive cancer cells, cancer-associated fibroblasts that collectively enable tumor growth and progression. Cell-specific mechanisms operate at the level of different cell types, and include regulation of their recruitment and activation. The most frequently found immune cells within the tumor microenvironment are tumor-associated macrophages (TAMs) and T cells and are an important source of cytokines (Balkwill et al., 2005; Balkwill & Mantovani, 2010; Mantovani et al., 2008). TAMs mostly promote tumor growth and may be obligatory for angiogenesis, invasion, and metastasis and high TAM content generally correlates with poor prognosis (B. B. Aggarwal & Gehlot, 2009; Balkwill et al., 2005; Balkwill & Mantovani, 2010; S. I. Grivennikov & Karin, 2010b). Moreover, most cancers contain an inflammatory infiltrate that is hijacked by tumor cells to promote angiogenesis, tissue invasion and cell proliferation. Also, overnutrition and obesity activate the immune system which at long-term switches to chronic inflammatory condition which is a fertile soil for cancer development (Bharat B. Aggarwal, 2010; Anand et al., 2008; Hotamisligil, 2010; Mandl et al., 2009; Olefsky, 2009; E. J. Park et al., 2010; Solinas & Karin, 2010; Zhang et al., 2008).

#### **4.2 Regulation of transcription factors in cancer-related inflammation**

Production of tumor-promoting cytokines by immune/inflammatory cells that activate transcription factors in premalignant cells to induce genes that stimulate cell proliferation and survival, is a major tumor promoting mechanism. Among all the mediators and cellular effectors of inflammation, NF $\kappa$ B is perhaps the central transcription factor, which regulates expression of more than 400 genes (Chaturvedi et al., 2011; L. F. Chen & Greene, 2004; Ghosh & Hayden, 2008; Karin & Greten, 2005). NF $\kappa$ B family transcription factors are rapidly activated in response to various stimuli, including cytokines, infectious agents, overnutrition (metabolic stress, endoplasmic reticulum stress) or danger signals (bacteria, viruses, chemicals, pathogen associated molecular patterns (PAMPs), danger associated molecular patterns (DAMPs), and radiation-induced DNA double-strand breaks. Furthermore, the NF $\kappa$ B pathway is regulated by many other pathways, i.e. EGFR/Her2-PI3K-Akt/IKK $\alpha$ , RSK2, MSK1, TP53 PTEN, Akt-mTOR, Ras, Raf, Wnt-catenin, hypoxia, oxidative stress (Chaturvedi et al., 2011). In nonstimulated cells, NF $\kappa$ B TFs are bound to inhibitory (I) $\kappa$ B proteins and are thereby sequestered in the cytoplasm. Activation leads to phosphorylation of I $\kappa$ B proteins and their subsequent recognition by ubiquitinating enzymes. The resulting proteasomal degradation of I $\kappa$ B proteins liberates NF $\kappa$ B transcription factors, which translocate to the nucleus to drive expression of target genes. Two protein kinases with a high degree of sequence similarity, I $\kappa$ B kinase (IKK) $\alpha$  and IKK $\beta$ ,

mediate phosphorylation of I $\kappa$ B proteins and represent a convergence point for most signal transduction pathways leading to NF $\kappa$ B activation. Most of the IKK $\alpha$  and IKK $\beta$  molecules in the cell are part of IKK complexes that also contain a regulatory subunit called IKK $\gamma$  or NF $\kappa$ B-essential modulator (NEMO). Alternative to IKK, various additional kinases have been identified which modulate transcriptional nuclear activity of NF $\kappa$ B, including mitogen- and stress-activated protein kinase (MSK), protein kinase (PK)Ac, phosphoinositide 3-kinases PI3K and AKT (L. F. Chen & Greene, 2004; Edmunds & Mahadevan, 2006; Vanden Berghe et al. 2011; Vermeulen et al., 2009; Vermeulen et al., 2003; Viatour et al., 2005). Members of the NF $\kappa$ B family of dimeric transcription factors regulate expression of a large number of genes involved in immune responses, inflammation, metabolic stress, cell survival, cell proliferation and cancer. At the same time, it is responsible for many aspects of inflammatory disease and malignancy by inducing transcription of soluble mediators that amplify inflammation, angiogenesis and neoplastic cell proliferation, and affect progression to more aggressive disease states (S. I. Grivennikov & Karin, 2010b). Furthermore, constitutive activity of NF $\kappa$ B/IKK has been observed in many cancer cells, inflammatory disorders, obesity and insulin resistance (Arkan et al., 2005; Ghosh & Hayden, 2008; Hotamisligil, 2010; Karin, 2006; Karin & Greten, 2005; Mandl et al., 2009; Nakanishi & Toi, 2005; Olefsky, 2009; E. J. Park et al., 2010; Perkins, 2007). Besides constitutively activated NF $\kappa$ B found in several human cancer cell lines, including lymphomas and carcinomas of the breast, prostate, lung, colon, pancreas, head and neck and oesophagus, activated NF $\kappa$ B has also been noted in tissue samples from cancer patients (Baud & Karin, 2009; Chaturvedi et al., 2011; Dey et al., 2008). Studies of cancer-associated mutations have also reported that mutations in the upstream signal components of NF $\kappa$ B or p53 mutations could direct constitutive NF $\kappa$ B activation in cancer cell lines and patient samples (Dey et al., 2008; Dijsselbloem et al. 2007; Weisz et al., 2007). Therefore, inhibition of NF $\kappa$ B activity has been found to be a useful addition to chemotherapy regimens of a variety of cancers (Baud & Karin, 2009; Karin et al., 2004). Although quite a number of genes contain NF $\kappa$ B-responsive elements in their regulatory regions, their expression pattern can significantly vary from both a kinetic and quantitative point of view (Ghosh & Hayden, 2008; Hayden & Ghosh, 2008; Medzhitov & Horng, 2009; G. Natoli et al., 2005; O'Dea & Hoffmann, 2010; Perkins, 2007; Ramirez-Carrozzi et al., 2009; Vanden Berghe et al., 2006b; Werner et al., 2005). At the transcription level, selectivity is conferred by the expression or activation of specific NF $\kappa$ B subunits and their respective posttranslational modifications, and by combinatorial interactions between NF $\kappa$ B and other transcription factors, such as activator protein (AP-1), signal transducers and activators of transcription (STAT3), nuclear factor erythroid 2-related factor (NRF2), peroxisome proliferator-activated receptor-  $\gamma$  (PPAR  $\gamma$ ), estrogen receptor (ER), liver X receptor (LXR), hypoxia inducible factor-1 (HIF-1), p53 which are involved in angiogenesis, chemoresistance, stem cell survival, cancer invasion and tumour progression (B. B. Aggarwal & Gehlot, 2009; Dey et al., 2008; Eferl & Wagner, 2003; Reuter et al., 2010; Rohwer et al., 2010; van Uden et al., 2011). Various naturally occurring phytochemicals such as withaferin, curcumin, resveratrol, mangiferin hold promise as anti-cancer drugs by interfering with NF $\kappa$ B, STAT3, AP1, HIF, PPAR, ER, LXR, p53 activities and gene expression programs (Dijsselbloem et al., 2007; Garcia-Rivera et al., 2011; Harvey, 2008; Kaileh et al. 2007; Kontogiorgis, C. et al. 2010; J. W. H. Li & Vederas, 2009; Surh, 2003; Vanden Berghe et al., 2006 &2011; Suttana et al., 2010) (Figure 4)



Abbreviations used: ARE, antioxidant response element; ARNT, aryl hydrocarbon receptor nuclear translocator; NHR, nuclear hormone receptor; HRE, hypoxia responsive element; IKK, inhibitor of I $\kappa$ B kinase; Jak, Janus kinase; JNK, cjun N terminal kinase; MAPK, mitogen-activated protein kinase; STAT3, signal transducer and activator of transcription

Fig. 4. Modulation of tumor-promoting transcription factor pathways by dietary phytochemicals (adapted from Sung et al. 2011).

#### 4.3 Chromatin dynamics in cancer-inflammation

Since transcription factors bind very poorly or not at all to nucleosomal DNA, their activation is coordinated to recruitment of noncoding RNAs (Gupta et al., 2010; Tsai et al., 2010), DNMTs (Hervouet et al., 2009) and epigenetic writer, reader or eraser proteins (Chi et al., 2010), including ATP-dependent chromatin-remodeling factors [switch/sucrose non fermentable SWI/SNF, Brahma (Brm), brahma-related gene Brg1], histone-enzyme complexes such as kinases [IKK, MSK, ataxia telangiectasia mutated (ATM), AKT, PI3K], poly(ADP-ribose) polymerase (PARP), methylases (EZH2, coactivator-associated arginine methyltransferase (CARM)1, protein arginine methyltransferases (PRMT)), demethylases (lysine specific demethylase (LSD)1, Jumonji C family histone demethylase (JMJD)3), prolyl isomerase (PIN1), acetylases (p300, CREB binding protein (CBP), p300/CBP-associated factor (p/CAF)), deacetylases (HDAC, SIRT) and DNMTs (Dong et al., 2008; Ghosh & Hayden, 2008; Perissi et al., 2010; Rosenfeld et al., 2006; Vanden Berghe et al., 2006b; Vermeulen et al., 2009). Parallel posttranslational modifications (phosphorylation, acetylation, methylation, ribosylation, sumoylation, ubiquitination) of histone and non-

histone transcription factor and cofactor complexes in response to signalling components allow displacement of polycomb complexes and formation of dynamic enhanceosome complexes which establish a distinct chromatin structure (Bracken & Helin, 2009; Dawson et al., 2009; Gehani et al., 2010; N. Ndlovu et al., 2009; Schreiber & Bernstein, 2002; Vanden Berghe et al., 1999a; Vermeulen et al., 2009; Vermeulen et al., 2003). These epigenetic settings are the ultimate integration sites of both environmental and differentiative inputs, determining proper expression of each target gene (Ford & Thanos, 2010; Ghosh & Hayden, 2008; Hayden & Ghosh, 2008; G. Natoli & De Santa, 2006; Vanden Berghe et al., 2006b). Investigation of epigenetic regulation of cancer-inflammation genes, revealed different subclasses according to chromatin activation mode and gene expression profile (Ramirez-Carrozzi et al., 2009; Ramirez-Carrozzi et al., 2006). A major class of primary response genes is characterized by CpG-island promoters, which facilitate promiscuous induction from constitutively active chromatin without a requirement for SWI/SNF nucleosome-remodeling complexes. The low nucleosome occupancy at promoters in this class can be attributed to the assembly of CpG-islands into unstable nucleosomes, which may lead to SWI/SNF independence. Another major class consists of non-CpG-island promoters that assemble into stable nucleosomes, resulting in SWI/SNF dependence and the requirement for transcription factors that promote selective nucleosome remodeling. Some inflammatory stimuli, exhibit a strong bias toward activation of SWI/SNF-independent CpG-island genes. In contrast interferon (IFN) $\beta$  preferentially activates SWI/SNF-dependent non-CpG-island promoters. At the level of CpG methylation, changes in DNA methylation of IKK, I $\kappa$ B and RelB promoters (G. Maeda et al., 2007; O'Gorman et al., 2010; Puto & Reed, 2008) affect gene induction properties upon re-exposure to a inflammatory stimulus (Ashall et al., 2009; El-Osta et al., 2008; El Gazzar et al., 2009; El Gazzar et al., 2007; Puto & Reed, 2008). Furthermore, CpG-methylation of certain genes enables some cells to acquire new capabilities needed for tumorigenesis (Widschwendter & Jones, 2002)(Figure 2). Cells which accumulated DNA methylation at various loci as a function of time (age) and as a function of exposure to growth factors or chronic inflammation gain novel capabilities to promote carcinogenesis, i.e. limitless replicative potential, selfsufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, sustained angiogenesis and tissue invasion and metastasis (Teschendorff et al., 2010; Widschwendter & Jones, 2002)(Figure 2).

## 5. Cancer-inflammation, cancer metabolism and epimutations: cause or consequence?

Since inflammatory gene expression dynamics is highly dependent on epigenetic control mechanisms (De Santa et al., 2007; Medzhitov & Horng, 2009; Messi et al., 2003; G. Natoli & De Santa, 2006; G. Natoli et al., 2005; Vanden Berghe et al., 2006b), we have previously characterized chromatin organization in weak or strong inflammatory cancer cell types with inducible or constitutive interleukin (IL)6 gene expression patterns. Upon investigation of autocrine IL6 gene expression production in aggressive myeloma cells or metastatic breast cancer cells, we observed euchromatin-like properties and highly accessible chromatin at the IL6 gene promoter (Gerlo et al., 2008; N. Ndlovu et al., 2009). Furthermore, recruitment of CBP/p300 acetylases and MSK kinase seems to prevent heterochromatinisation and recruitment of heterochromatin protein (HP)1 upon phosphacetylation of transcription factor and histone components (Boeke et al., 2010; N. Ndlovu et al., 2009; Vanden Berghe et

al., 1999a; Vermeulen et al., 2009; Vermeulen et al., 2003). Along the same line, the kinase MSK kinase was found to displace polycomb repressor complexes during gene activation (Gehani et al., 2010). Interestingly, promoter-binding activity of Sp1 and AP1 Fra1 are responsible for priming IL6 promoter chromatin relaxation, which further promotes binding of NF $\kappa$ B transcription. Interestingly, complementation of low invasive cancer cells with Fra1 seems to convert the promoter chromatin architecture in a highly accessible chromatin configuration. Reciprocally, highly accessible chromatin in invasive cancer cells can be silenced with anti-inflammatory phytochemicals, or following silencing of AP1/NF $\kappa$ B transcription factors, demonstrating reversible epigenetic changes towards a less aggressive phenotype (Dijsselbloem et al., 2007; N. Ndlovu et al., 2009; Vanden Berghe et al., 2006a). Furthermore, we and others observed DNA hypermethylation at the IL6 gene promoter in cancer cells with low NF $\kappa$ B/AP1 activity and inducible IL6 gene expression, as compared to DNA hypomethylation in cancer cells with hyperactivated NF $\kappa$ B/AP1 transcription factors and elevated constitutive IL6 gene expression (Armenante et al., 1999; Dandrea et al., 2009). Similarly, various transcription factors (p53, cmyc, ER, GR, NF $\kappa$ B p65 and others) were found to recruit DNMTs and modulate promoter enhancer function in a time-dependent and signal specific fashion (Aaltonen et al., 2008; Brenner et al., 2005; Hervouet et al., 2009; Kangaspeska et al., 2008; Y. Liu et al., 2011; Metivier et al., 2008; Santourlidis et al., 2001; Wiench et al., 2011). Reciprocally, depletion of NF $\kappa$ B can also trigger DNA demethylation and gene reactivation, illustrating gene-specific epigenetic effects which may further depend on posttranslational NF $\kappa$ B modifications (Dong et al., 2008; X. Liu et al., 2010b; Y. Liu et al., 2011). Also, chronic exposure to chemotherapeutic agents may epigenetically reprogram cancer cell metabolism and gene expression and trigger chemoresistance (Blair et al., 2011; Kujo et al., 2011; S. V. Sharma et al., 2010; W. Suttana et al., 2010). As such, this demonstrates that cancer-inflammation pathways and transcription factors are able to rewire epigenetic settings and amplify gene expression in an autocrine fashion (Hervouet et al., 2009; S. Liu et al., 2008).

Of special note, despite promising (pre)clinical studies with epigenetic drugs (azacytidine, suberoylanilide hydroxamic acid (SAHA)) for reactivation of silenced tumor suppressor genes in cancer treatment, one should also be cautious, as these compounds may also boost gene expression of inflammatory oncogenes such as IL6, which promote aggressive carcinogenesis, cancer stem cell proliferation, metastasis and hormone resistance (B. B. Aggarwal & Gehlot, 2009; S. Grivennikov et al., 2009; D. Iliopoulos et al., 2009; S. Maeda et al., 2009; Min et al., 2010; Naugler & Karin, 2008; W. Suttana et al., 2010; H. Wang et al., 2009; Yu et al., 2009). In line with this, knocking out DNMT1 or treatment of tumors with a global DNA hypomethylating agent was found to promote aspects of tumor progression and was accompanied by increased invasion *in vitro* and increase tumor growth *in vivo* (Eden et al., 2003; Gaudet et al., 2003; Christopher A. Hamm & Costa, 2011; C. A. Hamm et al., 2009). Furthermore, the inflammatory cytokine IL6 is able to trigger epigenetic changes of tumor suppressor genes via regulation of DNMTs (Gasche et al., 2011; Hodge et al., 2007; Hodge et al., 2005b; Peng et al., 2005; Pompeia et al., 2004), microRNAs (Braconi et al., 2010; D. Iliopoulos et al., 2009; Meng et al., 2008) and histone methyltransferases (Ezh2) (Croonquist & Van Ness, 2005). Another regulatory circuit involving NF $\kappa$ B, STAT3, IL6, and let7, miR-21 and miR-181b-1 triggers an epigenetic switch driving tumor progression (D. Iliopoulos et al., 2009 & 2010). Remarkably, expression of enzymes central to cellular methylation, Sadenosylmethionine synthetase and S-adenosylhomocysteine, as well levels of specific

metabolites associated with cellular methylation reactions are significantly altered during inflammation, which results in a global change in DNA/histone methylation during inflammation (Kominsky et al., 2011). This suggests that epigenetic regulators themselves and methylation of tumor suppressor genes are also susceptible to dynamic inflammatory control (Braconi et al., 2010; Hodge et al., 2005a; Hodge et al., 2001; D. Iliopoulos et al., 2009; Kawasaki et al., 2008; Mathews et al., 2009; Meng et al., 2008; Peng et al., 2005), which adds an extra level of complexity to the cancer-inflammation link.

Furthermore, besides epigenetic changes in neoplastic cells, inflammatory stimuli in the tumor microenvironment can also epigenetically reprogram tumor-associated immune cells, as demonstrated for the NF $\kappa$ B-dependent histone demethylase JMJD3 which determines cell fate and transdifferentiation of tumor-associated macrophages (De Santa et al., 2007; K. C. Kim et al., 2009b). Reports on epigenetic events in cancer are traditionally produced from analyses on "bulk" tumor samples, i.e. without distinction between neoplastic cells on one hand and the tumoral stroma on the other. The pro-inflammatory micro-environment that drives many tumor types is as such capable of triggering these epigenetic alterations within cancer progenitor cells, alterations which can substitute for genetic defects later in tumour progression (D. Iliopoulos et al., 2009; S. V. Sharma et al., 2010). However, also tumor stromal components (which include bone-marrow-derived cells, tumor-associated macrophages) are a target of epigenetic events (De Santa et al., 2007; Dijsselbloem et al., 2007; Messi et al., 2003). Besides inflammatory factors, the micro-environment also contains free radicals produced by neutrophils, macrophages, endothelial and other cells. Reactive Oxygen Species (ROS) such as  $\bullet\text{O}_2$ ,  $\bullet\text{OH}$ ,  $\text{H}_2\text{O}_2$  and Reactive Nitrogen Species (RNS) such as  $\bullet\text{NO}$  and  $\bullet\text{NO}_2$  can injure cellular biomolecules such as nucleic acids, enzymes, carbohydrates, and lipid membranes, causing cellular and tissue damage, which in turn augments the state of inflammation. In addition, reactive ROS and RNS intermediates, indirectly also modulate activity of epigenetic machinery which finally will affect chromatin dynamics and DNA (hydroxyl)methylation in tumor-associated immune cells (Brewer, 2010; Carta et al., 2009; Forneris et al., 2008; Franco et al., 2008; Illi et al., 2009).

## 6. Nutri-epigenomics: Lifelong remodelling of our epigenomes

Human epidemiological studies and appropriately designed dietary interventions in animal models have provided considerable evidence to suggest that maternal nutritional imbalance and metabolic disturbances, during critical time windows of development, may have a persistent effect on the health of offspring and may even be transmitted to the next generation (Aguilera et al., 2010; Burdge & Lillycrop, 2010; Cooney, 2006; Gallou-Kabani et al., 2007; Godfrey et al., 2010; Weaver, 2009; Youngson & Whitelaw, 2008). This has led to the hypothesis of "fetal programming" and new term "developmental origin of health and disease" (DOHaD): common disorders, such as cancer, obesity, cardiovascular disease (CVD), diabetes, hypertension, asthma and even schizophrenia, take root in early nutrition during gestation and continues during lactation (Anway et al., 2005; Anway & Skinner, 2006; Barker & Martyn, 1992; Burdge & Lillycrop, 2010; Chmurzynska, 2010; Gluckman et al., 2008; Hochberg et al., 2011; Jackson et al., 2010; Jirtle & Skinner, 2007). Similarly, there is increasing evidence in animals that nutritional intervention (caloric, iron and protein restriction, polyphenol-, folate-, micronutrient-, fat- or carbohydrate-rich diet) and maternal diabetes occurring during pregnancy and the lactation period, affects health in following

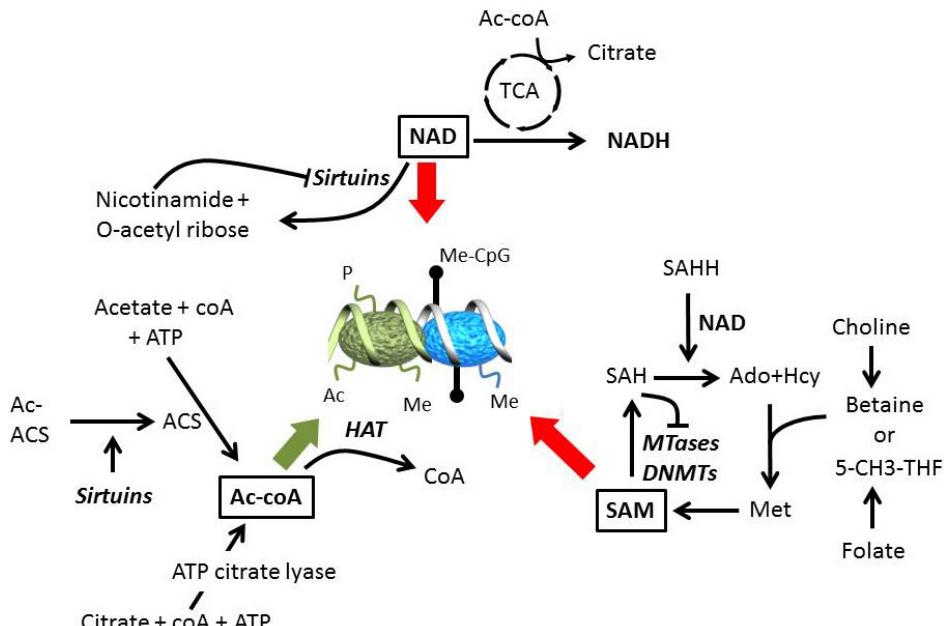
generation(s) (Dolinoy & Jirtle, 2008; Jirtle & Skinner, 2007; Kirk et al., 2008; Waterland, 2009; Waterland & Jirtle, 2004; Waterland et al., 2008; Youngson & Whitelaw, 2008). The various non-Mendelian features of metabolic disease, cancer or chronic inflammatory disorders, clinical differences between men and women or monozygotic twins and fluctuations in the course of the disease are consistent with epigenetic mechanisms in the influence of fetal and/or lifelong nutrition or stochastic events on adult phenotype (Aguilera et al., 2010; Bell & Spector, 2011; Burdge & Lillycrop, 2010; Cooney, 2006; Gallou-Kabani et al., 2007; Godfrey et al., 2010; Kaminsky et al., 2009; Petronis, 2006; Weaver, 2009; Youngson & Whitelaw, 2008). Thus, lifetime shapes the multitude of epigenomes not only within, but also across generations (Anway & Skinner, 2006; Burdge & Lillycrop, 2010; Chong et al., 2007; Godfrey et al., 2010; Hochberg et al., 2011; Skinner et al., 2011; Youngson & Whitelaw, 2008). Interest in transgenerational epigenetic effects of food components has been fueled by observations in Agouti mice fed with a soy polyphenol diet, which revealed epigenetic changes in DNA methylation patterns in their offspring. This in turn protected them against diabetes, obesity and cancer across multiple generations (Dolinoy & Jirtle, 2008; Dolinoy et al., 2006; Waterland, 2009). Furthermore, maternal nutrient supplementation with soy polyphenols was found to counteract xenobiotic-induced DNA hypomethylation in early development (Dolinoy et al., 2007; Dolinoy & Jirtle, 2008; Dolinoy et al., 2006; Jirtle & Skinner, 2007; Kujjo et al., 2011; Skinner et al., 2011).

Recently, evidence emerged that also timing (preconception, pregnancy, lactation, neonatal life, early life, pre-/post-menopause, puberty) of various dietary exposures may be vitally important in determining health beneficial effects, as epigenetic plasticity changes continually from conception to death (Burdge et al., 2009; Faulk & Dolinoy, 2011). Studies of human populations following famine have suggested that pathologies in later life are dependent on the critical timing of nutritional insult during pregnancy (Lumey & Stein, 2009; Painter et al., 2008; Roseboom et al., 2006). In principle, epigenetic changes occurring during embryonic development will have a much greater impact on the overall epigenetic status of the organism because, as they can be transmitted over consecutive mitotic divisions, alterations occurring in single embryonic stem cells will affect many more cells than those occurring in adult stem and/or somatic cells during postnatal development (Aguilera et al., 2010). In addition to epigenetic imprinting during crucial periods of development, stochastic or genetically and environmentally triggered epigenomic changes (epimutations) occur day after day and accumulate over time, as maximal differences in DNA methylation profiles are observed in aged monozygotic twins with a history of non-shared environments (Christensen et al., 2009; Fraga et al., 2005). Although it has long been thought that the epigenomic profile is wiped clean in the embryo shortly after fertilization, with the exception of imprinted genes, methylation clearing is not complete after fertilization and on a global DNA level is reduced to 10% (Hajkova et al., 2008; Surani et al., 2004). Remarkably, recent evidence suggests that DNA methylation is rather converted into hydroxymethylation than erased (Ficz et al., 2011; Iqbal et al., 2011; Wossidlo et al., 2010; Wossidlo et al., 2011). Alternatively, it can not be excluded that transgenerationally inherited nutritional effects may also depend on polycomb proteins (Blewitt et al., 2006; Bracken & Helin, 2009; Chong et al., 2007; Youngson & Whitelaw, 2008), miRNA profiles (Guil & Esteller, 2009; Y. Li et al., 2010) or epigenetic capacitor properties of hsp proteins present in the fertilized embryo (Ruden et al., 2005a; Ruden et al., 2005b; Sollars et al., 2003).

## 7. Epigenetic targets of bioactive dietary components for cancer prevention and therapy

A next challenge will be to determine which adverse epigenomic marks in cancer-inflammation are reversible or can be prevented by specific diets, natural phytochemicals or lifestyle changes (Burdge & Lillycrop, 2010; Burdge et al., 2009; Godfrey et al., 2010; Kirk et al., 2008). Numerous botanical species and plant parts contain a diverse array of polyphenolic phytochemicals which exert cancer-chemopreventive effects in man by its anti-inflammatory, anti-oxidant, phytohormonal, homeostatic effects (hormesis) in immune cells and/or cancer (stem)cells (Bickford et al., 2006; Blanpain & Fuchs, 2009; Crea et al., 2009; Dijsselbloem et al., 2004; Kawasaki et al., 2008; Shytle et al., 2007; Surh, 2003; Zhou et al., 2008). Upon re-exploration of its biological activities, various nutritional natural compounds (including epigallocatechingallate, resveratrol, genistein, curcumin, isothiocyanates, withanolides, ...) were found to interfere with enzymatic activity of DNMT, Class I, II, IV HDAC, HAT and Class III HDAC sirtuins (SIRT) which modulate cancer-inflammation ((Arasaradnam et al., 2008; Burdge & Lillycrop, 2010; Delage & Dashwood, 2008; Fang et al., 2007; Folmer et al., 2010; Hauser & Jung, 2008; Jackson et al., 2010; Kirk et al., 2008; Kontogiorgis et al., 2010; Link et al., 2010; Suzuki & Miyata, 2006; Szarc vel Szic et al., 2010; Vaquero & Reinberg, 2009), and references included). HDACs are zinc metalloproteins which rely on Zn<sup>2+</sup> for their activity and are divided into 4 classes based on their homology with yeast HDACs. Class III HDACs, called sirtuins are zinc-independent but nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent. Class I, II, IV HDAC inhibitors characteristically contain a Zn<sup>2+</sup> chelating group consisting of a thiolate, thiol, hydroxamate, carboxylate, mercaptoamide, epoxide or ketone group. Natural HDAC inhibitors can be divided in following groups based on their chemical characteristics: carboxylates, organosulfides, isothiocyanates, hydroamates, cyclic tetrapeptides and macrocyclic depsipeptides (Folmer et al., 2010). In contrast to natural HDAC inhibitors, only few natural products (i.e. niacin, vitamin B3, dihydrocoumarin) have been identified as inhibitors of class III HDACs. Reciprocally, various natural flavonoids have been identified as activators of class III HDACs (SIRTs). Turmeric and green tea have been identified as sources of natural inhibitors of p300/CBP HAT. Finally DNMT inhibitors work mainly through one of the following mechanisms, either covalent trapping of DNMT through incorporation into DNA (i.e. nucleoside analogues decitabine, 5-azacytidine), non-covalent blocking of DNMT catalytic active site (i.e. EGCG, parthenolide), interruption of binding site of DNMT to DNA (i.e. procaine), degradation of DNMT (i.e; decitabine), or suppression of DNMT expression (i.e. miRNAs). Specific epigenetic effects of natural phytochemicals may be the result of a superposition of combined concentration-dependent actions of the compound as a nuclear hormone receptor ligand and/or modulator of histone-modifying enzymes and DNMTs (Darbre & Charles, 2010; Denison & Nagy, 2003; Kuniyasu, 2008; Mai et al., 2008; Newbold et al., 2009) which may target chromatin dynamics of specific gene clusters. Although effects of dietary factors and extracts have frequently been demonstrated in *in vitro* experiments at concentrations which can never be achieved *in vivo*, "epigenetics" sheds a more realistic light on dietary studies, as longlife exposure at physiological concentrations can remodel the epigenome in a cumulative fashion by repetitive effects on the epigenetic machinery (Manach & Donovan, 2004; Manach et al., 2005a; Manach et al., 2004; Manach et al., 2005b; Williamson & Manach, 2005). Furthermore, it should be evaluated which epigenetic changes are stable over time. Interestingly, even transient exposure to a specific dietary component can induce

long-lasting epigenetic changes in the promoter of the NF $\kappa$ B subunit p65, which acts as a master switch in cancer-inflammation (El-Osta et al., 2008). Alternatively, compounds may chemically interfere with histone mark interacting effector domains (such as chromo-, bromo- or tudor domains) (Seet et al., 2006; Wigle et al.; Zheng et al., 2008). Though, upon performing *in vitro* compound screenings in cofactor activity assays based on peptide-protein interactions, one should be careful with interpretation as peptide interactions may not always represent true targets *in vivo* (Altucci & Minucci, 2009; Pacholec et al., 2010).



NAD, acetyl-coenzyme A (Acetyl-coA) and S-adenosylmethionine (SAM) are elemental for epigenetic control of transcription including methylation of DNA and posttranslational modifications of histones and non-histone chromatin factors (not shown). NAD contributes to transcriptional control mainly via the activity of the protein deacetylase sirtuin (SIRT), which uses NAD as one of the substrates. Sirtuins are also important for maintaining the activity of the acetyl-coA acetyltransferases. Ac-coA is synthesized by acetyl-coA-synthetase and ATP-citrate lyase that use acetate and citrate as the precursor, respectively. Citrate is an intermediate/product of the TCA cycle. SAM is the methyl donor for DNA, RNA, histones and non-histone protein methylation. SAH generated in each round of methylation reaction is a potent inhibitor of methyltransferases and has to be cleared by SAH hydrolase. NAD is an essential coenzyme for SAHH. Synthesis of methionine from homocysteine is achieved through extracting the methyl group from betaine, derived from choline, or 5-methyl-THF, a derivative of folic acid. Metabolism of phospholipids and folic acid may thus indirectly contribute to epigenetic regulation. Likewise, the abundance of NAD and citrate is linked to the cellular energy flux, e.g. the TCA cycle. Changes in the expression of certain genes may therefore be influenced significantly. Abbreviations used: Ac-coA, acetyl-coenzyme A; ACS, acetyl-coA-synthetase; AC-ACS acetylated-ACS; Ado, adenosine; HAT, histone acetyltransferase; Hcy homocysteine; MTases, methyltransferases; NAD, Nicotinamide adenine dinucleotide; SAH, S-adenosyl homocysteine; TCA tricarboxylic cycle; THF tetrahydrofolate.

Fig. 5. Global shifts in cancer epigenome regulation depend on metabolic shifts in cofactors for epigenetic enzymes (adapted from Luo et al. 2009)

From another perspective, chemopreventive phytochemicals may indirectly modulate chromatin dynamics and epigenetic effects upon interference with global cancer metabolism. As such, epigenetic changes may follow biochemical metabolisation of dietary factors, which can deplete cellular pools of acetyl-CoA, NAD<sup>+</sup> and methyldonors, resulting in unbalanced DNA methylation and/or protein acetylation or methylation (Imai & Guarente, 2010; Ladurner, 2009; Vaquero & Reinberg, 2009) (Figure 1&5). For example, flavanol-rich diets interfere with the methyldonor metabolism and the available pool of Sadenosylmethionine, resulting in changes in DNA methylation (Bistulfi et al., 2010; N. C. Chen et al., 2010; Ghoshal et al., 2006; J. M. Kim et al., 2009a; Ulrich et al., 2008) and histone methylation, which is also affected by alterations in SAM/SAH ratios (Pogribny et al., 2007; P. Sharma et al., 2006). Furthermore, even without nutritional deficiency of methyl groups, impaired synthesis of SAM and perturbed DNA methylation can happen when the need for the synthesis of the detoxification enzyme glutathione transferase (GSH) synthesis increases (D. H. Lee et al., 2009). Diets or nutritional compounds which affect energy metabolism or mitochondrial respiration can have global epigenetic effects upon changes in NAD<sup>+</sup> availability and SIRT activity (Whittle et al., 2007). Since SIRT activation has been linked to longevity (increased lifespan and healthy aging) and mimics a caloric restricted diet, SIRT activators such as resveratrol represent a major class of caloric mimetic epigenetic modulator phytochemicals which could reverse metabolic disease (Imai & Guarente, 2010). Along the same line, flavanol-rich diets which interfere with the methyldonor metabolism and the available pool of S-adenosylmethionine will result in (Global) changes in DNA and histone methylation (Bistulfi et al., 2010; Ghoshal et al., 2006; J. M. Kim et al., 2009a; Pogribny et al., 2007; P. Sharma et al., 2006; Ulrich et al., 2008). As such, specific dietary classes of functional food maybe designed as therapeutic epigenetic modulators in cancer-inflammation.

## 8. Conclusion & future perspectives

The phenotype of an individual is the result of complex gene-environment interactions in the current, past and ancestral environment, leading to lifelong remodelling of our epigenomes. In recent years, several studies have demonstrated that disruption of epigenetic mechanisms can alter immune function and contribute to many cancer types. Various replication-dependent and -independent epigenetic mechanisms are involved in developmental programming, lifelong recording of environmental changes and transmitting transgenerational effects. It is likely that understanding and manipulating the epigenome, a potentially reversible source of biological variation, has great potential in chemoprevention or stabilization of cancer. Much attention is currently focusing on modulating DNA hyper/hypomethylation of key inflammatory genes by dietary factors as an effective approach to cure or protect against cancer-inflammation (Burdge & Lillycrop, 2010; Delage & Dashwood, 2008; Fang et al., 2007; Folmer et al., 2010; Hauser & Jung, 2008; Jackson et al., 2010; Kirk et al., 2008; Kontogiorgis et al., 2010; Link et al., 2010; Suzuki & Miyata, 2006; Vaquero & Reinberg, 2009). In this respect, "Let food be your epigenetic medicine" could represent a novel interpretation of what Hippocrates said already 25 centuries ago. As such, it will be a challenge for future anti-inflammatory therapeutics and preventive cancer research to identify novel epigenetic targets which allow selective modulation of the inflammatory signaling network in the diseased tumor microenvironment (Bremner & Heinrich, 2002; Deorukhkar et al., 2007; Karin et al., 2004; Khanna et al., 2007; Paul et al.,

2006; Rios et al., 2009; Surh, 2003). Given several encouraging trials, prevention and therapy of age- and lifestyle-related diseases by individualised tailoring of optimal epigenetic diets or supplements are conceivable. However, these interventions will require intense efforts to unravel the complexity of these epigenetic, genetic and environmental interactions. Another goal is to evaluate their potential reversibility with minimal side effects as diet components may reprogram malignant cells as well as the host immune system and HPA-axis depending on the bioavailability of the dietary compounds (Burdge & Lillycrop, 2010; Dijsselbloem et al., 2007; Manach et al., 2005b; N. Ndlovu et al., 2009; Vanden Berghe et al., 2006a; Williamson & Manach, 2005). There is some concern that epigenetic therapy with dietary inhibitors of DNMT, HDAC, histone (de)methylases in longterm treatment setups may suffer from lack of specificity (Altucci & Minucci, 2009; Mai et al., 2008; Zheng et al., 2008). As such, the possible alternative is to combine nonselective epigenetic therapies with more targeted approaches (Hervouet et al., 2009). For example, combined treatment of specific transcription factor inhibitors and/or hormone receptor ligands with epigenetic drugs may trigger synergistic activities at subsets of inflammatory genes (Biddie et al., 2010; Di Croce et al., 2002; Fiskus et al., 2009; Hervouet et al., 2009; Perissi et al., 2010). An excellent example of cooperation between a dietary vitamin A-derivative targeting a nuclear receptor and the HDAC inhibitor butyrate has been described in the treatment of acute promyelocytic leukemias (Delage & Dashwood, 2008). Finally, microRNA and long ncRNA pathways also hold promise to join soon the arsenal of epigenetic combination therapies, as their target sequence specificity may bridge the gap between genetic and epigenetic changes (De Santa et al., 2010; Guil & Esteller, 2009; Gupta et al., 2010; Parasramka et al., 2011; Tsai et al., 2010). In conclusion, cancer-inflammation studies are revealing a dazzling complexity in the mechanisms leading to dynamic alterations of the epigenome and the need of combination therapies targeting different chromatin modifiers, to reverse disease prone epigenetic alterations for chemoprevention. Medical benefits of dietary compounds as epigenetic modulators, especially with respect to their chronic use as nutraceutical agents in cancer chemoprevention, will rely on our further understanding of their epigenetic effects during embryogenesis, early life, aging, carcinogenesis as well as through different generations.

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## **Part 3**

### **Nutritional Value**



# From Nutrition to Health: The Role of Natural Products – A Review

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## 1. Introduction

The word *natural* literally means something that is present in or produced by nature and not artificial or man-made (Spainhour, 2005). Although, many effective poisons are natural products (Schoental, 1965). When the word *natural* is used in written or verbiage form, many a times refer to something good or pure (Spainhour, 2005). Today, the term *natural products* are commonly understood to refer to herbs, herbal concoctions, dietary supplements, traditional medicine including Chinese traditional medicine, or other alternative medicine (Holt, and Chandra, 2002). In general, natural products are either of prebiotic origin or originate from microbes, plants, or animal sources (Nakanishi, 1999a; Nakanishi, 1999b). As chemicals, natural products include such classes of compounds as terpenoids, polyketides, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acid bases, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and so forth. Natural products are an expression of organism's increase in life complexity by nature (Jarvis, 2000).

It is good to understand what 'nutrition' and 'a nutrition perspective' mean. It should be understood that food and nutrition are not the same. Nutrition is both the outcome and the process of providing the nutrients needed for health, growth, development and survival. Although food—as the source of these nutrients—is an important part of this process, it is not by itself sufficient. Good health management services, as well as good caring practices are other important and necessary inputs needed in maintaining good health apart from good nutrition (SCN, 2004).

## 2. World status of nutrition and health

During the past two to three decades there have been rapid changes in global economy accompanied by several significant changes in the nutritional status and health of developing countries (Evans et al., 2002). Significant reductions in the prevalence of undernutrition are some of the good changes recorded. The increased prevalence of obesity and diet-related chronic disease are some of the negative changes related to human nutrition (Hoffman, 2001). More also, on the increase is the data suggesting the existence of a biological link between poor nutrition in early life and chronic diseases in adulthood (Jackson et al., 2010; Langley-Evans and McMullen, 2010).

The prevalence of undernutrition in today's world varies greatly base on region and country, there has also been a decrease in global trends in wasting and stunting. However, the situation is still different in countries with extremely unstable governments and those with civil strife, with records of higher prevalence rate up to 20-30% (Bryce et al., 2008). Undernutrition in developed nations happen to be a problem among people living in rural areas, this could be due to the fact that inhabitants of rural areas mostly suffer from poor nutrition in terms of micronutrient deficiencies. Low income and poor access to nutritious foods is a common factor in poor urban societies leading to undernutrition (Shetty, 2009; Black et al., 2008). The prevalence of undernutrition remains moderate to high in developing nations, depending on the relative degree of economic development. These countries have relatively higher prevalence rate of children wasting and stunting of approximately 30-40% (Gutierrez-Delgado and Guajardo-Barron, 2009; Delisle, 2008).

### **3. Natural products and body anatomy**

Most of the body structures like bones and muscle tissues are formed and nourished by natural products like calcium, phosphorus, vitamin D, proteins and so forth. Bone formation consists of a biological cascade through mesenchymal proliferation, chondrogenesis, osteogenesis, and remodelling (Reddi, 1994). For optimal bone mineral accrual in the developing skeleton, calcium and vitamin D are very important. Human skeleton is rich in calcium supply, with finely tuned mechanisms for release of calcium as needed. Calcium homeostasis is maintained during either low calcium intake or vitamin D status, through the regulation of parathyroid gland and kidneys, at the expense of bone. Adolescents are at risk for poor nutritional status in both calcium and vitamin D. Lack of calcium accumulation in the skeleton of an adolescent or a growing child can have negative consequences for achievement of peak bone mass (Bailey et al., 2000; Bachrach, 2001; Harkness and Bonny, 2005). Differentiation of mesenchymal stem cells into osteoblast to produce new bone tissue was capably induced by bone morphogenetic proteins (BMP), a phenomenon known as osteoinduction (Urist, 1965; Wozney, 2002). Growth factors contained in platelet-rich plasma (PRP) have been proposed to enhance bone grafts maturation and to support repair in the treatment of small bone defects in maxillofacial surgery, when in combination with an organic bovine bone (Roldan et al., 2004). The composition of collagens and noncollagenous matrix proteins defined the organic phase of mineralized tissues. Bone, dentin, and cementum contains collagen type I, cartilage contains collagen type II, and enamel is virtually free of collagen (Sommer et al., 1996). Proteins are the most important nutrients for maintaining body structures, they are the major component of muscles, it is generally believe that flesh makes flesh (Bischoff and Voit, 1860). Skin and bone contain a fibrous protein. Keratinocytes undergo a series of morphological and biochemical changes including the expression of large quantities of proteins which constitute cytoplasmic filamentous networks, keratohyalin granules and cornified envelopes in the course of differentiation (Manabe et al., 1997). Stiffness and rigidity to fluid biological components is provided by structural proteins most of which are fibrous proteins, actin and tubulin are globular and soluble as monomers, which upon polymerization form long, stiff fibres that comprise cytoskeleton, which allows the cell to maintain its shape and size. Connective tissue like cartilage has collagen and elastin as its main components, and keratin is an important component of hard or filamentous structures such as hair, nails, feathers, hooves, and some animal shells (Van Holde and Mathew, 1996).

#### **4. Natural products and body physiology**

Natural products are the potential sources of organic nutrients like carbohydrate, fats, proteins or amino acids and vitamins, as well as dietary minerals necessary for normal physiological functioning of the body.

Body physiological reactions involving nerve, muscle, blood, bone, endocrine and visceral tissues require constant renewal, a delicate balance of which human health is depended upon. Vital exchanges constantly occur, involving many enzymatic systems activated by minerals or trace elements (Speich et al., 2001). The contraction of skeletal, cardiac and smooth muscles aided by calcium ions and involving neuronal and hormonal interplay are responsible for a lot of vital functions in the body such as movements, heartbeats, blood circulation, respiration, parturition, micturition, digestion, and so forth. These activities are important for life maintenance, some of the crucial roles of natural products in maintaining body physiology such as cell signalling electrophysiology, muscle contraction, enzymes and hormones synthesis, etc. are outline below:---

#### **5. Cell signalling**

Natural products play important roles in a lot of cellular activities such as cell signalling that aids in cellular communications. Calcium and vitamin D are necessary for many cellular processes. Primarily, the role of calcium is to serve as a second messenger in virtually all cells. The most common signal transduction is the ionized calcium due to its ability to reversibly bind to proteins. Vitamin D receptors have been identified in most body cells such as the small intestine, colon, brain, skin, prostate, gonads, breast, lymphocytes, osteoblasts, B-islet cells, and mononuclear cells (Holick, 2004). At intracellular level, 1,25-dihydroxyvitamin D interacts with vitamin D receptors and retinoic acid X receptor to enhance or inhibit the transcription of vitamin D-responsive genes, including calcium-binding protein. Stimulation of many noncalcemic physiological functions including insulin production, thyroid hormone secretion, and activated T and B lymphocyte function has been shown to be promoted by 1, 25-dihydroxyvitamin D (Harkness and Bonny, 2005).

Apart from Vitamin D and calcium, Proteins are also involved in several cellular processes. Being the chief actors within the cell, proteins are said to be carrying out the duties specified by the information encoded in genes (Lodish et al., 2004). Most of the biological molecules are relatively inert elements upon which proteins act, with the exception of certain types of RNA (Voet and Voet, 2004). Within the cell proteins acts as enzymes that catalyse chemical reactions. Due to specific nature of enzymes they accelerate only one or few chemical reactions. Most of the reactions involved in metabolism, as well as manipulating DNA in processes such as DNA replication, DNA repair, and transcription are carried out by enzymes (Bairoch, 2000).

Cell signalling and signalling transduction are among the numerous processes involved by many proteins. Insulin is a good example of extracellular proteins that transmit a signal from a cell in which it was synthesized to other cells in distant tissues. Some proteins are membrane proteins that act as receptors, which function mainly to bind a signalling molecule and induce a biochemical response in the cell (Branden and Tooze, 1999). The protein components of adaptive immune system are the antibodies, whose main function is to bind antigens or foreign substances in the body, and target them for destruction. Antibodies are either secreted into the extracellular environment or anchored in the

membranes of specialized B cells called the plasma cells (Van Holde and Mathews, 1996). This phenomenon assists in maintaining body immunity.

## 6. Electrophysiology

Electrophysiological properties of some natural products serve as a means of communication or cell signalling in several body cells and glands. Many cellular functions, such as electrical signal generation in nerve and muscle cells, contraction in muscle cells, and secretion in nerve and gland cells depends on the significant role of widely distributed voltage-dependent calcium channels (Hagiwara, 1983; Hagiwara and Byerly, 1981). They exist not only in fully differentiated cells but also already in oocytes (Okamoto et al., 1977) and in developing nerve and muscle cells (Spitzer, 1979). There are several reports that calcium channels are restricted to, or more prominent in, the less differentiated states of excitable cells such as skeletal cells (Kano, 1975; Kidokoro, 1973; Kidokoro, 1975) and nerve cells (Matsuda et al., 1978; Mori-Okamoto et al., 1983; Spitzer and Baccaglini, 1976).

The involvement of calcium channels as well as sodium channels in generating action potentials in embryonic chick skeletal muscle cells has been established. (Fukuda et al., 1976; Kano, 1975; Kano and Yamamoto, 1977). The involvement of a chloride component has also been shown in addition to the sodium and calcium component of the action potential, in particular in the long-lasting plateau phase of the action potential in these muscle cells (Fukuda et al., 1976). The availability of ATP in mammalian neurones is due to its well-known role as a major energy carrier for cellular metabolism and has been reported to act as a fast transmitter in mammalian brain (Edwards and Gibb, 1993). An action potential is a transient depolarization of the membrane potential of excitable cells. They serve two main functions: to transmit and encode information, and to initiate cellular events such as muscular contraction. An action potential results from a transient change to the properties of the cell membrane, from a state where it is much more permeable to  $K^+$  than  $Na^+$ , to a reversal of these permeability properties. Thus during the action potential an influx of  $Na^+$  is responsible for the rapid depolarization and an efflux of  $K^+$  causes repolarization. Changes to membraneionic permeability are due to the opening and closing of voltage-gated ion channels, and the properties of such channels explain additional phenomena such as refractoriness, threshold and cellular excitability. Action potentials conduct with a finite velocity along nerve axons, and the actual velocity depends on a number of factors that include: fibre radius, temperature, functional ion channel number and the presence of a myelin sheath (Fry and Jabr, 2010). The cations  $Na^+$ ,  $K^+$ ,  $Mg^{2+}$ , and  $Ca^{2+}$ , are involved in the propagation of nerve impulses and in muscle and heart contraction. Phosphorus (P), in phosphoric ester form (ATP), results from the third step of cell respiration. The active transport of  $Na^+$  and  $K^+$  through the plasma membrane involves the energy of ATP hydrolysed by  $Na^+ K^+$  ATPase and activated by  $Mg^{2+}$ , constituting an essential cell function requiring around 25% of the energy metabolism of man at rest (Lehninger et al., 1994). The formation and the use of energy-rich bonds require  $Mg^{2+}$  (Durlach et al., 2000).

## 7. Muscle contraction

Although there are hormonal and neuronal interplay, diverse functions in the body like movement, respiration, digestion, blood circulation, heartbeat, micturition, parturition etc.

are facilitated by muscle contraction. Communication between muscle cells that lead to muscle contraction results from formation of action potential which is due to the electrophysiological properties of these tissues. It is an established fact that all muscle fibres use  $\text{Ca}^{2+}$  as their main regulatory and molecule signalling. Therefore, the variable expression of proteins involved in  $\text{Ca}^{2+}$  signalling and handling play a key role in the contractile properties of muscle fibres. Contraction and relaxation properties of a muscle fibre are largely determined by molecular diversity of the main proteins in the  $\text{Ca}^{2+}$  signalling apparatus otherwise known as the calcium cycle. The  $\text{Ca}^{2+}$  signalling apparatus includes: the ryanodine receptor that is the sarcoplasmic reticulum  $\text{Ca}^{2+}$  release channel, the troponin complex that mediates the  $\text{Ca}^{2+}$  effect to the myofibrillar structures leading to contraction, the  $\text{Ca}^{2+}$  pump responsible for  $\text{Ca}^{2+}$  reuptake into the sarcoplasmic reticulum, and calsequestrin--the  $\text{Ca}^{2+}$  storage protein in the sarcoplasmic reticulum. In addition, a multitude of  $\text{Ca}^{2+}$  binding proteins is present in muscle tissue including paryalbumin, calmodulin, S100 proteins, annexins, sorcin, myosin light chains,  $\beta$ -actin, calcineurin, and calpain. These  $\text{Ca}^{2+}$  binding proteins may either exert an important role in  $\text{Ca}^{2+}$  triggered muscle contraction under certain conditions or modulate other muscle activities such as protein metabolism, differentiation, and growth. Muscle diseases have been shown to be associated with alteration of several  $\text{Ca}^{2+}$  signalling and handling molecules. Pathophysiological conditions like malignant hyperthermia, dystrophinopathies and Brody's disease seem to be associated with functional alterations of  $\text{Ca}^{2+}$  handling. These also underline the importance of the affected molecules for correct muscle performance (Berchtold et al., 2000).

## 8. Body electrolytes and homeostasis

The major electrolytes found in the body are sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), calcium ( $\text{Ca}^{2+}$ ), magnesium ( $\text{Mg}^{2+}$ ), chloride ( $\text{Cl}^-$ ), bicarbonate ( $\text{HCO}_3^-$ ), phosphate ( $\text{HPO}_4^{2-}$ ), and sulphate ( $\text{SO}_4^{2-}$ ) (Ahmad and Ahmad, 1993). For humans to be in an adequate physical condition and a highly efficient state, stable volume, osmotic concentration and electrolyte composition of internal fluids are necessary prerequisites (Zorbas et al., 2002). The macroelements Ca, Mg, K, Na, and phosphorus (P) are generally integrated into anatomic structures (bone elements, nucleic acids, membranes, proteins, enzymes) although they are also involved in the ionized active form and regarded as essential trace elements, as in voltage-gated ionic channels (Allain, 1996). In active form, they are of particular importance for metabolic balance in sports and during physical exercise (Maughan, 1999).  $\text{Na}^+$  contributes to the maintenance of osmotic pressure, water regulation, and acid-base balance.  $\text{Ca}^{2+}$  controls vascular tonicity and coagulation of the blood (Lehninger et al., 1994).

## 9. Blood composition

Among the components of blood are protein (albumin, globulin, and fibrinogen), fat cholesterol, carbohydrate glucose, calcium, phosphorus, sodium chloride ( $\text{NaCl}$ ), urea, uric acid, nonprotein nitrogen (N.P.N) compounds, and creatine. These natural products component are distributed by the blood to body cells and tissues for necessary physiological activities.

## 10. Red blood cells maturation

Red blood cells formation is important for maintaining normal red blood cells count and blood volume. The erythropoietic cells of the bone marrow are among the most rapidly growing and reproducing cells in the entire body, due to the continuing need to replenish red blood cells. Their maturation and rate of production are affected greatly by a person's nutritional status. For final maturation of the red blood cells two vitamins, vitamin B12 (cyanocobalamin) and folic acid are important. Both of these are essential for the synthesis of DNA, because each in a different way is required for the formation of thymidine triphosphate, one of the essential building blocks of DNA. Abnormal and diminished DNA and, as well as failure of nuclear maturation and cell division could be caused by lack of either vitamin B<sub>12</sub> or folic acid (Guyton, 2006).

## 11. Haemoglobin formation

Haemoglobin serves as oxygen transportation medium through the formation of oxyhaemoglobin in the blood, which is distributed to other body cells and tissues. Maintenance of this role is achieved by maintaining the normal haemoglobin count. Initially, to form a pyrrole molecule, succinyl-CoA formed in the Krebs metabolic cycle, binds with glycine. In turn, to form the *heme* molecule, four pyrroles combine to form protoporphyrin IX, which then combines with iron. Finally, each heme molecule combines with a long polypeptide chain, a *globin* synthesized by ribosomes, forming a subunit of haemoglobin called a haemoglobin chain. Four of these chains in turn bind together loosely to form the whole haemoglobin molecule. The different types of chains base on the amino acid composition of the polypeptide portion are designated alpha chains, beta chains, gamma chains, and delta chains. The most common form of haemoglobin in the adult human being, haemoglobin A, is a combination of two alpha chains and two beta chains (Guyton, 2006).

## 12. Heredity

The role of natural products like deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in heredity is too important to be overlooked. It is a widely accepted hypothesis that deoxyribonucleic acid (DNA) is the genetic carrier of information and that ribonucleic acid (RNA) is an essential component in the expression of this information in polypeptide synthesis (Hurwitz and August, 1963). DNA molecule is a repository of genetic information. Therefore, there must be some precise mechanism for duplicating DNA so that the information within it can be handed down unchanged from one generation to the next. Inherited information resides in the precise sequence of bases in DNA and that this information is transferred to messenger RNA which can then specify the sequence of amino acids in some particular protein (Davern and Cairns, 1963).

## 13. Enzymes and hormones synthesis

Almost all process in a biological cell needs enzymes to occur at significant rates, the set of enzymes made in a cell determines which metabolic pathways occur in that cell. Pancreatic adaptation to the diet is a phenomenon due to the dietary induced modifications in enzyme composition of pancreatic tissue and secretion, which has been described in many species

(Poort and Poort, 1980; Corring, 1977; Ben Abdeljilil and Desnuelle, 1964; Reboud et al., 1966). A carbohydrate-rich diet results in an increase in the specific activity of amylase with a concomitant decrease in the specific activity of chymotrypsinogen. The converse is true for a protein-rich diet (Ben Abdeljilil and Desnuelle, 1964) and the same phenomenon has been described for the lipase-colipase system (Bazin et al., 1978). Such modifications of pancreatic content are now thought to be induced by changes in the biosynthetic rate of individual enzymes rather than by non-parallel secretion (Palade, 1975; Dagorn, 1978) or differential enzyme catabolism (Kramer and Poort, 1972). Changes in the individual rates of enzyme biosynthesis have been shown to occur in the developing embryonic pancreas (Kemp et al., 1972) and also after 30 days (Reboud et al., 1966) and more recently after 5 days of dietary adaptation in the adult rat (Poort and Poort, 1980). These long-term adjustments in enzyme synthesis have been correlated to concomitant adaptive modifications in pancreatic content (Reboud et al., 1966). However, no data are available to extend these conclusions to the short-term modifications in enzyme content that have been reported to occur within 24 hours (Deschondt-Lanckman et al., 1971). It became evident that rapid modulation of pancreatic enzyme synthesis was possible. Hormonal stimulation (Dagorn and Mongeau, 1977) or enteral administration of a product of digestion (Dagorn et al., 1977) produced changes in the biosynthetic rates of amylase, chymotrypsinogen and lipase within 15-30 min. It thus becomes possible that a meal may have an immediate regulatory function on pancreatic enzyme synthesis (Dagorn and Lahaie, 1981).

The entire organs functions are controlled by hormones; affecting diverse processes as growth and development, reproduction, and sexual characteristics. Energy storage and uses are also influence by hormones as well as controlling the volume of fluid and levels of salts and sugars in the blood. Large responses in the body could be triggered by very small amounts of hormones. Secretin and glucagon are members of a family of peptides, the vasoactive intestinal polypeptide (VIP)-secretin-glucagon family, which also includes pituitary adenylate cyclase-activating polypeptide (PACAP), gastric inhibitory polypeptide (GIP), parathyroid hormone (PTH), growth hormone-releasing hormone (GHRH), and exendins (Chow et al., 1997; Paul and Ebadi, 1993). All these peptides possess a marked amino acid sequence homology, are widely distributed in the body, and exert pleiotropic physiological effects, in many instances acting in a paracrine manner. The effects of these peptides are initiated by their specific interaction with cell-surface receptors, belonging to the superfamily of G-proteins-coupled receptors. These receptors are glycoproteins with a large hydrophilic extracellular domain followed by 7 highly conserved hydrophilic transmembrane helices, and their signalling mechanism primarily involves the activation of adenylate cyclase (AC)/protein kinase A (PKA) and phospholipase C (PLC)/PKC cascades (Arimura and Shioda, 1995; Chow et al., 1997; Fahrenkrug, 1989; Harmar and Lutz, 1994; Paul and Ebadi, 1993; Nussdorfer et al., 2000).

#### **14. Natural products as nutritional supplements**

Apart from being the major sources of nutrition in form of carbohydrate, protein, fat, etc. natural products are also used as nutritional supplements. According to the Dietary Supplement Health and Education Act of 1994 of the United States (Public Law 103-417, DSHEA), a dietary supplement is a product that is meant to supplement one's diet. Dietary

supplements contain one or more of the following ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, or another dietary substance, or a combination of these ingredients or their extracts. By definition, a dietary supplement is intended for ingestion in pill, capsule, tablet, or liquid form, but it is not for parenteral use. The most commonly used dietary supplement products are: echinacea, ginseng, ginkgo, garlic, glucosamine, st. John's worth, peppermint, fish oils/omega-3 fatty acids, ginger, soy and so forth (Low Dog and Markham, 2007). Elemental analysis of garlic indicated that the powdered plant material contained mainly potassium, phosphorus, iron and calcium among others. While its phytochemical screening revealed presence of chemical compounds like saponins, steroids, tannins, carbohydrates and cardiac glycosides (Mikail, 2010).

Vitamin/mineral supplements can be defined as products that are formulated to supply vitamin and/or mineral nutrients. They are often categorized as vitamin A, vitamin E, vitamin B-complex, multi-vitamins, calcium (Ca), iron (Fe), and multi-vitamins with mineral supplements (Kim, 1997; Kim-Park et al., 1991). In general, it has been found that school children select multi-vitamins with minerals and multi-vitamin supplements more frequently than other types of supplements (Bowering and Clancy, 1986). Vitamin/mineral supplement use has been reported to be influenced by several factors. With respect to young children, daily eating habits can be a particularly significant factor affecting supplement use, as mothers of children often adopt vitamin/mineral supplements as an insurance against possible poor or unbalanced meals. Supplements are also often given to promote appetite in young children (Song and Kim, 1998). This is perceived to be an important issue, as it is thought that young children have numerous eating problems including skipping meals, eating small meals and a strong dislike for some foods (Pipes, 1992). According to demographic characteristics, females, individuals in high socioeconomic categories, and individuals living in large cities tend to take vitamin/mineral supplements more often than their contrasting groups (Bowering and Clancy, 1986; Kim, 1997; Slesinski et al., 1995; Schellhorn et al., 1998; Kim and Keen, 2002).

Nutritional supplements aimed at improving physical performance or altering body compositions have become readily available worldwide. Athletes have been the greatest consumers of many of these products (Burk et al., 2006; Erdman et al., 2006; Huang et al., 2006) and their habits may be followed by other groups of individuals (Sobel and Marquart, 1994; Striegel et al., 2006), mainly those who exercise in gyms regularly (Morrison et al., 2004). The desire for achieving quick results has made the use of such substances very attractive (Rocha and Pereira, 1998). However, it is known that, in general, physically active people do not need additional nutrients apart from those obtained from a balanced diet (Rockwell et al., 2001; Costill, 2003; Silva, 2005; Goston and Correia, 2010).

## **15. Natural products roles in sanitation and personal care/cosmetics**

Not only use as nutritional supplements, rather natural products have interesting roles in sanitation, personal care and cosmetic surgery. Large variety of products and formulations are considered as personal care products in the United States (US) and cosmetics in the European Union (EU), these products includes soaps, shampoos and shower products, sunscreens, skin and hair care products, hair dyes, make ups, lip sticks, toothpastes, dental care products, deodorants, personal hygiene products and many others(Antignac et al.,

2011). Decorative cosmetics are principally used to beautify or cover minor, visible imperfections. Shiny, oily, inhomogeneous colourings, as well as slight imperfections on skin surface are corrected by these kinds of cosmetics. These products play an important role, creating the effect of youthfulness and wholesomeness which are becoming more and more important in our society today (Valet et al., 2007).

Personal care products (PCP) from botanical ingredients include a variety of preparations, such as plant extracts, expressed juices, tinctures, waxes, vegetable oils, lipids, plant carbohydrates, essential oils, as well as purified plant components, such as vitamins, antioxidants or other substances with biological activity (Allemann and Baumann, 2009). For thousands of years, soap has been used as probably the oldest skin and cloth cleanser. Soap is produced from the saponification of fats and oils by alkali. The manufacturing process of soap involved saponification by which triglycerides (fats and oils) or fatty acids are transformed into the corresponding alkali salt mixtures of fatty acids (Friedman and Wolf, 1996). From the pulp industry soft and liquid soap are prepared from tall oil as tall resin by-products (Rappe et al., 1990). Products designed to improve the appearance of the aging face by altering the structure and function of the skin in ways that are important for cosmetic surgeons are termed cosmeceuticals. Thus, cosmeceuticals aids cosmetic operations, these products include alpha hydroxy acids, beta hydroxy acids, polyhydroxy acids, vitamins, retinoid, skin lightening agents, and sunscreens (Draelos, 1999).

## 16. Natural products as sources of drugs

Natural products have played an important role throughout the world for thousands of years in human diseases treatments and preventions. Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates (Newman et al., 2000). An analysis of the origin of the drugs developed between 1981 and 2002 showed that natural products or natural product-derived drugs comprised 28% of all new chemical entities (NCEs) launched onto the market (Newman et al., 2003). In addition, 24% of these NCEs were synthetic or natural mimic compounds, based on the study of pharmacophores related to natural products (Newman et al., 2000). This combined percentage (52% of all NCEs) suggests that natural products are important sources for new drugs, are also good lead compounds suitable for further modification during drug development (Chin et al., 2006).

Natural products can come from anywhere. People most commonly think of plants first when talking about natural products, but trees and shrubs can also provide excellent sources of material that could provide the basis of a new therapeutic agent. Animals too, whether highly developed or poorly developed, whether they live on land, sea, or in the air can be excellent sources of natural products. Bacteria, smuts, rusts, yeast, moulds, fungi, and many other forms of what we consider to be primitive life can provide compounds or leads to compounds that can potentially be very useful therapeutic agents (Spainhour, 2005).

Some of the provision of nature to human kind over the years includes the tools for the first attempts at therapeutic intervention (Nakanishi, 1999a; Nakanishi, 1999b). The *Nei Ching* is one of the earliest health science anthologies ever produced and dates back to the thirtieth century BC (Nakanishi, 1999a; Nakanishi, 1999b). Some of the first records on the use of

natural products in medicine were written in cuneiform in Mesopotamia on clay tablets and date to approximately 2600 BC (Cragg and Newman, 2001a; Cragg and Newman, 2001b; Nakanishi, 1999a; Nakanishi, 1999b). Indeed, many of these agents continue to exist in one form or another to this day as treatments for inflammation, influenza, coughing, and parasitic infestations (Holt and Chandra, 2002; Spainhour, 2005). The best known natural products documentation is the Ebers Papyrus, which documents nearly 1000 different substances and formulations, most of which are plant-based medicines (Nakanishi, 1999a; Nakanishi, 1999b; Spainhour, 2005). More also, to date natural products continue to be the potential sources of new compounds or molecules that awaits further scientific elucidations, like the newly isolated alkylresorcinols from *Urginea indica* (Mikail et al., 2010).

The World Health Organization estimates that approximately 80% of the world's population relies primarily on traditional medicines as sources for their primary health care (Farnsworth et al., 1985). Over 100 chemical substances that are considered to be important drugs are either currently in use or have been widely used in one or more countries in the world have been derived from a little under 100 different plants. Approximately 75% of these substances were discovered as a result of chemical studies focused on the isolation of active substances from plants used in traditional medicine (Cragg and Newman, 2001a; Cragg and Newman, 2001b, Spainhour, 2005). A lot of natural products medications are derived from polyketides, which includes antibiotic, antifungal, anticancer, anthelmintic and immunosuppressant compounds such as erythromycins, tetracyclines, amphotericins, daunorubicins, avermectins, and rapamycins. These derivatives are used in treatment of host of disease situations affecting various body systems such as central nervous system, cardiovascular system, renal system, visual system and common integument.

## 17. Natural products as sources of antibiotics

Antibiotic is a word originally coined for those natural compounds with antimicrobial properties (Strohl et al., 2001; Overbye and Barrett, 2005; Palaez, 2006). The word antibiotic now a day no longer refers only to natural compounds; most of the marketed antibiotics are based on natural chemotypes. In the years 1982-2002 seventy out of the ninety antibiotics marketed originated from natural products (Newman et al., 2003; Palaez, 2006). The discoveries of sulphonamide antibiotics in the 1930s and penicillin in the 1940s have reduced the fatality rates of bacterial infections (Sneader, 2005; Newman et al., 2000; Drews, 2000). These has led to the discovery of most of the classes of antibacterial drug known today, many of which derived from natural products (Sneader, 2005; Walsh and Wright, 2005; Finch et al., 2003). The natural products-derived drugs mostly belong to five different structural classes namely: the  $\beta$ -lactam, the streptogramin, the macrolide, the tetracycline and the daptomycin (Butler and Buss, 2006).

Natural product-derived antibacterial drug prevalence may be due to the evolution of secondary metabolites as biologically active chemicals which conferred some selectional advantages to the producing organisms. There is likelihood of natural products to have evolved to penetrate the cell membranes and interact with specific proteins targets (Stone and Williams, 1992). The structural complexity of many natural products is required for the inhibition of many antibacterial protein targets (Butler, 2004; Koehn and Carter, 2005; Butler and Buss, 2006).

Some of the representative antibiotics of natural product origin include Daptomycin, Vancomycin, Ramoplanin, Tetracycline, Streptomycin, Erythromycin, Chloramphenicol, Penicillins, Cephalosporins, Carbapenems (Singh and Barret, 2006). However, as reported by Palaez (2006), Penicillins, Cephalosporins, Erythromycin, Thienamycin, Vancomycin, Fosfomycin, Mupirocin, Fusidic acid, Streptogramins, and Daptomycin are some of the marketed antibiotics that originated from microbial natural products. Some of the natural product-derived antibiotics currently in clinical development include the followings: Daptomycin, Dalbavancin, Telavancin, Oritavancin, Ramoplanin, Efiprestin, Lyostaphin and so forth (Clardy, et al., 2006).

## 18. Antivirals derived from natural products

Numerous compounds have been revealed through antiviral testing from structural classes that include peptides, terpenoids, polysaccharides, steroids and alkaloids that potentially inhibit both RNA and DNA viruses (Abad Martinez et al., 2008). Infections with viruses are counteracted by the host natural defences which prevent or limit the extent of these diseases. Interferon, a protein moiety produced in virus infected cells provides one of these defences. Interferon causes the production of a new protein that shuts off virus replication when attached to the cell membrane and consequently may lead to suppression of the viral infection. Therefore, antiviral compounds could be produced by the synthesis of drugs that induce interferon production before or after the infection in the sense that they activate certain host defence mechanisms (Becker, 1980). Marine compounds are good sources of Pharmacological agents. In the market today, there are over 40 pharmacological compounds, including alternative antiviral medicines or those being tested as potential antiviral drugs at preclinical and clinical stages (Yasuhara-Bell and Lu, 2010).

Some of the marine-derived antiviral agents circulating in market and are on clinical development include: Acyclovir, Ara-A (vidarabine), Ara-C (cytarabine), Avarol, Azidothymidine (zidovudine), and Cyanovirin-N (Yasuhara-Bell and Lu, 2010). Triterpenoids isolated from plants are biologically active natural products attracting considerable interest due to their variety of structures and their broad range of biological activities. Some compounds having significant anti-tumor activities in an *in vivo* assay have been reported. Some of these compounds are useful in the development of novel drugs with pharmacological actions (Barquero et al., 2006). Triterpenoidal saponins family of which Avicins is a member reduce both oxidative and nitrosative cellular stress, which result in developmental suppression of malignancies and other related diseases (Haridas et al., 2001).

Milk has been reported to contain antiviral agents (Matthews et al., 1976; Newburg et al., 1992). Lactoferrin is one of such agents that later shown to *in vitro* inhibit the human immunodeficiency virus (HIV-1), human herpes simplex virus (HSV-1 and -2), human cytomegalovirus, respiratory syncytial virus, poliovirus and rotavirus (Marchetti et al., 1999). Assayed chemically modified proteins presented antiviral activity against HSV-1 before, during and after infection. Higher concentrations of modified proteins are required if present before infection as compared to during or after infection. This therefore, suggests that targeted chemical modification of some natural products might provide antiviral compounds effective against HSV-1 infection (Oevermann et al., 2003).

## 19. Antiprotozoal potentials of natural products

Parasitic diseases are major public health problem especially in tropical developing countries, affecting hundred millions of people (Tagboto and Townson, 2001).

During phagocytosis reactive oxygen species are generated by neutrophilic granulocytes as a means of natural defence against invading microorganisms (Baehner et al., 1982). It is believed that this oxygen radicals formed by electron transfer processes have significant role in xenobiotic mechanism of action (Eberson, 1985; Halliwell and Gutteridge, 1985). Some antiprotozoal agents have been tested to possess this form of mechanism of action (Kovacic et al., 1989) although others may act in different ways.

Among the established antiprotozoal drugs from natural sources used in treating human protozoan infections are quinine from *Cinchona* species, artemisinin from *Artemisia annua* for malarial treatment and *Psychotria ipecacuanha* for treating amoebiasis (Tagboto and Townson, 2001). Three alkaloids namely quinidine, cinchonine, and cinchonidine together with quinine have significant antimarial activity. All of these compounds were isolated from *Cinchona* trees. Totaquine is an antimalarial agent containing all the four alkaloids used in the past as a cheap alternative to quinine sulphate (Dobson, 1998). Seven out of 14 anti-parasitic drugs approved from 1981-2006 are natural products derivatives including artemisinin (Newman and Cragg, 2007). Apart from the established antiprotozoal drugs, natural products still possess the potentials of providing more alternative sources of antiprotozoal medications that needs further scientific elucidations. *Allium sativum* has been shown to possess trypanocidal activity both in vitro and in vivo (Mikail, 2009a). There are several other plants that possess this activity (Mikail, 2009b).

## 20. Antifungal property of natural products

Antifungal drugs are used in treating any of the following disease conditions: allergic reactions to fungal proteins, toxic reactions to toxins present in certain fungi and infectious mycoses which is the most serious and difficult to diagnose and treat due to the fact that mycoses come in many forms (Barret, 2002). Polyene natural product amphotericin B is the most commonly drug used in treating these disease conditions (Gallis et al., 1990; Wingard et al., 1999). Other various newer lipid formulations are also used in handling such disease conditions (Hiemenz and Walsh, 1996). Griseofulvin which was first isolated from the fungus *Penicillium griseofulvin* has been used in the treatment of dermatophyte infections for the past 30 years (Finkelstein et al., 1996). Polyene antifungal antibiotic nystatin, is used for prophylaxis and treatment of candidiasis of the skin and mucous membranes (Waugh, 2008). In Brazil, many plants biomes, such as the Cerado (savannah), the Atlantic and the Amazon rain-forest, have been used in the treatment of several tropical diseases, such as leishmaniasis, malaria, schistosomiasis, fungal and bacterial infections. These are mostly used by local populations as natural medicines (Alves et al., 2000).

## 21. Anthelminthic activity of natural products

More than 1 billion people are reported by the World Health Organization to suffer from neglected tropical diseases such as helminthiasis. This disease condition is a major health problem throughout developing countries and is also a food safety issue worldwide (Savioli, 2009). Th2 immunity is the key for protective immunity to all helminths, although the final

effector mechanisms for helminths expulsion are distinct for each helminths, which could be due to the different invasion strategy of each helminth (Shigeo Koyasu et al., 2010). Worm expulsion is dependent on Th2 immune responses. Critical for worm expulsion are Th2 cytokines, IL-4 and IL-13. Both of these cytokines significantly delays worm expulsion (Finkelman et al., 2004). Anthelmintic act rapidly and selectively on neuromuscular transmission of nematodes, agonism at nicotinic acetylcholine receptors of nematode muscle and cause spastic paralysis, organophosphorus cholinesterase antagonism, increasing the opening of glutamate-gated chloride (GluCl) and produce paralysis of pharyngeal pumping, increasing calcium permeability, while other anthelmintics have a biochemical mode of action (Martin, 1997).

Ivermectin was discovered from a microorganism, *Streptomyces avermitilis*, isolated from an Oceanside golf course soil in Japan, it was found to have potent bioactivity. It has systemic anti-parasitic activities, effective against helminths, arachnids, and insects, but not against protozoa, bacteria, flatworms or fungi (Omura, 2008) Ivermectin was the first 'endoectoparasites' discovered due its proven activity against endo- and ectoparasites, at unprecedented low doses it could be easily used orally, topically and parentally (Arena et al., 1992; Omura, 2002). *Digenea simplex* and *Chondria armata* are two Japanese red algae, which have been used for their potent anthelmintic properties for more than 1000 years. Elimination of intestinal worms, such as parasitic round worms (*Ascaris lumbricoides*), tape worms (*Taenia* spp), and whip worms (*Trichuris trichura*) are some of the anthelmintic properties of these Japanese algae. Domoic acid and kainic acid are two closely related compounds isolated from these red algae, which are responsible for these curative properties (Gerwick et al., 2007). A wide range of plant and plant extracts has been used as potential alternative anthelmintic strategy for the treatment of helminth infections. Many species of fig tree from the genus *Ficus* and the papaya tree, *Carica papaya*, the crude latex was extracted and used successfully against ascarids, tapeworms, whipworms and hookworms in the early 19<sup>th</sup> century (Berger and Asenjo, 1940; Waller et al., 2001; Tagboto and Townson, 2001; Stepek et al., 2004).

## 22. Role of natural products in treating non infectious diseases

Although natural products are the origin of several drugs used in the treatment of many infectious diseases, they also play a significant role in the treatment of several non-infectious disease conditions.

## 23. Diabetes mellitus and obesity

Hyperglycaemia is the unifying feature of this heterogeneous endocrine disorder. Every year the number of diabetic patients is rising by 4-5% (Wagman and Nuss, 2001). Plant extract and complex microbial secondary metabolites of natural products have attracted the attention of scientific world for their potential use as drugs for the treatment of chronic diseases such as Type II diabetes (Bedekar et al., 2010). Acarbose was discovered from compounds isolated from *Actinomycetes* species, which are potent inhibitors of digestive enzymes such as  $\alpha$ -amylase, sucrase, and maltase. Acarbose is the most widely used digestive enzyme inhibitor among the numerous antidiabetic drugs used for the treatment of Type-II diabetes (Bedekar et al., 2010). Maglitol which was derived from 1-deoxynojirimycin is one of the widely used  $\alpha$ -glucosidase inhibitors used in the treatment of Type II diabetes. Nojirimycin, deoxynojirimycin, and their derivatives are new compounds

with inhibitory properties derived from various *Bacillus* and *Streptomyces* strains (Schmidt et al., 1979; Tan, 1997). Another  $\alpha$ -glucosidase inhibitor used as antidiabetic drug mostly in Asia is voglibose, which is synthesized from valiolamine isolated from fermentation broth of *Streptomyces hydroscopicus* subsp. *Limoneus* (Matsuo et al., 1992; Goke et al., 1995).

Interestingly, polyphenols natural compounds such as flavonoids have demonstrated numerous health benefits, by addressing the issue of obesity and diabetes due to their digestive enzyme inhibition activity, induction of apoptosis in adipose tissue, etc. (Nelson-Dooley et al., 2005). A subgroup of flavonoids, the anthocyanins is water-soluble plant pigments responsible for the blue, purple and red coloration of many plant tissues. Anthocyanidins are extracted mostly from plants or plant waste in a form of a mixture. Anthocyanidins are the aglycon forms of anthocyanins of which 17 of them are found in nature. Anthocyanins have antioxidant and antihypertensive activities, they have also demonstrated the inhibition of lipid oxidation. Anthocyanins specifically inhibit  $\alpha$ -glucosidase activity and have the potential to reduce blood glucose levels after starch-rich meals. (Matsui et al., 2001a; Bedekar, 2010). Through influencing signalling molecules natural products can prevent both adult and childhood obesity. During physiological conditions such as exercise, hypoxia, the presence of reactive oxygen species (ROS), and ischemia/reperfusion activated protein kinase (AMPK) is activated, which is the master regulator of metabolic processes. Natural products also activate AMPK to reduce obesity through the regulation of fatty acid metabolism-related proteins such as acetyl-coenzyme A (CoA) carboxylase (ACC), sterol regulatory element-binding protein (SREBP), fatty acid synthase (FAS) and so forth (Hwang et al., 2011).

## 24. Hypertension

The hypothesis that meat is a source of peptides that are effective in preventing and reducing chronic life style-related diseases (CLSRDS) such as hypertension has been tested. Empowering hypertensive people in quality life such as offering nutritional food rich in antioxidant vitamins, and proteins or biologically active peptides, can lower blood pressure, possibly by preventing an underlying cause of the condition. Provision of these forms of functional food is useful even to the normotensive individuals nutritionally. The underlying aetiology to clinical hypertension may be due to a deficiency in proteins from meat origins, along with abnormalities in carbohydrate and fat metabolism. Proteolysis of meat muscle generate multiple number of amino acid peptides with nutrafunctional roles, the have strong angiotensin-converting enzyme inhibitory activity, which perhaps lower blood pressure (Ahmed and Muguruma, 2010). Dietary supplements promote cholesterol-lowering benefits, some of these supplements reported to have significant low-density lipoprotein-cholesterol (LDL-C) lowering properties are soluble plant fibre (oats, psyllium, pectin, flaxseed, barley, guar gum, cellulose, lignins, wheat bran), plant sterols, soy proteins, nuts (almonds, pecan, walnut) and red yeast rice supplements (Nijjar et al., 2010).

## 25. Analgesia and recreation

Pain is simply an undesirable physical or emotional experience. For the past 7000 years ago natural products have been used to treat pain disorders. A good example is opium poppy (*Papaver somniferum*) and the bark of willow tree (*Salix* spp.). In 19<sup>th</sup> century some individual components of different natural products remedies were identified and purified. One of the

most widely used and available compounds for the management of mild pain is Aspirin or acetylsalicylic acid derived from salicylic acid, which is extracted from Willow tree (*Salix alba*). Opioid is a name given to all compounds having the same mechanism of action as the constituents of opium. These are derived from opium juice from *Papaver somniferum*; examples of these groups of drugs include morphine, codeine and thebaine. These drugs are also used for recreational purposes apart from their use as analgesics (McCurdy and Scully, 2005). Cocaine interacts with voltage-gated ion channels and blocks sodium channels which is responsible for its local anaesthetic activity. Cocaine has the ability to block the dopamine transporter due to its ability to create a euphoric state, meaning it is also used for recreational purposes (McCurdy and Scully, 2005). Caffeine is the most widely used psychoactive drug in the world, found in a number of plant sources. Coffee (*Coffea arabica*, native to Africa), tea (*Camellia sinensis*, native to China), and cacao (*Theobroma cacao*, native to South and Central America), from which chocolate is made. Other caffeine-containing plants include kola (*Cola acuminata*), guarana (*Paullinia cupana*), and yerba mate (*Ilex paraguariensis*). Theophyllines found in tea and theobromines found in cacao are other botanical methylated xanthines closely related to caffeine with psychoactive effects. Nicotine from tobacco plant (*Nicotiana tabacum*, *Nicotiana rustica*) and related species are other psychoactive substances. *Cannabis* plant is the source of cannabinoids such as marijuana and hashish which also possess psychoactive effects in form of relaxation, sedation, intensification of thoughts and feelings, alterations of perception, and increased appetite (Presti, 2003).

## 26. Antipsychotics

From ancient times to present in Indian ayurvedic medicine, extracts of the snakeroot plant, *Rauwolfia serpentine*, have been used to treat psychotic symptoms. Reserpine was isolated and identified in the 20th century from *R. serpentine*, and was found to cause decreases in the activity of monoaminergic neurons using the neurotransmitters dopamine, norepinephrine, and serotonin (Presti, 2003). Polygalasaponins is an extract of a plant (*Polygala tenuifolia* Willdenow) that has been used as antipsychotic for hundred years in Korean traditional medicine. Polygalasaponin has been shown to have dopamine and serotonin receptor antagonism properties *in vivo*, suggesting its possible utility as an antipsychotic agent (Chung et al., 2002).

## 27. Antidepressants

Saint John's wort (*Hypericum perforatum*) for centuries in Europe, the extract of this plant has been used for their antidepressant effects (Presti, 2003). Pharmacophores are natural products derived from chemically defended marine organisms related to serotonin or clinically utilized antidepressant drugs. Aaptamine and 5,6-dibromo-*N,N*-dimethyltryptamine are two marine natural products which produced significant antidepressant-like activity in the forced swim tests. In the tail suspension test antidepressant-like effects of 5,6-dibromo-*N,N*-dimethyltryptamine were confirmed, whereas aaptamine has not produce significant results (Diers et al., 2008).

## 28. Wound healing (angiogenesis)

The plant Saint John's wort when used in topical preparations facilitates wound healing. Its healing properties were mentioned in the ancient medical texts of Hippocrates, Pliny, and

Galen (Presti, 2003). Honey has been shown to possessed various antimicrobial activities in addition to its wound healing effect, a good example is the manuka honey (*Leptospermum scoparium*). Preparations of aloe vera (*Aloe barbadensis*), cocoa and oak bark extracts have been used to treat various ailments especially those of the skin (Davis and Perez, 2009). Upon injury papaya and fig trees produce latex rich in proteolytic enzymes, the juices extracted from the stem or fruit of plants such as the pineapple, contain large amount of cysteine proteinases (Rowan et al., 1990). Chymopapain, is one of these enzymes used in medicine to treat intervertebral disc prolapse with a similar success rate to surgery (Smith and Brown, 1967). For burn injuries ananain and comosain are used as debriding agents (Rowan et al., 1990). Bromelain, papain, and ficin have been used to replace glucocorticoids and non-steroidal anti-rheumatics as anti-inflammatory drugs (Lotz-Winner, 1990; Maurer, 2001; Stepek et al., 2004).

## 29. Role of natural products in preventive medicine

Natural products like medicinal plants and foodstuffs are used for their preventive effects against life-style related diseases such as coronary heart diseases, hypertension, thrombosis, allergic inflammation, arteriosclerosis, diabetes and cancer, although clinical basis and experimental evidence are insufficient and unclear. However, biochemical and pharmacological study of isolated natural compounds from various medicinal plants and foodstuffs indicate that fucoidan (polysaccharides), carp oil (fatty acids) and triterpenoids inhibited the tumour growth and/or metastasis in the liver, through the inhibition of tumour induced neovascularization, in tumour-bearing mice. The inhibition of thrombin-induced adhesion molecule through protein C kinase activation inhibition has been demonstrated by baicalein (flavones) isolated from *Scutellaria baicalensis* roots. Furthermore, through endothelium-dependent nitric oxide production, xanthoangelol (chalcones) isolated from *Angelica keiskei* roots inhibited catecholamine-induced vasoconstriction (Kimura, 2005). Many plants are used in traditional medicine as active agents against various effects induced by snakebite. *Baccharis trimera* (Asteraceae), known in as *carqueja* in Brazil, has been shown to inhibit haemorrhagic and proteolytic activity caused by *Bothrops* snake venoms (Januario et al., 2004). Biologicals like vaccines and antisera have a great role in preventive medicine. Polyclonal antibodies are mixture of antibody specificities which all recognize the same antigen. Blood serum that contains polyclonal antibodies is known as antiserum. Polyclonal antibodies are used in medicine to confer passive immunity to certain diseases. For instance, transfusion of serum antibodies from a human survivor of Ebola virus is the only effective treatment for the viral infection. Antiserum is also used in medicine as antitoxin or antivenin, which contain antibodies specific for venom from poisonous reptiles, arachnids and insects. People who have been bitten or stung by these animals are treated with this antiserum. Vaccine is a biological preparation that improves immunity to a particular disease made from attenuated or killed forms of a microbe or its toxins. Vaccines are used for either prophylaxis or therapy against certain disease conditions such as smallpox. Smallpox vaccine was the first successful vaccine to be developed in 1796 by Edward Jenner (Stewart and Devlin, 2006).

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# **Effect of Fruit and Vegetable Intake on Oxidative Stress and Dyslipidemia Markers in Human and Animal Models**

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## **1. Introduction**

In the last 40 or 50 years mankind, particularly in the western world, has modified its diet to include a reduced amount of fruits and vegetables, while increasing its intake of processed foods with a high content in fats and simple carbohydrates. This change has brought on consequences such as the development of a vast array of conditions like the metabolic syndrome (also known as syndrome X or insulin resistance syndrome), which is known to include dyslipidemia and oxidative stress among other physiologic disturbances, and be itself a risk factor for the development of diabetes and cardiovascular disease (CVD). On the other hand, studies suggest that a diet rich in fruits and vegetables can have a positive impact on dyslipidemia and oxidative stress markers, as well as other components of the metabolic syndrome. These positive effects are mediated mainly by the antioxidant vitamins (A, C and E), carotenoids, polyphenols and other important phytochemicals.

## **2. Diet, the metabolic syndrome, dyslipidemia and oxidative stress**

In 2007, the World Health Organization (WHO) estimated that about 1.7 billion people worldwide were overweight (including at least 155 million children) (Hossain et al., 2007), these numbers have transformed obesity and its related maladies into a worldwide epidemic. Even though obesity is almost always assumed to be a necessary condition for the development of dyslipidemia, metabolic syndrome, diabetes and CVD, and these terms are sometimes used as if they all occur alongside each other, this may not always be true, since overweight people may in fact present normal lipid and glucose concentrations, as well as normal blood pressure, normal insulin metabolism and low inflammatory markers (Barter et al., 2007) (the term marker in this chapter is to be understood as measurable markers such as cholesterol concentration and not genetic markers). Therefore it is the clustering of factors

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defined as metabolic syndrome that confers the highest risk for CVD mortality, although many of the isolated conditions are also risk factors for CVD (Isomaa et al., 2001).

Since diet is a major contributor to metabolic syndrome, some authors have noted the particular relationship between the two. Fructose has been consumed mainly from fruits and honey at about 16-20 g/day since early times, while having a recent and significant increase to 85-100 g/day with the addition of high-fructose corn syrup (HFCS) as a sweetener in products such as soft drinks, fruit juices and many other items commonly included in western diets; this rise in consumption has important metabolic consequences for the individual (Basciano et al., 2005). When adequate amounts of fructose are ingested it can have a positive effect on glucose metabolism, like the reduction in glycemic response and a better tolerance to glucose, however, when fructose is ingested in greater quantities it directs the liver (the main organ responsible for metabolizing it) to increase *de novo* lipogenesis, in other words, it deviates the metabolism in favor of storing energy in the form of triacylglycerols while increasing their plasma levels, which in turn translates into obesity and insulin resistance, all of which part of the metabolic syndrome (Basciano et al., 2005).

A high fat diet can also have important effects on the individual, including induction of oxidative stress. A study compared the effects of the consumption of a high fat breakfast (fast food style) in patients with metabolic syndrome, to a meal recommended by the American Heart Association (control). An increase in triacylglycerols, with a concomitant decrease in HDL, was found in the fast food breakfast group; at the same time, biomarkers of oxidative stress rose significantly in this group compared to the control (Devaraj et al., 2008). These effects have also been noted on animal models, for example, when New Zealand White rabbits were subjected to a diet rich in cholesterol for six months their lipid profiles changed; their triacylglycerols, total and LDL cholesterol rose while HDL cholesterol was reduced; oxidative stress markers were also elevated when cholesterol was administered in the diet, these effects were not apparent when coconut or sunflower oil were administered instead of cholesterol (Sabitha et al., 2010). The aforementioned experiments show how dyslipidemia and oxidative stress can occur simultaneously as consequences of a high fat diet; however this apparently simple relationship can become more intertwined thereafter, since the products of oxidative stress can oxidize lipid-containing molecules such as LDL to form oxLDL (which can be considered a marker of oxidative stress), and in turn, generate an inflammatory response. The organism cannot cope with these extenuating circumstances for an extended period of time and cardiovascular disease takes place (Rizzo et al., 2009). It is therefore possible to argue that initially both conditions take place side by side, and after a while the two become part of the same cumulative disorder, in which oxidative stress can synergize with altered lipid levels and aid in the development of cardiovascular disease. Sachidanandam in 2009 (Sachidanandam et al., 2009) shows that in rats fed a high fat diet, the collagen patterns were altered favoring matrix accumulation, as well as increased constriction and impaired relaxation of the vascular tissue (Sachidanandam et al., 2009). Similar results were obtained in mice by Poirier in 2005, who reported that a high fat diet can lead to the development of obesity, insulin resistance and other symptoms similar to metabolic syndrome in humans (Poirier et al., 2005). This evidence shows that the dyslipidemia and oxidative stress (initially brought on by diet) can lead to the development of CVD by impacting specific tissues like the arteries.

Dyslipidemia is so called because it involves a high serum triacylglycerol concentration ( $\geq 200$  mg/dL), low high-density lipoprotein cholesterol (HDL) concentration ( $< 35$  mg/dL), small dense low-density lipoproteins (sdLDL) and an increased concentration of inflammatory markers (Adiels et al., 2008; Barter et al., 2007; Bestehorn et al., 2010; Rader, 2007). The first three biomarkers have even been referred to as the "lipid triad", and because they have been found in patients with CVD, it is also called the "atherogenic lipoprotein phenotype" (Bestehorn et al., 2010; Grundy, 1998; Rizzo & Berneis, 2005).

Oxidative stress can be described as an imbalance in the REDOX homeostasis of the cell, although it lacks a precise definition. Under normal circumstances the cell produces an assortment of oxidant molecules that can become extremely detrimental if their concentrations are not kept under strict regulation and whose elimination takes place via enzymatic and non-enzymatic systems. The synthesis of these oxidant molecules, usually known as reactive oxygen species (ROS) is an unfortunate byproduct of having an aerobic metabolism and underlies the so called oxygen toxicity (Auten & Davis, 2009). Nevertheless, the cell has an impressive array of defense mechanisms that, in healthy individuals and under an optimal diet, can be quite effective in maintaining ROS under adequate concentrations. This balance of oxidation and reduction can be a fragile one, and when it is broken cells and tissues are led to oxidative stress. This can occur by an acute or chronic overproduction of ROS brought on by trauma, toxicity (by the consumption of ethanol, cigarette smoke, xenobiotics and others), genetic factors, stress, poor or unhealthy eating habits (something already acknowledged) and many other factors (Augustyniak & Skrzyllewska, 2009; Studzinski et al., 2009; Talukder et al., 2011). It can also be caused by a deficient antioxidant defense system even if the oxidative molecules are present in normal quantities. In any case, this brittle balance is overwhelmed to the side of the oxidative molecules, and it is at this point where the negative effects can become apparent in the progression of CVD as well as other conditions like different types of cancers, diabetes and the progression of aging (Agalliu et al., 2010; Gupta et al., 2010; Kenyon, 2010). Another interesting point is that oxidative stress is a condition that cannot be completely neutralized, since there is constant damage being inflicted on the cell from its own metabolism as well as environmental sources (Costantini & Verhulst, 2009).

Oxidative stress is a complex condition, and it can affect the organisms in many different ways, there are various ways that it can be measured; these can be grouped into four general categories: free radical production, antioxidant mechanisms, oxidative damage and repair mechanisms (Monaghan et al., 2009).

Thiobarbituric acid reacting substances (TBARS) is one of the most popular techniques used to evaluate oxidative stress in living organisms (Alturfan et al., 2009). It is also used to evaluate oxidation of food systems. TBARS are used to determine malondialdehyde (MDA) and other molecules that are generated as byproducts of lipid oxidation, (it is therefore a measure of oxidative damage) although it can be somewhat unspecific since other compounds also react with thiobarbituric acid (Caprioli et al., 2011).

Antioxidant capacity of plasma can also be used as a marker of oxidative stress or REDOX status of live systems. A number of methods are used to measure plasma antioxidant capacity, some of the most popular are ferric reducing ability of plasma (FRAP), oxygen radical scavenging capacity (ORAC), total radical-trapping antioxidant parameter (TRAP) and trolox equivalent antioxidant capacity (TEAC) among others (Grigelmo-Miguel et al., 2009).

Other indicators of oxidative stress or REDOX status include activity and expression of antioxidant enzymes and concentration of non-enzymatic antioxidants in plasma and tissues (Beltowski et al., 2008).

## 2.1 Oxidative stress and antioxidants

The main molecules implicated in the occurrence of oxidative stress are free radicals and ROS. Free radicals are defined as species having one or more unpaired electrons which make them unstable and highly reactive. Among the most common oxygen free radicals are the superoxide anion ( $O_2^{\cdot -}$ ), the hydroxyl radical ( $\cdot OH$ ) and peroxy radicals ( $ROO^{\cdot}$ ). Other kinds of ROS are not free radicals, the most important one being hydrogen peroxide ( $H_2O_2$ ). The sources of the  $O_2^{\cdot -}$  are dioxygen-reducing enzymes such as NADPH oxidases, xanthine oxidase, monoamine oxidase, prostaglandin synthases (Peyrot & Ducrocq, 2008) and cytochrome P450-dependant oxygenases, some of which are located in the cell membrane of polymorphonuclear cells, macrophages and endothelial cells (Turrens, 2003). The mammalian mitochondria complexes I and III have been shown to produce  $O_2^{\cdot -}$ , however, these are not the only sites inside the mitochondria that produce it, the others are mainly enzymes that interact with the matrix NADH pool and/or the CoQ pool (Murphy, 2009). Non-enzymatic sources of the anion have also been reported, mainly by the action of coenzymes (flavins), prosthetic groups (iron-sulfur clusters) or enzyme-reduced xenobiotics (adriamycin) (Turrens, 2003).

Some of the negative effects that ROS can exert is DNA and RNA damage which can lead to an increased number of mutations, membrane lipid peroxidation, and protein tyrosine nitration (this last one being a marker for so called nitrosative stress), and after the cell sustains too many of these insults, it may become mutated or direct itself to apoptosis, giving way to the development of the previously mentioned oxidative stress-related diseases, like CVD, cancer diabetes and aging (Corpas et al., 2008). Some ROS molecules are, under certain conditions, non-toxic, but can react with other free radicals such as  $NO^{\cdot}$  to form the peroxy nitrate ion (Corpas et al., 2008; Peyrot & Ducrocq, 2008; Valko et al., 2007):



This reaction has been known to take place in situations such as hyperglycemia, atherosclerosis, inflammation and others, while at the same time modulating important signaling pathways such as the one mediated by the serine/threonine protein kinase Akt, which is involved in cell growth, glycogen synthesis, cell proliferation and apoptosis (Song et al., 2007). Since the production of ROS is largely inevitable, the cell has evolved mechanisms to cope with them and maintain an acceptable REDOX state, they can be divided into two broad groups; enzymatic and non-enzymatic.

The enzymatic antioxidant mechanisms are sensitive to the REDOX state of the cell, and are thus activated when an overproduction of free radicals starts to become a threat to the wellbeing of the cell. Their function is to neutralize a free radical or potentially toxic molecule by either converting it to an innocuous compound, or to another one that is less reactive and/or less toxic. Among them are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST). These enzymes work in synergy with non-enzymatic compounds, such as phytochemicals, obviously ingested in the diet.

SOD (E.C. 1.15.1.1) is an enzyme whose function is to catalyze the dismutation of the  $O_2^{\cdot\cdot}$  into  $H_2O_2$ . This important reaction has been known to take place on both the cell's interior and exterior, mediated however by different isoenzymes. SOD1 is present in the cytosol, nucleus and intermembrane space in the mitochondria; it is also called the Cu/Zn SOD because it requires these two metals in order to be catalytically active. SOD2 is dependent not on Cu/Zn but on Mn, and it is found on the mitochondrial matrix. SOD3 contains Cu and it is found on the extracellular matrix of tissues, being therefore called EC-SOD (Gao et al., 2008; Juarez et al., 2008; Wilcox et al., 2009). These enzymes play an important role in maintaining the  $O_2^{\cdot\cdot}$  concentration in a range of about  $10^{-10}$  M, nevertheless they do it by transforming it into  $H_2O_2$  that is still toxic; it is at this point that CAT and GPX becomes active. Catalase (E.C. 1.11.1.6) is a heme-containing enzyme that is almost ubiquitously expressed in aerobic organisms. It is an oxidoreductase that is capable of converting the toxic  $H_2O_2$  into water and molecular oxygen without the parallel synthesis of free radicals, a critical feature since  $H_2O_2$  itself may have been formed from the  $O_2^{\cdot\cdot}$ ; a free radical. It is also known for having one of the fastest catalytic rates ( $\sim 10^7$  M/s) (Goyal & Basak, 2010; Odajima et al., 2010; Prakash et al., 2009). GPX (E.C. 1.11.1.9 and 1.11.1.12) is another enzyme that also performs a similar function as CAT, while at the same time having broader substrate range, therefore being able to remove other organic peroxides and converting them into water and the corresponding alcohols while utilizing glutathione as an electron donor. GPX is a family of enzymes that may be Se-dependent (in the form of a selenocysteine); and in mammals there are four isoenzymes. GPX1 is found in all tissues, and is abundant in erythrocytes, liver, lungs and kidneys; GPX2 or gastrointestinal GPX is found in the gastrointestinal tract, GPX3 is present in plasma, and GPX4, or phospholipid GPX is ubiquitous, as GPX1, but is selective towards lipid hydroperoxides (Arsova-Sarafinovska et al., 2009; Margis et al., 2008). When the toxic hydroperoxides have been neutralized by GPX, glutathione is oxidized in the process and in order to return it to its reduced state, GR (E.C. 1.8.1.7) must participate at this time. GR is a flavoprotein capable of reducing glutathione by oxidizing NADPH, therefore contributing to maintaining a pool of reduced glutathione and helping to preserve the REDOX state of the cell (Marty et al., 2009; Meister, 1988). Finally, GST (E.C. 2.5.1.18) is a family (containing at least the  $\alpha$ ,  $\mu$ ,  $\omega$ ,  $\pi$ ,  $\theta$ , and  $\zeta$  isoforms) pertaining to the Phase II detoxification enzymes. They are responsible for transferring glutathione to electrophilic molecules of diverse nature, such as xenobiotics, environmental toxins, a number of drugs and of course products of oxidative stress; thereby making them less reactive and easier to excrete (Burmeister et al., 2008; Carlsten et al., 2008; D. M. Townsend & Tew, 2003; Danyelle M. Townsend et al., 2009). All of these enzymes work in unison to maintain the ROS and free radicals under tolerable concentrations; a simplified scheme of how they do so is exemplified in Fig. 1

Although the enzymes are quite efficient in maintaining the cell's REDOX state they must be aided by non-enzymatic molecules. These can be of endogenous (glutathione) and of exogenous origin (ascorbate, tocopherols, carotenes, retinols and polyphenols among others) and their functions are to neutralize free radicals by either acting on their own or in conjunction with the enzymatic systems. Glutathione is a tripeptide ( $\gamma$ -glutamylcysteinylglycine) synthesized in the cell by a two-step process involving  $\gamma$ -glutamyl cysteine synthetase and glutathione synthetase, both of which are ATP-dependent. This molecule is found in the millimolar concentration range in the cytoplasm and within many organelles. Since it has a thiol functional group, it can become oxidized and reduced and thus function

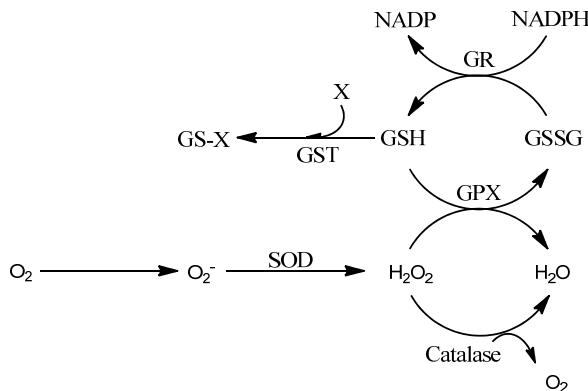


Fig. 1. The relationships between the different enzymatic antioxidant mechanisms. GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; GPX: glutathione peroxidase; SOD: superoxide dismutase; X: xenobiotic; GS-X : glutathione-conjugated xenobiotic. Equations are not balanced.

as an antioxidant, donating an electron and sacrificing itself to neutralize ROS in addition to many other molecules of diverse natures. The neutralization of oxidative molecules can take place without an enzymatic component by a direct interaction of the oxidant and the antioxidant, but when other molecules such as hydroperoxides are involved, an enzymatic system such as GPX is crucial to the process. Since reduced glutathione is oxidized during the course of these reactions, the enzyme GR is in charge of maintaining the pool of reduced glutathione consuming NADPH (from the pentose phosphate shunt) in the process. There are plasma membrane transporters that can also modulate the cell's REDOX state by importing and exporting reduced glutathione, oxidized glutathione and many conjugates of glutathione. The depletion of glutathione can be an important indicator of oxidative stress via the ratio of reduced to oxidized glutathione (Ballatori et al., 2009; Diaz Vivancos et al., 2010; Foyer & Noctor, 2011; Martin & Teismann, 2009).

Ascorbate, also known as vitamin C, is one of the exogenous molecules obtained through the diet. It is present in a diverse variety of vegetable foodstuffs such as apples, papaya, mango, guava oranges and others (Oliveira et al., 2010; Suárez-Jacobo et al., 2011). It is known as a free radical scavenger that does not act as a pro-oxidant under normal conditions, and it has been mentioned to function in parallel with glutathione (Cuddihy et al., 2008; Foyer & Noctor, 2011; Frei & Lawson, 2008; Valero et al., 2009). Because of its ability to neutralize free radicals it has been mentioned as having a possible therapeutic use in disorders such as ischemic stroke, Alzheimer's, Parkinson's and Huntington's diseases (Harrison & May, 2009), and it is sometimes consumed by athletes to neutralize the ROS produced during exercise (Gomez-Cabrera et al., 2008), it can neutralize protein radicals (Domazou et al., 2009) and its role in the improvement of endothelial function (a precursor of atherogenesis) has also been noted (Frikke-Schmidt & Lykkesfeldt, 2010; May & Qu, 2011; Sabharwal & May, 2008).

Vitamin E is the collective term for at least eight structurally related molecules: four tocopherols and four tocotrienols, of which  $\alpha$ -tocopherol is the most studied (Ravaglia et al.,

2008). Since tocopherols are of non-polar nature, their main function lies in the hydrophobic environment of cell and organelle membranes, protecting these structures from free radicals and also by stabilizing them (Atkinson et al., 2008; Naumowicz et al., 2009). Tocopherols induce a protective effect against oxidative stress linked to metabolic syndrome as well as other sources (Chung et al., 2010; Devaraj et al., 2008; Grattagliano et al., 2008; Roberts & Sindhu, 2009).

Carotenoids are other important molecules with antioxidant activity. They are hydrophobic vegetal pigments derived from isoprenoid units with up to 15 conjugated double bonds (Costantini & Moller, 2008; Tanaka et al., 2008). This large family of molecules contains more than 700 different structures identified so far, and are thought to play an essential role in the life cycle of the plants that produce them (Nishino et al., 2009; Tanaka et al., 2008). Even though some animals like birds, fishes and invertebrates use them in order to generate colorations for structures such as their skin and feathers, none of them have the ability to synthesize them and must therefore obtain them from exogenous plant sources. Of the most relevant ones we find  $\alpha$  and  $\beta$  carotene (the later one being the one with the most provitamin A activity, and also one of the most thoroughly studied), lutein, lycopene, zeaxanthin and  $\beta$ -cryptoxanthin (Britton & Khachik, 2009; Costantini & Moller, 2008). These pigments have been shown to interact with virtually all the radicals present in biological systems and are, consequently, significant antioxidants. In animals they have the potential to not only act upon free radicals, but also to modulate critical functions such as immunostimulation, and inhibition of the tumorigenesis process (Krinsky & Yeum, 2003; Nishino et al., 2009).

The last exogenous antioxidant to be mentioned is retinol (Vitamin A), a molecule that is related to the previously mentioned carotenoids.  $\beta$ -carotene can in fact be transformed to retinol, and this can in turn be transformed into retinal and retinoic acid, all of which have different biological functions on many tissues (Bremner & McCaffery, 2008). This compound, as well as its other metabolites, affects important processes such as immunity, reproduction, growth, development and, perhaps its best known function, it holds a vital role in the visual cycle (Pasquali et al., 2008; Redmond, 2009; Redmond et al., 2010).  $\beta$ -carotene has been characterized as an antioxidant, nevertheless, it can have toxic effects and has been named as a pro-oxidant when it is administered at higher doses and on certain types of cell cultures (Pasquali et al., 2008; Roehrs et al., 2009). When it functions as an antioxidant it has been positively linked to diverse ailments related to oxidative stress, such as diabetes (Ramakrishna & Jailkhani, 2008), obesity (Botella-Carretero et al., 2010), low sperm motility (Kao et al., 2008), hearing loss (Michikawa et al., 2009) and others.

Aside from the "antioxidant vitamins" the polyphenol group is another very diverse family of molecules present in fruits and vegetables. They are plant secondary metabolites that can be subdivided into many groups according to their molecular structure. Some of the representative molecules included here are quercetin, coumaric acid, proanthocyanidins resveratrol among others. Although traditionally considered antioxidants, they can also have antimicrobial, antiviral and anti-inflammatory properties (Ignat et al., 2011); they have consequently gained attention because of these *in vivo* health-promoting properties (Bridselli et al., 2009; Chong et al., 2010; Huang et al., 2010; Massaro et al., 2010).

Since fruits and vegetables are rich in all types of exogenous antioxidant molecules, scientists have linked these bioactive molecules to the positive health effects of a fruit and

vegetable-rich diet. However, fruit and vegetable intake cannot be replaced by a single antioxidant molecule or supplement; that is, ingesting vitamins or polyphenols in a purified form is not the same as acquiring them from the diet, since other nutrients present in fruits and vegetables may synergize with each other in order to elicit an effect (Liu, 2004).

## 2.2 The role of vegetable foodstuffs

Up until this point we have established that diet can have a profound impact in the development of conditions like oxidative stress and dyslipidemia, which are in turn related to metabolic syndrome and CVD. We have also defined oxidative stress and the ways the cell protects itself from it, and described some of the molecules which may be found in fruits and vegetables that contribute to mitigate oxidative stress in animal cells and tissues. The discussion now turns to the *in vivo* effects of fruits and vegetables, and the scientific evidence that shows their potential role (either positive, negative or neutral) on human health as well as on animal models.

There are epidemiological studies which underlie the relationship between the consumption of vegetable foodstuffs with a number of positive health effects. One of those is a sixty year follow up study that associates the consumption of fruits during childhood with a protective effect against cancer in adulthood (Maynard et al., 2003). Other authors have noted the link between consuming plant-derived products with a reduced risk for developing CVD and other non-communicable diseases (Liu, 2004), while some have found a correlation between a diet containing fruits and vegetables with a lower LDL concentration in both men and women (Djousse et al., 2004). Although most of the evidence supporting a cardioprotective effect of fruit and vegetable consumption comes from observational epidemiological studies, many of these studies have reported either weak or non significant associations (Dauchet et al., 2009). These weak associations may indicate that, aside from consuming fruits and vegetables, other factors may contribute to their positive health effects, for example, people who regularly consume fruits and vegetables tend to smoke less, exercise more and be better educated than those who do not (Dauchet & Dallongeville, 2008). Therefore, diet would not be the only factor for the prevention of CVD; exercise, smoking and drinking habits, genetic variability and many other influences must surely impact on heart health (Dauchet et al., 2009). In addition to epidemiologic studies, many dietary interventions have been carried out focused on individual fruits, vegetables or bioactive molecules isolated from them, in order to establish a direct effect of those particular foodstuffs on specific markers of health and disease. Table 1 briefly lists some experiments which have measured the effect of fruits and vegetables on oxidative stress and dyslipidemia markers in human and animal models.

From the first two studies shown in the table, it can be assumed that strawberries have the ability to regulate both dyslipidemia and oxidative stress markers. In the study by Jenkins et al., a very high dose of fruit (1 lb. daily) was administered to patients in a cholesterol-lowering diet. Strawberries improved the oxidative stress markers but had no effect on dyslipidemia markers additional to the cholesterol-lowering diet alone. Although it can be argued that such a regimen would seldom be followed by a healthy subject with a non-restrictive diet, the authors claim that this was actually a point in their favor since the inclusion of strawberries made the cholesterol-lowering diet, which limits or prohibits the ingestion of certain items, more palatable (Jenkins et al., 2008). This diet would therefore be a viable option in patients who may complain about the dullness in flavor of their available

Fruit or Vegetable	Model	Effect on dyslipidemia markers	Effect on oxidative stress markers	Author
Strawberries	Human subjects on a cholesterol lowering diet	- TC, LDL, and TAG	↓ TBARS and LDL oxidation	(Jenkins et al., 2008)
Strawberries	Human subjects with metabolic syndrome	↓ TC, LDL and small LDL particles		(Basu et al., 2010)
Different berries	Human subjects	- TC and TAG ↑ HDL	↑ Vitamin C and polyphenols	(Erlund et al., 2008)
Blueberries	Male pigs	↓ TC, LDL and HDL - TAG		(Kalt et al., 2008)
Mango	Normolipidemic human subjects	↓ VLDL and TAG	↑ Plasma AC	(Robles-Sánchez et al., 2011)
Crude mango pulp extract	Male albino mice		↓ ROS ↑ SOD1, CAT, GR and GST	(Prasad et al., 2008)
Mango extract	Hyper-cholesterolemic mice		↓ Mitochondrial oxidative stress	(Pardo-Andreu et al., 2008)
Grape seed oil	Rats	↓ TC, LDL and atherogenic index		(Kim et al., 2010)
Grape seed extract	Rats on a hyperlipidemia-inducing diet	↓ TC, LDL and VLDL ↑ HDL	↑ Enzymatic and non-enzymatic AOX systems	(Thiruchendran et al., 2010)
Grape seed proanthocyanidins	Rats on a high-fat diet	↓ TAG, LDL Modulated gene expression		(Quesada et al., 2009)
Grape seed proanthocyanidins	Healthy human subjects		↑ Plasma AC	(Natella et al., 2002)
Banana	Healthy human subjects	↑ TAG - TC	↓ Lipid peroxides	(Yin et al., 2008)
Banana polyphenols	Rats	↓ TC, TAG, free fatty acids, ↑ HDL		(Vijayakumar et al., 2009)
Turmeric and garlic	Type 2 diabetic human subjects with dyslipidemia	↓ TC, LDL and TAG		(Sukandar et al., 2010)
Garlic and aged black garlic	C57BL/KsL mice		↓ TBARS ↑ SOD, GPX and CAT	(Lee et al., 2009)
Garlic extracts	Type 2 diabetic human subjects with dyslipidemia	↓ TC and LDL ↑ HDL - TAG		(Ashraf et al., 2005)

Fruit or Vegetable	Model	Effect on dyslipidemia markers	Effect on oxidative stress markers	Author
Tomato juice	Healthy human subjects	↓ TC	↓ TBARS	(Jacob et al., 2008)
Tomato extract	Rats		↓ TBARS ↓ Glutathione depletion and tissue damage	(Jamshidzadeh et al., 2008)
Spinach and tomato	Healthy human subjects		↑ Lymphocyte intracellular carotenoid concentrations ↑ resistance to H <sub>2</sub> O <sub>2</sub>	(Porrini et al., 2002)
Broccoli sprouts	Healthy human subjects	↓ TC and LDL ↑ HDL (in women)	↓ Plasma phosphatidyl choline hydro peroxides, urinary 8 isoprostane and 8-hydroxy deoxy guanosine	(Murashima et al., 2004)
Orange juice, apples and pears	Smokers and non-smokers	↑ TC, LDL, HDL in non-smokers. ↓ TC and LDL in smokers (higher initial values)	↑ Plasma AC in non-smokers. - Plasma AC in smokers	(Alvarez-Parrilla et al., 2010)
Potatoes	Rats	↓ TC and TAG	↑ Plasma, urine and tissue AC ↑ Vitamin E	(Robert et al., 2008)

Table 1. Different studies on fruit and vegetable consumption, and their effect upon dyslipidemia and oxidative stress markers in human and animal models. – indicates no difference, ↓ decrease, ↑ increase. TC, total cholesterol; TAG, triacylglycerols; AC, antioxidant capacity; AOX, antioxidant.

eating options. In another study done by Basu et al., they administered a more modest dose of strawberries (2-3 cups daily) to obese patients with metabolic syndrome and found that this lower dose of strawberries modulated dyslipidemia markers in a positive way by decreasing cholesterol, LDL and LDL particles (Basu et al., 2010). This second study was a randomized controlled trial, in which patients continued with their regular diet and lifestyle, strawberries were added as a drink and water was used as a control. Therefore, it can be argued that the large dose of strawberries used in the study of Jenkins showed no further improvement on the lipid markers because the dyslipidemia of those patients was already controlled. In contrast, the patients in the study of Basu did not receive any additional treatment for their dyslipidemia, making the strawberries' effects more prominent.

To further scrutinize the family of berries, Erlund et al., administered an assortment of them to human subjects and detected no effect on total cholesterol or triacylglycerols, a fact that may be put aside by an important (5 %) increase in HDL. They also determined that the concentrations of vitamin C and polyphenols in plasma were increased, and, although they did not measure plasma antioxidant capacity, it seems likely that ascorbate and polyphenols would have had a positive effect on this marker (Erlund et al., 2008). Another study with berries, specifically blueberries, was done on pigs and found a decrease in total cholesterol, LDL and HDL, while having no effect on triacylglycerols. While the cholesterol lowering effect is a positive one, a reduction in HDL is not, since the decrease in this molecule is a feature of dyslipidemia (Kalt et al., 2008). Taken together these studies suggest that berries have the ability to influence lipid metabolism in a complex manner, depending on the model of study, the type of berry and the specific lipid. Effects may be regarded generally as positive or non-existing, for example triglycerides, total and LDL cholesterol might be reduced or unaltered. Only in one study, performed in pigs, a negative result was observed as a reduction in HDL. Berry consumption showed a consistent improvement of different oxidative stress markers, although very few studies evaluated them. The effects of strawberries (as well as other berries) may be mediated by their high content of compounds such as  $\alpha$ -carotene, vitamin C and phenolic antioxidants (ellagic-acid, proanthocyanidins, quercetin, kampferol, anthocyanins, p-coumaric acid and others), which give them their characteristic bright color (Azzini et al., 2010; Pineli et al., 2011).

Mangoes have also been found to have an effect on both humans and animals. Robles et al., administered mangoes to healthy human subjects and found a decrease in both triacylglycerols and VLDL particles, while at the same time increasing plasma antioxidant capacity; in other words, they can have a positive effect upon dyslipidemia and oxidative stress markers (Robles-Sánchez et al., 2011). Since this effect was demonstrated in healthy adults, it can be argued that mangoes can be an important tool in preventing the onset of CVD, that is, they can be a preventive measure rather than a remedial one. Previous studies on mango consumption were done in mice, showing a positive effect on different markers of oxidative stress. Prasad et al. (2008), using an extract from the pulp of the mango in mice, suggested that the decrease in the production of ROS molecules in the prostate could be related to a protective role of this extract against prostate cancer, since its onset has been linked to oxidative damage when the REDOX balance is altered. The previously stated effects of mangoes can be attributed to the many antioxidant molecules present in the fruit, namely, antioxidants such as  $\beta$ -carotene, vitamins C and E and polyphenols (particularly mangiferin xanthone) (Masibo & He, 2008; Shah et al., 2010).

The antioxidant potential of mango extracts has been tested on several *in vitro* and cell-based assays. Ajila & Rao (2008) found that mangoes can protect the cell lipids and proteins from oxidative damage induced by H<sub>2</sub>O<sub>2</sub> in erythrocytes; the cells morphology was also preserved, which indicates the major role of this fruit's antioxidant activity in live cells (Ajila & Rao, 2008). Mangiferin, a polyphenolic antioxidant particularly abundant in mango, has been shown to inhibit the generation of O<sub>2</sub><sup>-</sup> in polymorphonuclear cells and in hypoxanthine-xanthine oxidase cell-free system; this result may pinpoint the precise enzymatic system affected by mangoes in whole organisms (Peyrot & Ducrocq, 2008). In mice, mangiferin protected mitochondria from oxidative stress, another finding of crucial importance since these organelles are the main source of oxidant molecules as a normal consequence of aerobic metabolism.

Grapes are an important crop grown commercially for winemaking as well as to be processed into juice; after they have been manufactured the seeds may be discarded but are sometimes further treated to make grape seed oil. The effects of the oil have been studied in rats; it was established that the oil has the ability to reduce total cholesterol and LDL (Kim et al., 2010). Another study notes similar results in the same animal model using grape seed extract. They showed that the extract is capable of inducing not only a decrease in cholesterol and LDL, but also in VLDL while increasing HDL and antioxidant systems (Thiruchenduran et al., 2010). This last observation may be explained by the fact that the compounds present in the seeds, namely phenolic acids, anthocyanins and proanthocyanidins, are of antioxidant nature, however the relationship between the phenolic antioxidants and the modulation of serum lipids is still not well understood.

In order to clarify this issue, the role of grape seed proanthocyanidins has been further studied in a rat model in which the animals were fed either a standard diet or a high fat diet (that the authors dub the cafeteria diet). After 13 weeks on this diet, the animals were subdivided into two groups, one of which consumed grape seed proanthocyanidins extract. It was determined that the treatment normalized plasma triacylglycerol and LDL levels, while also decreasing fatty liver (Quesada et al., 2009). The authors also evaluated the expression of some genes related to hyperlipidemia, like the SREBP1 (sterol regulatory element binding protein 1), DGAT2 (diacylglycerol O-acyl transferase 2) and MTP (microsomal transfer protein) genes, all of which are involved in the synthesis of VLDL, free fatty acids and triacylglycerols. Those genes were found to be overexpressed by the cafeteria diet and normalized with the proanthocyanidins, which in turn contributed to the stabilization of the lipid profile. Therefore this study suggests a possible mechanism of action of these phenolic phytochemicals in modulating lipid levels. Proanthocyanidins (in the form of commercially-available capsules; Leucoselect) are also capable of reducing postprandial oxidative stress in healthy human subjects after ingesting a meal rich in oxidized and oxidizable lipids (Milanese meat). Immediately after a meal is ingested the individual's antioxidant status is expected to change, one way this can be determined is by measuring LDL's susceptibility to become oxidized; this susceptibility was lower in the antioxidant-enriched meal than the control one, albeit, not statistically significant (Natella et al., 2002). Although the results in this study were not statistically significant, other publications have determined a significant effect over LDL's susceptibility to oxidation. Chopra, et al. found that after supplementing smoker's and non-smokers diets with green ( $\beta$ -carotene and lutein-rich) or red (lycopene-rich) vegetables for a period of 7 days, the red vegetables significantly reduced LDL's susceptibility to oxidation (Chopra et al., 2000). Stein, et al. showed that patients with coronary artery disease (CAD) who consumed grape juice for a period of 14 days reduced their LDL's susceptibility to oxidation as well as presenting an improvement in other parameters (Stein et al., 1999). Another publication by Upritchard, et al. found similar results; they supplemented the diet of type 2 diabetic patients with tomato juice, vitamin E or vitamin C and found that after four weeks of this regimen LDL's susceptibility to oxidation was significantly decreased. The effect was more prominent with tomato juice and vitamin E; vitamin C however, showed no such effect which the authors attribute to the fact that lycopene and vitamin E are both non polar and therefore transported by LDL, while vitamin C is water soluble and does not get incorporated into LDL (Upritchard et al., 2000). Contrasting these publication with the one by Natella indicates that phytochemicals can have an effect in a short period of time,

however, not statistically significant, while other authors report a significant effect on longer timeframes (7 days, 14 days and 4 weeks); which may indicate that the phytochemicals present in the consumed foodstuffs may require subsequent doses in order for their effects to become more evident.

The effect of bananas, one commonly consumed fruit in the western world, in short term regulation of plasma lipid levels and oxidative stress has also been explored. In one study, healthy subjects drank a single banana-containing drink and after two hours their triacylglycerol levels rose, while plasma lipid peroxidation was decreased; it was also shown that LDL was more resistant to oxidation after the treatment, suggesting that bananas were capable of excreting a short-term antioxidant effect *in vivo* (Yin et al., 2008). The effect of bananas is apparently due to the flavonoids they contain; an experiment done with rats estimates the effect these phytochemicals may have on the medium or long-term lipid metabolism. The animals were fed a banana-derived polyphenol-enriched diet for a period of 45 days, after this time it was found that cholesterol, phospholipids, free fatty acids and triacylglycerols in various tissues were lowered, as well as an increase in HDL (Vijayakumar et al., 2009). Although these results suggest the flavonoids are exerting a hypolipidemic effect, it should be noted that a dose over 5 mg/100 g of body weight/day of polyphenols resulted in an increase in cholesterol, mediated (at least in part) by an increase in 3-hydroxy-3-methyl-glutaryl-CoA reductase activity (HMG CoA) in the liver, an enzyme responsible for cholesterologenesis, meaning the rate of cholesterol synthesis seemed to be amplified. Even so the overall effect of a banana-rich diet tended to a total cholesterol reduction, explained by an increase in lecithin-cholesterol acyltransferase (LCAT) activity, an enzyme that removes tissue cholesterol in order to degrade it; bile acids and neutral sterols in the feces were also increased. This experiment points to a possible role of banana flavonoids in regulating lipid metabolism in favor of a total cholesterol decrease; however, it also indicates the importance of regulating the ingested doses, due to the possibility of finding dose-dependent negative effects.

Garlic has been used in herbal medicine to treat various ailments for centuries. A study administered turmeric and garlic in combination in the form of capsules of the extracts prepared to obtain the main bioactive compounds to determine their effect on type 2 diabetes mellitus patients that also presented dyslipidemia. The patients were divided in three groups that ingested 1.2, 1.6 or 2.4 g extract/day (groups A, B and C respectively). It was found that after eight weeks of treatment, group A significantly decreased their total cholesterol concentration, while LDL also decreased, although the change was not statistically significant. After 10 and 12 weeks groups A and C respectively showed a significant increase in HDL. Two of the groups (B and C) decreased their triacylglycerol concentrations, but once again it was not statistically significant (Sukandar et al., 2010). The authors of this study suggest that the observed effects could be related to the ability of the compounds present in garlic to inhibit liver lipogenic or cholesterologenic enzymes, and also by delaying lipid absorption in the gastrointestinal tract. Turmeric on the other hand may affect cholesterol catabolism by means of an increase in the activity of cholesterol 7α-hydroxylase activity, hindering cholesterol synthesis by inhibiting HMG CoA reductase, increasing the levels of LDL receptors (which aid in its elimination from blood) and by inhibiting dietary cholesterol absorption. This study points out a fact not previously mentioned, how two (or more) foodstuffs can interact with one another in order to synergize a particular effect, that is, both turmeric and garlic may have hypolipidemic effects by

themselves but taking them together may improve this effect. Garlic and aged black garlic have also been studied in an animal model of type 2 diabetes, mice were fed diets containing 5 % of either garlic or aged black garlic for a 7 week period; it was determined that the animals' hepatic TBARS were significantly decreased with the treatments; being the aged black garlic group levels significantly lower than both the control group and the garlic group. Hepatic SOD and GPX activities were significantly increased by both treatments while CAT was significantly increased in the aged black garlic group. These results indicate garlic may also have antioxidant effects as determined by an increase in the hepatic endogenous antioxidant systems, being the aged black garlic the one with the most effect. The better *in vivo* antioxidant effect of aged black garlic may be related to its higher *in vitro* antioxidant activity (4.5 times higher TEAC value). One of the most important bioactive compounds found in garlic is  $\gamma$ -glutamyl cysteine, which is converted to alliin and then to allicin by alliinase upon crushing, cutting, chewing or dehydrating the bulb (Pal et al., 2006); this last compound has also been studied by other authors. In an article by Ashraf, they administered 300 mg garlic tablets (or a placebo to the control group) containing 1.3 % allicin twice daily to type 2 diabetic human subjects with newly diagnosed dyslipidemia for a 12 week period. After the treatment they found a significant reduction in total cholesterol and LDL with a simultaneous increase in HDL, triacylglycerols were not affected. They explain that the effect of the garlic extract may be due to the presence of allicin and note the importance of the delivery method used to administer this compound.

Tomatoes are grown commercially for the preparation of many different products, one of which is tomato juice. The consumption of this beverage was able to reduce total cholesterol and TBARS in healthy human subjects (Jacob, 2008) indicating a positive effect of this vegetable product in both dyslipidemia and oxidative stress. Furthermore, it was established by Jamshidzadeh that a tomato extract can have cell protecting effects against drug-induced toxicity in rats, that is, it can reduce TBARS, prevent glutathione depletion and tissue damage (Jamshidzadeh et al., 2008). These experiments seem to highlight the antioxidant-promoting ability of tomatoes, something that has been linked by some authors to lycopene, an important carotenoid molecule present in them.

The role of lycopene was established in a study where the purified compound was administered in capsules to healthy humans, they found lymphocyte DNA damage was reduced, but without significantly affecting other markers of oxidative stress or antioxidant health (Devaraj et al., 2008). The effect of lycopene and other carotenoids has also been analyzed *in vivo*. Porrini et al., found that after ingesting a tomato and spinach diet the intracellular concentrations of the three main carotenoids present in both foodstuffs (lutein,  $\beta$ -carotene and lycopene) were significantly increased in lymphocytes (Porrini et al., 2002). At the same time, vegetable puree consumption provided the cells with an increased resistance to oxidative damage from H<sub>2</sub>O<sub>2</sub>. However there was no correlation between the cell's lycopene concentration and its resistance to oxidative damage. The authors conclude that even though vegetables rich in carotenoids are considered to elicit an antioxidant effect, it is yet to be clarified if that antioxidant capacity is related to carotenoids or to other molecules; it is also possible that the relationship is not simple and cannot be described with a linear model. Another possible explanation to these results is that carotenoids do not behave like direct antioxidants, but rather act by stimulating the cell's endogenous enzymatic antioxidant mechanisms such as CAT or GPX, both of which are capable of

removing H<sub>2</sub>O<sub>2</sub>; in other words, these molecules may be acting on a genetic level to stimulate the cell's own defense systems.

Murashima et al., studied the effects of broccoli sprouts upon metabolism and oxidative stress markers of healthy human subjects. They found that after a week of consuming 100 g/day of fresh broccoli sprouts total and LDL cholesterol concentrations were significantly lower and HDL was increased but only in the female subjects, the triacylglycerol concentration of three subjects shifted to a normal concentration (out of six who had abnormal values). Oxidative stress markers were also affected as determined by a reduction in phosphatidylcholine hydroperoxides, 8-isoprostane (a product of non-enzymatic tissue phospholipid oxidation) and 8-hydroxydeoxyguanosine (a product of oxidative DNA damage). This study remarks how gender differences in lifestyle and dietary habits, as well as hormonal levels can influence how the individuals respond to a treatment such as this one; and highlights how heterogeneous a response may be within a population.

Similar observations were made by Alvarez-Parrilla et al., (2010) who investigated the effect of consuming orange juice, a pear and an apple in both smokers and non-smokers healthy adults. They found that in non-smokers total cholesterol, LDL and HDL were significantly increased, while in smokers initial cholesterol and LDL values were higher than those of non-smokers, but after the intervention significantly decreased to values similar to those of non-smokers; triacylglycerols were not affected in either group. Plasma antioxidant activity (measured by the ORAC and FRAP techniques) was also differently affected by the dietary intervention: in non-smokers it was either increased (ORAC) or not affected (FRAP), while in smokers it was not affected (ORAC) or decreased (FRAP) (Alvarez-Parrilla et al., 2010). This study suggested that plasma antioxidant activity was not a sensitive marker to detect oxidative stress in smokers and that the habit of smoking can be an important variable that can alter the way the phytochemicals present in fruits and vegetables interact with the individual and affect health-related physiological responses. This can once again point to how synergy occurs not only among the items that we eat but also with other environmental stimuli.

The interaction between carbohydrates and phytochemicals, and their effect on lipid levels and antioxidant status has also been studied. A recent study determined the effect of consuming a diet containing simple carbohydrates (sucrose), one of complex carbohydrates (starch) and a third containing complex carbohydrates and antioxidant micronutrients (potatoes) in a rat model. It was found that after consuming the diets for a period of three weeks, the animals' plasma and liver cholesterol levels were significantly lower with the potato-based diet; the triacylglycerol levels in the liver were also decreased. The plasma antioxidant levels were also increased. This study suggests that simple carbohydrates initiate liver *de novo* lipogenesis which in turn increases plasma triacylglycerol levels, an effect that is avoided when carbohydrates are ingested in the form of starch from potatoes, which in addition to carbohydrates contain other important non-energetic micronutrients. Some of the reported micronutrients from potatoes are vitamins C and E, carotenoids and phenolic acids, which may account for the *in vivo* antioxidant effect of this vegetable. In addition to starch and antioxidants, potatoes contain fiber in the form of resistant starch, molecules that have the ability to induce the fecal excretion of cholesterol, bile acids and other lipids, another mechanism that may explain the observed hypolipidemic effect.

### 3. Conclusion

Regular consumption of a fruit and vegetable rich diet has undeniable positive effects on health, although the markers to evaluate these effects are sometimes hard to demonstrate. We analyzed the effects of fruit and vegetable consumption on dyslipidemia and oxidative stress markers, two hallmarks of metabolic syndrome and CVD risk, among other diseases. We have found mostly positive effects of certain fruit and vegetable products. Interestingly, the effects can be found after short term (hours) and midterm (several weeks) interventions; and they are, in general, more clearly related to attenuating oxidative stress than to regulating lipid levels, in fact the effects of fruit and vegetable products on lipid levels are heterogeneous, usually positive or non-existent. We may conclude that oxidative stress markers are useful tools to evaluate the effects of a fruit and vegetable rich diet, however since oxidative stress is a complex condition, several markers should be analyzed in order to provide a better understanding of it. Dyslipidemia markers are probably more valuable for assessment of risk groups than for healthy individuals. In some cases the effects of fruit and vegetable consumption can be traced to a specific molecule(s) and the mechanisms of action of these compounds seem to involve regulation of gene expression. However, since food is not made up of individual molecules but rather a vast number of them, it makes sense that the isolated compounds won't have the same effect as eating the whole fruit or vegetable, because the different bioactive molecules present in the foodstuff can interact with one another and with innumerable cell targets; therefore, altering their individual bioactive properties. It is highly difficult to describe the biochemical mechanisms of action of whole fruit or vegetable products. Moreover variation in individual responses to food-derived phytochemicals is also great in human populations due to genetic and environmental variations. Consequently, the search for these factors may help to better understand how to take advantage of the many potential benefits of a healthy diet rich in fruits and vegetables.

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# ***Acanthopanax trifoliatus*, a Potential Adaptogenic Thai Vegetable for Health Supplement**

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## **1. Introduction**

Nowadays, people are struggling with stress, either from environmental pollutions and daily life routines of urgency and competition. From the risk of exposure to toxic or pathogenic substances from various routes, our bodies have to adapt and maintain a systemic balance for physiological functions. Obtaining the supplements or substances that promote good health is the recommended choice for people in this century. This is the beginning of the word "adaptogens" or "balancing material" that will help the body adjust and increase the tolerance to physical, emotional and environmental stresses as well as promote the metabolic system and homeostasis of the body.

An adaptogen was previously defined as a substance that had to; show some nonspecific effect, such as increasing bodily resistance to physically, chemically, or biologically noxious agents or factors; had a normalizing influence on a pathologic state, independent of the nature of that state; and was innocuous and not disturb body function at a normal level (Lasarev, 1947). Panossian and Wagner (2005) suggested that the adaptogenic substance from plants was a substance that increased the ability of an organism to adapt in various factors in the environment and to prevent damage caused by such factors. Some plants have been used for adaptogenic purposes as shown in Table 1.

Panossian (2003) suggested that most of the active phytochemicals separated from the adaptogenic plants were in 3 main chemical groups; phenolic compounds such as phenylpropanoids, phenylethane derivatives and lignans; tetracyclic triterpenes; and unsaturated trihydroxy or epoxy fatty acids. Chemical structures of some adaptogenic compounds are shown in Figure 1.

To demonstrate the adaptogenic effects of medicinal plants, related pharmacological activity determinations were studied including *in vivo* effects for decreasing the stress, promote physical performance, anabolic efficacy, and brain metabolism. Some *in vitro* studies were also conducted such as antioxidative and anti-inflammatory effect experiments. Adaptogenic plants can promote anti-stress effects via several mechanisms including

increasing of body temperature, improvements of body function, enhancing of cognitive abilities, promoting of locomotor and exploration activities, and moderating the emotional behavior (Wagner et al., 1994).

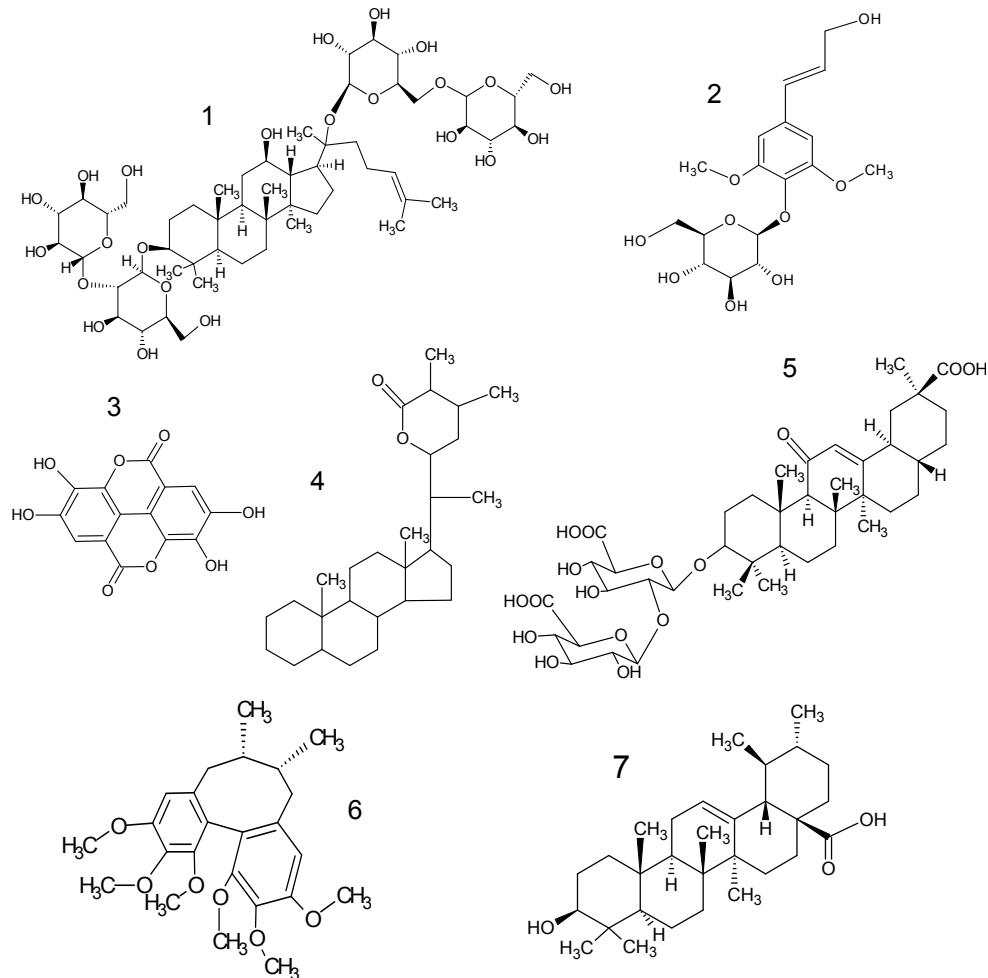


Fig. 1 Adaptogenic compounds; 1=ginsenoside Rb1, 2=eleutheroside B, 3= ellagic acid, 4= withanolide, 5= glycyrrhetic acid, 6= schisandrin, 7= ursolic acid.

Free radicals and reactive oxygen species are some chemical species that have odd number of electrons which have high reactivities and are capable to cause reversibly or irreversibly oxidative damages to compounds of all biochemical classes including nucleic acids, proteins and free amino acids, lipids and lipoproteins, carbohydrates and connective tissue macromolecules (Halliwell and Gutteridge, 1999). Oxidative stress is considered some importance for many ailments and pathologies including cardiovascular diseases, cancers, rheumatoid arthritis and Alzheimer's disease (Cross, 1987). Inflammation and oxidative

Common name	Scientific name	Family	Part used
American ginseng	<i>Panax quinquefolius</i>	Araliaceae	Root
Indian gooseberry	<i>Emblica officinalis</i>	Euphorbiaceae	Fruit
Ashwagandha	<i>Withania somnifera</i>	Solanaceae	Root
Korean ginseng	<i>Panax ginseng</i>	Araliaceae	Root
Siberian ginseng	<i>Eleutherococcus senticosus</i>	Araliaceae	Root, stem bark
Indian tinospora	<i>Tinospora cordifolia</i>	Menispermaceae	Root, stem
Holy basil	<i>Ocimum sanctum</i>	Lamiaceae	Whole plant
Jiaogulan	<i>Gynostemma pentaphyllum</i>	Cucurbitaceae	Whole plant
Licorice	<i>Glycyrrhiza glabra</i>	Fabaceae	Root
Reishi	<i>Ganoderma lucidum</i>	Ganodermataceae	Mushroom
Schisandra	<i>Schisandra chinensis</i>	Schisandraceae	Fruit, seed

Table 1. Reported adaptogenic plants (Winston and Maimes, 2007).

stress are key components of the pathology of chronic neurodegenerative conditions, in particular Alzheimer's disease, Down syndrome, multiple sclerosis and dementia (Auroma et al., 2003). Previous studies indicated that oxidative injury is present in the brains of patients with Alzheimer's disease may play important role in the development of the disease (Grundman and Delaney, 2002, Pratico and Delanty, 2000, Rottkamp et al., 2000, Smith et al., 2000). Excessive lipid peroxidation and increasing of malondialdehyde concentrations have been found in the patients' brains of Alzheimer's disease (Grundman and Delaney, 2002). From ethnomedical intellectual, various plants have been used to enhance cognitive function and to relieve other symptoms associated with Alzheimer's disease. The effects of traditional herbal drugs may not only be relevant in managing the cognitive decline that can be associated with general aging but may also be relevant in the treatment of specific cognitive disorders such as Alzheimer's disease. Therefore, plants with anti-aging or memory enhancing activities can be considered for potential efficacy in cognitive dysfunction including conditions that feature dementia. The activities of plants that show the effects in relation to cognitive disorders are including anti-cholinesterase (anti-AChE), anti-inflammation and antioxidant (Howes and Houghton, 2003).

Health promotion by Thai wisdom has been conducted from the Thai traditional practice in prevention, diagnosis, and treatment of imbalancing in human bodies and minds. The process is accompanied with folk medicine which herbs and natural products in each region all over the country are main constituents. The knowledge and medical procedures have been transferred from generation to generation until nowadays. In Thai traditional medicine, herbs with adaptogenic or antioxidative effects should relate to plants that exhibit detoxification, blood purifying, jaundice curing, hepatoprotection, tonic and haematomic, aphrodisiac, anti-fatigue, nourishment and longevity promotion (Faculty of Pharmacy, Mahidol University, 1998).

Eleven indigenous plant species traditionally used as ginseng-like agents or adaptogens were collected from the north and northeastern parts of Thailand and evaluated for adaptogenic-related properties including antioxidant and anti-anxiety activities as well as total phenolic and total flavonoid contents. Along with the rhizome extract of *Smilax corbularia*, leaf decoction extract of *Acanthopanax trifoliatus* (*A. trifoliatus*) exhibited strong antioxidant activity with high amount of phenolic and flavonoid contents (Sithisarn et al., 2010). The results suggested that *A. trifoliatus* is an interesting plant that could promote

significantly pharmacological activities related to adaptogenic properties, which supported the ethnomedical uses of this plant.

## 2. Botanical characteristics of *A. trifoliatus*

*A. trifoliatus* is a shrub that belongs to the family Araliaceae. Its taxonomic position is as follows:

Order	Apiales
Suborder	Apiineae
Family	Araliaceae (ginseng family)
Genus	Acanthopanax (Eleutherococcus)
Species	trifoliatus

In Flora of China (2004), *A. trifoliatus* was described as a shrub, scandent or climber that usually reaches a height of 7 m. The branches are scattered with recurved prickles. The glabrous and prickly petioles are 2-6 cm long with the 2-8 mm long petiolules. The papery, adaxially glabrous or slightly setose on midvein and veins, secondary veins 5 or 6 pairs, base cuneate, margin serrulate, apex acute or acuminate leaves are 4-10 x 2-4.5 cm and the ovate, elliptic-ovate, or oblong leaflets which number up to 3 or 5. The inflorescence is terminal raceme, umbel or compound umbel which borne on leafy shoots and 3-10 umbels. The peduncles are 2-7 cm long with the 1-2 cm long pedicels. The glabrous calyx has 5 teeth. The ovary has 2 carpellates, the styles are united to middle. The fruit is globose and laterally compressed with the size of 3-4 mm. The flowering period is from August to November. The fruiting time is during September to December.

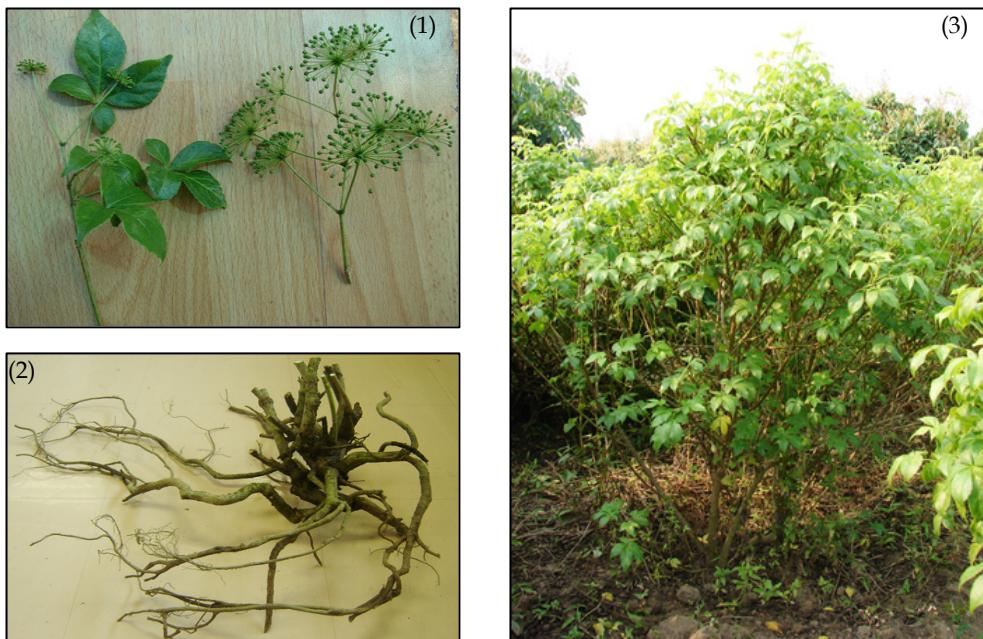


Fig. 2. *Acanthopanax trifoliatus*. 1= leaves and flowers, 2= roots, 3= whole plant.

Normally *A. trifoliatus* thrives in shrub fields, roadsides, forest margins, in valleys or on mountain slopes; below 1000 m in the East and 3200 m in the West part of range. This plant is widely distributed in India, Japan, the Philippines, Thailand, Vietnam, and many provinces of China including Anhui, Fujian, Guangdong, Guangxi, Guizhou, Hunan, Hubei, Jiangsu, Jiangxi, Sichuan, Taiwan, Yunnan and Zhejiang. The photos of *A. trifoliatus* from Chiang Mai province, Thailand are shown in Figure 2.

### **3. Ethnomedical uses and previous reported biological activities of *A. trifoliatus***

The leaves of *A. trifoliatus* have been used as a tonic to improve general weakness, the leaves and young shoots are also used for treatments of tuberculosis, lung hemorrhages, bruises, ulcers and, contusion (Perry and Metzger, 1981a, Perry and Metzger, 1981b, Chi, 1997, Loi, 2000). The stem bark is used as antiflatulent agent and is used for treatments of emaciation and neurosis (Petelot, 1954). The root bark and stem bark are used in the treatments of rheumatism, lumbago, ostealgia and impotence (Nguyen and Doan, 1989). The bark was reported to be used as tonic, CNS stimulant, and memory enhancer (Nguyen and Doan, 1989). In Chinese medicine, this plant was used for the treatments of cold, cough, neuralgia and rheumatism (Duke and Ayensu, 1985). Moreover, young leaves and the shoots are popularly consumed in Northern Thai traditional cuisine as vegetables. Antioxidant and anti-inflammatory activities of the extracts from *A. trifoliatus* have been previously reported and suggested that young leaves provided the most active sample (Sithisarn and Jarikasem, 2009, Sithisarn et al., 2009). Anti-anxiety effects in animal models of leaf extract from *A. trifoliatus* were also reported (Sithisarn et al., 2010).

### **4. Chemical constituents of *A. trifoliatus***

Some chemical components including terpenoids, phenylpropanoids, phenolics, flavonoids, lactones and essential oils were separated and identified from various parts of *A. trifoliatus* as shown in Table 2.

### **5. Antioxidant activity of *A. trifoliatus***

The extracts from some parts of *A. trifoliatus* including leaves, stems, stem barks, roots and root barks with various extraction methods were tested for free radical scavenging activity using DPPH scavenging method and inhibitory effect to lipid peroxidation of rat brain homogenate by thiobarbituric acid reactive substances (TBARS) method. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical is a stable radical with a deep violet color. When DPPH radical receives a proton from antioxidants, it converts to a colorless protonated DPPH molecule. After 15 minutes of DPPH scavenging reaction, the absorbance at 517 nm was determined then % of inhibition and EC<sub>50</sub> value were calculated (Yamasaki et al., 1994). It was found that the leaf decoction extract, the root bark decoction and 75% ethanolic refluxing extracts and the stem bark 75% ethanolic refluxing extract showed high radical scavenging activity with EC<sub>50</sub> values of 14.50 ± 1.04, 34.24 ± 5.01, 34.51 ± 2.74 and 37.85 ± 0.85 µg/ml, respectively (Sithisarn and Jarikasem, 2009). TBARS method is popular single assay for the measurement of lipid peroxidation. The sample under test is treated with thiobarbituric acid (TBA) at low pH, and a maximum absorption of pink chromogen is

Group of chemical	Chemical compounds (plant part)	Reference
Terpenoid	- acanthoic, continentalic, kaurenoic acids (root, stem, leaves) - acantrifoic acid A, acantrifoside C (leaves) - acantrifoside D (stem bark) - 16 $\alpha$ H, 17-isovalerate-ent-kauran-19-oic acid, ent-Kaur-16-en-19-oic acid, ent-Primara-8(14), 15-dien-19-oic acid (stem bark) - acantrifoside A (leaves) - lupane triterpene glycoside (leaves) - 24-nor-11 $\alpha$ -hydroxy-3-oxo-lup-20(29)-en-28-oic acid 28-O-alpha-L-rhamnopyranosyl-(1 $\rightarrow$ 4)-beta-D-glucopyranosyl-(1 $\rightarrow$ 6)-beta-D-glucopyranosyl ester (leaves) - kaur-16-en-19-oic acid, taraxerol, taraxerol-acetate (leaves)	Phuong, 2006 Kiem, 2004 Kiem, 2004 Kiem, 2004 Yook, 1998 Yook, 1999 Kiem, 2003a Du, 1992
Phenylpropanoid	- 1- $\beta$ -D-glucopyranosyl-2,6-dimethoxy-4-propenylphenol, 1-[ $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranosyl]-2,6-dimethoxy-4-propenylphenol (stem bark)	Kiem, 2003b
Polyphenolic and flavonoid	- syringin, quercitrin (stem bark) - nevadensin (leaves)	Kiem, 2003b Du, 1992
Lactone	- eleutheroside E, (2R,3R)-2,3-di-(3,4-methylenedioxybenzyl)-butyrolactone (stem bark)	Kiem, 2003b
Essential oil	- $\alpha$ -pinene, sabinene, terpinen-4-ol, $\beta$ -pinene, <i>p</i> -cymene, carissone (leaves)	Muselli, 1999

Table 2. Reported chemical constituents in *A. trifoliatus*.

measured at 532 nm (Punchard, 1996). Inhibitory effects to lipid peroxidation of rat brain homogenate of various extracts from *A. trifoliatus* ranged from EC<sub>50</sub> of 11.18 ± 2.60 to 75.35 ± 7.52 µg/ml. Root and leaf decoction extracts showed significantly higher effects (EC<sub>50</sub> of 11.18 ± 2.60 and 16.11 ± 0.29 µg/ml, respectively) (Sithisarn and Jarikasem, 2009).

## 6. Anti-cholinesterase activity of *A. trifoliatus*

Cholinergic deficit was reported to associate with memory loss and the severity of Alzheimer's (Bierer, 1995). To restore cholinergic function in AD, many mechanisms are studied including stimulation of cholinergic receptors or prolonging the availability of acetylcholine (ACh) released into neuronal synaptic cleft by inhibiting ACh hydrolysis by acetylcholinesterase (AChE) which can be developed by the applications of proper AChE inhibitors. Extracts from several parts of *A. trifoliatus* were tested for AChE inhibitory activity using Ellman's colorimetric method in 96-well microplate. According to Vinutha et al. (2007), at the concentration of 100 µg/ml, most tested extracts exhibited moderate acetylcholinesterase inhibitory effect (30-50% inhibition). All root extracts including 95% ethanolic, decoction and 75% ethanolic extracts showed the highest effects among tested samples with AChE inhibition of 40.24 ± 3.56, 42.06 ± 5.29 and 47.41 ± 3.52 %, respectively. The results of inhibitory effect of *A. trifoliatus* extracts to AChE are shown in Table 3.

Sample	AChE inhibitory activity*
LM	15.51 ± 9.95 <sup>a,e,g,h,i,j</sup>
LR	22.82 ± 8.71 <sup>a,b,e,h,i,j</sup>
LD	31.76 ± 2.51 <sup>c,g</sup>
SBM	36.83 ± 3.34 <sup>b,c,d</sup>
SBR	22.51 ± 0.53 <sup>e</sup>
SBD	25.78 ± 1.92 <sup>i,j</sup>
SM	31.27 ± 3.54 <sup>a,c,j</sup>
SR	16.98 ± 4.36 <sup>e,h</sup>
SD	15.37 ± 1.68 <sup>h</sup>
RBM	36.26 ± 4.09 <sup>b,c,d</sup>
RBR	36.30 ± 1.50 <sup>a,c,d</sup>
RBD	20.65 ± 3.14 <sup>e,h,i</sup>
RM	40.24 ± 3.56 <sup>b,d</sup>
RR	47.41 ± 3.52 <sup>f</sup>
RD	42.06 ± 5.29 <sup>d,f</sup>
Galanthamine	Completely inhibit
Physostigmine	Completely inhibit

\* different letters in the same column are significantly different ( $P<0.05$ )

Table 3. Acetylcholinesterase inhibitory effect of extracts and standard compounds at 100  $\mu\text{g}/\text{ml}$  from various parts of *A. trifoliatus*. LM: leaf maceration, LR=leaf refluxing, LD=leaf decoction, SBM=stem bark maceration, SBR=stem bark refluxing, SBD=stem bark decoction, SM=stem maceration, SR=stem refluxing, SD=stem decoction, RBM= root bark maceration, RBR=root bark refluxing, RBD=root bark decoction, RM=root maceration, RR=root refluxing, RD=root decoction.

## 7. Anti-anxiety activity of *A. trifoliatus*

Adult male ICR mice with a weight range 30-35 g were used for determination of anti-anxiety effect of *A. trifoliatus* by light-dark task and hole-board test. Tested mice were received for corticosterone solution about 13 mg/kg per day via drinking water for 17 consecutive days. Then mice were tested for anxiety-like behavior in the light-dark task and hole-board test.

### 7.1 Light-dark task

Thirty mice were randomly divided into five groups of six mice. Each group of mice was orally administered with distilled water as a control group, phenobarbital (30 mg/kg) was used as a standard drug, and *A. trifoliatus* leaf decoction extract in 3 different doses of 500, 750 and 1000 mg/kg, respectively. Modified from Ardayfio and Kim (2006) and Krishna et al (2006), the apparatus consisted of a Plexiglas box with two compartments, one of which was illuminated with a white light while the other remained dark. One hour after drugs administration, each mouse was placed at one corner of the dark compartment, facing against of the light area. The time spent in illuminated and dark places, as well as the number of entries in each space, was recorded for 10 min. The administration of all 3 different doses of the extract in mice induced both significant increments ( $P<0.05$ ) of the

number of entries and time spent by mice in light chamber of the light-dark apparatus (Sithisarn et al., 2010).

## 7.2 Hole-board test

Applied from Brown and Nemes (2008), the apparatus consisted of a square plastic plate with 16 holes, regularly spaced on the surface. Each hole contains sensors for detecting when animal dips the head in a hole. Thirty mice were randomly divided into five groups of six mice. Each group of mice was orally administrated with distilled water as a control group, phenobarbital (30 mg/kg) was used as a standard drug, and *A. trifoliatus* leaf extract in 3 different doses of 500, 750 and 1000 mg/kg, respectively. Forty five minutes after drug administration, each mouse was placed on the centre of the board and the number of head dips was automatically counted for 3 min. Administration of the leaf decoction extract of *A. trifoliatus* in the concentration of 1000 mg/kg in mice significantly ( $P<0.05$ ) induced an increment of the number of head-dip of the animals, similar to the effect observed in phenobarbital treated group (Sithisarn et al., 2010).

## 8. Anti-inflammatory activity of *A. trifoliatus*

Leaf decoction extract from *A. trifoliatus* was tested for anti-inflammatory effect using rat paw edema model induced by carrageenan injection. Thirty adult male Wistar rats with a weight range of 180–200 g were randomly divided into five groups of six rats. Each group of rats was orally administrated with distilled water as a control group, indomethacin (20 mg/kg) was used as a standard drug, and *A. trifoliatus* leaf extract in 3 different doses of 100, 300 and 600 mg/kg, respectively. The method of edema induction described by Winters et al. (1987) was modified to induce inflammation in rats' paws. 1% carrageenan in 0.9% sodium chloride solution was injected into the right hind paw of each rat after 1 h of each treatment. The volumes of rat paw edema were determined using plethysmometer at 1, 2, and 3 h after edema induction. The percentage of edema inhibition was calculated (Palanichamy and Nagarajan, 1990). Two hours after edema induction, the extract showed dose-dependent inhibition and the extract at the dose of 600 mg/kg exhibited significantly anti-inflammatory activity (41% inhibition). While the standard drug, indomethacin in the dose of 20 mg/kg showed significant effects at both 2 h and 3 h with the percentages of inhibition of 35 and 26, respectively (Sithisarn et al., 2009).

## 9. Toxicity of *A. trifoliatus*

Acute toxicity of the decoction extract from the *A. trifoliatus* leaves collected from Sunpathong district, Chiang Mai province, of Thailand which contained total phenolic and total flavonoid of 16.26 and 1.31 g% CAE and g% RE was determined for an oral lethal dose in rats. Adult male Wistar rats with a weight range of 215–250 g and adult female Wistar rats with a weight range of 178–198 g were randomized into control and experimental groups. Each tested group of rats consisting of 5 males and 5 females was orally administered a dose of 2 g/kg of *A. trifoliatus* leaf decoction extract while the control animals were administered distilled water. The animals were observed for mortality or any signs of abnormalities periodically during the first 0.5, 1 and 3 h and once daily for 14 days thereafter. Clinical sign, morbidity and mortality of tested group was observed for 14 days and compared to the respective control group. Body weight of each animal was recorded on day 1, 8 and 15. The

position, shape, size and color of visceral organs, i.e., heart, kidneys, lungs, stomach, intestine, liver, pancreas and sex organ were visually observed for any signs of gross lesions.

Upon gross examinations of visceral organs, no abnormality sign was observed in all tested groups compared to the control. In addition, there was no significant difference in average body weights of treated and controlled animals. According to the common classification of chemicals (Auletta, 1995, Organization for Economic Co-operation and Development, 2001), the extract showed no sign of toxicity ( $LD_{50} > 2 \text{ g/kg}$  body weight).

## 10. Phytochemistry of *A. trifoliatus*

Extracts of the leaves and roots of *A. trifoliatus* prepared by different methods of extraction were chromatographic analyzed by thin layer chromatography. Flavonoids and polyphenolic compounds were detected with natural product/polyethylene glycol (NP/PEG) spraying reagent which showed fluorescence bands under UV 366 nm. Thin layer chromatographic fingerprints of *A. trifoliatus* extracts are shown in Figure 3. From TLC, extracts from the roots and leaves of *A. trifoliatus* showed some chromatographic bands positive to NP/PEG spraying reagent under UV 366 nm suggested the presence of phenolic and flavonoid compounds. TLC of the root extracts from refluxing with 75% ethanol and decoction as shown in track number 3 and 5 revealed bright blue fluorescence band corresponded to chlorogenic acid while those of the leaf extracts as shown in track number 2 and 4, other than chlorogenic acid, also contained orange fluorescence band corresponded to rutin. The leaf 95% ethanol maceration extract as shown in track number 2 also positively showed some chromatographic bands to the spraying reagent while the root 95% ethanol maceration extract revealed very weak chromatographic bands as shown in track number 1.

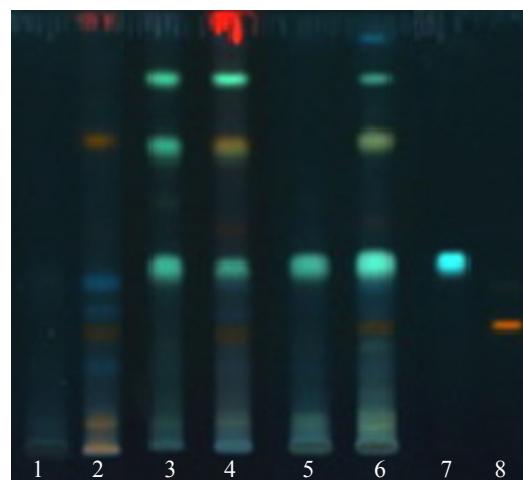


Fig. 3. TLC chromatogram of extracts from *A. trifoliatus*. Track : 1= root 95% maceration, 2= leaf 95% maceration, 3= root 75% refluxing, 4= leaf 75% refluxing, 5= root decoction, 6= leaf decoction, 7= standard chlorogenic acid, 8= standard quercetin, Stationary phase: silica gel GF254 Solvent system: ethyl acetate: acetic acid : formic acid : water 137: 11: 11: 26 Detection: NP-PEG spraying reagent / UV 366 nm.

### 10.1 Total phenolic and total flavonoid contents

Phenolics and flavonoids are known to be strong antioxidants especially through a free radical scavenging mechanism (Tyrrell, 1992). Total phenolic and total flavonoid contents in plant extracts can be determined colorimetrically using specific reagents such as Folin-Ciocalteu and aluminium chloride reagents, respectively. Extracts from various parts of *A. trifoliatus* were determined for total phenolic content using Folin-Ciocalteu reagent (Dasgupta and De, 2007) and showed high concentration of phenolic compounds especially 75% refluxing extracts from stem barks and stems and root bark decoction extract ( $21.79 \pm 0.23$ ,  $19.17 \pm 0.58$  and  $20.74 \pm 0.44$  g chlorogenic acid equivalent (CAE) in 100 g extract (Sithisarn and Jarikasem, 2009). Total flavonoid content of extracts was determined using the method adapted by Meda et al. (2005). Aluminium chloride solution was mixed with the same volume of the sample solution. Absorption readings at 415 nm were taken and total flavonoid content was calculated as g rutin equivalent (RE)/100 g extract. Among tested extracts, leaf 95% ethanolic maceration extracts contained the highest amounts of flavonoids ( $9.61 \pm 0.52$  g % RE) (Sithisarn and Jarikasem, 2009).

### 10.2 High performance liquid chromatography

Leaf decoction extract of *A. trifoliatus* was subjected for HPLC-DAD and HPLC-MS analysis, six main peaks were separated by column chromatographic technique and were identified by their mass spectra and UV absorption spectra as chlorogenic acid, 3,5-di-O-caffeoquinic acid, rutin, isoquercetin, 4,5-di-O-caffeoquinic acid and quercitrin, respectively (Sithisarn and Jarikasem, 2009). MS chromatogram and HPLC chromatogram of these compounds are shown in Figure 4. Chemical structures are shown in Figure 5.

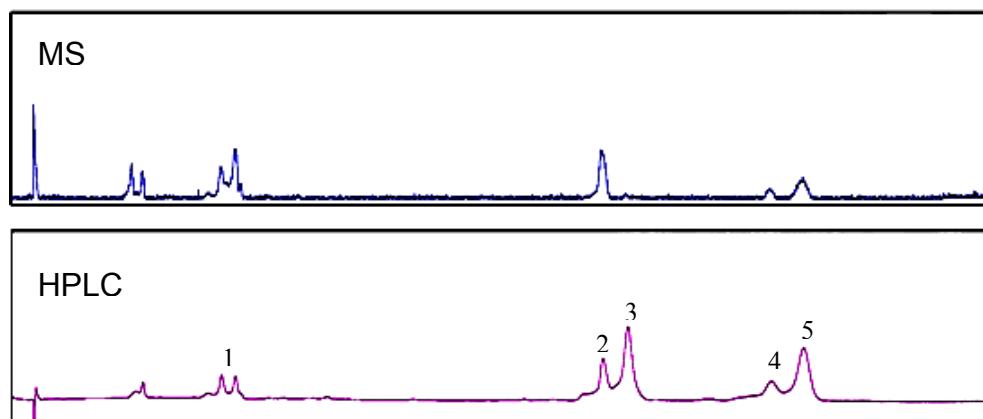


Fig. 4. HPLC chromatogram (UV 310 nm) and MS chromatogram of *n*-butanol fraction of leaf extract from *A. trifoliatus* (Sithisarn et al., 2008). No. of peak ( $t_R$ , min); 1= chlorogenic acid (16.6), 2= 3,5-di-O-caffeoquinic acid (47.2), 3= rutin and isoquercetin (49.1), 4= 4,5-di-O-caffeoquinic acid (60.7), 5= quercitrin (63.3).

## 11. Standardization of *A. trifoliatus*

Chemical and biological standardizations of plant extracts are also needed to be clarified before further studies in animal model or clinical trial. The major variables in plant geography are area, annual rain fall, ground water, types of soil, pH of soil, temperature, altitude, wind, light and local insecticides. These factors, especially rain fall and

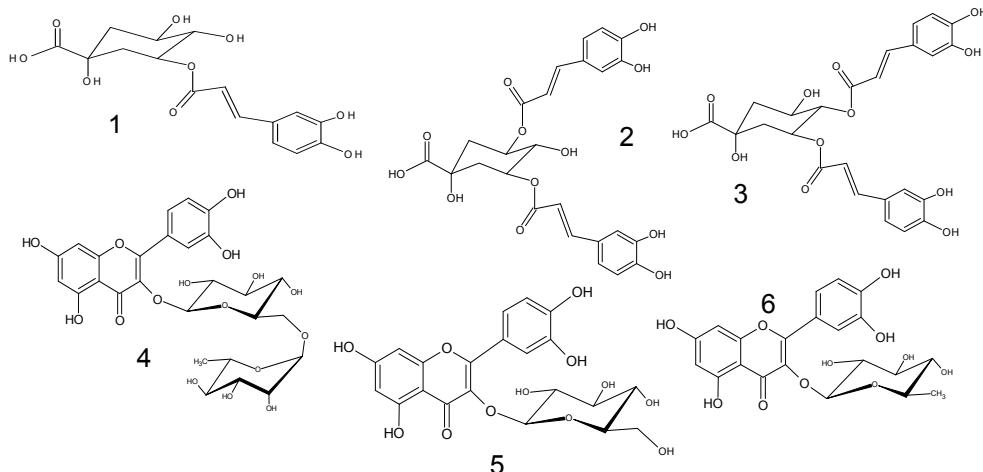


Fig. 5. Chemical structures of compounds from *n*-butanol fraction of leaf extract from *A. trifoliatus*: 1 = chlorogenic acid, 2 = 3,5-di-*O*-caffeoquinic acid, 3 = 4,5-di-*O*-caffeoquinic acid, 4 = rutin, 5 = isoquercetin, 6 = quercitrin.

temperature, could affect the biosynthesis of caffeoylquinic acid and flavonoid in *A. trifoliatus* (Sithisarn et al., 2011a). Chemical, physical and biological standardization of decoction extract of *A. trifoliatus* was set up by determination of antioxidant activity and quantitative analysis of the marker compositions of 11 *A. trifoliatus* leaf samples harvested in Chaing Mai province at different time intervals within a year. Using decoction, the most suitable extraction method for active *A. trifoliatus* leaf extract as previously reported (Sithisarn, and Jarikasem, 2009), it was found that samples collected in Winter and Fall contained significantly higher amount of total phenolic (12.72 – 14.66 g% CAE in dried extract) and total flavonoid (1.97 – 2.20 g% RE in dried extract) (Sithisarn et al., 2011b). HPLC analysis of marker components including monocaffeoylquinic acid (chlorogenic acid), dicaffeoylquinic acids (3,5-di-*O*-caffeoquinic acid and 4,5-di-*O*-caffeoquinic acid), and flavonoid glycosides (rutin, isoquercetin and quercitrin) revealed that samples collected in January and November significantly contained high amount of phenolic and flavonoid contents suggesting the harvesting period of *A. trifoliatus* leaf samples during Winter or in low temperature condition (Sithisarn et al., 2011a). HPLC fingerprints of all extracts as shown in Figure 6 showed the similar chromatographic characteristics suggested that this HPLC fingerprint could be used for both quantitative and qualitative analysis of leaf extracts of *A. trifoliatus*.

Free radical scavenging activity of the collected extracts was studied by DPPH scavenging assay, it was found that most of the extracts showed strong effects ( $EC_{50} < 50 \mu\text{g/ml}$ )

(Cervantes-Cervantes, 2005). Samples which contained high amount of total phenolic and total flavonoid contents significantly exhibited the strongest activity. The high correlations of total phenolic and total flavonoid contents with DPPH scavenging activity were found to be 0.863 and 0.831 ( $P < 0.05$ ), respectively. For the capacity to inhibit iron-induced lipid peroxidation, moderate correlations of 0.586 and 0.389 ( $P < 0.05$ ) were found between the inhibition and total flavonoid and total phenolic contents, respectively (Sithisarn et al., 2011b).

An excess of water in medicinal plant materials will lead to microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. Therefore, limits for the amount of water should be set for plant materials. This is especially important for materials which absorb moisture easily or deteriorate quickly in the presence of water (World Health Organization, 1992). Extracts and the leaves of *A. trifoliatus* collected at 11 different times were investigated for loss on drying as mentioned in BP (2004). Loss on drying of plant samples ranged from  $6.58 \pm 0.13$  to  $15.06 \pm 0.05$  % while the extract contained the loss on drying amount of  $8.28 \pm 3.30$  to  $11.51 \pm 0.52$  %.

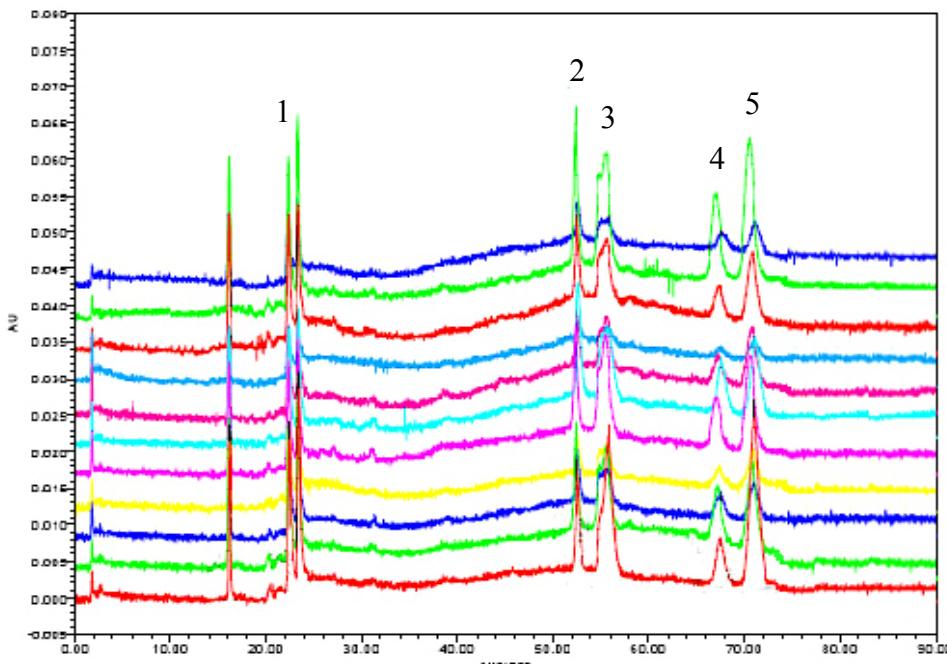


Fig. 6. HPLC chromatogram of leaf decoction extracts from *A. trifoliatus* collected from 11 different durations. No. of peak; 1= chlorogenic acid, 2= 3,5-di-O-caffeoquinic acid, 3= rutin and isoquercetin, 4= 4,5-di-O-caffeoquinic acid, 5= quercitrin.

## 12. Conclusion

As the mention of Teeguarden (1998), “an adaptogen is substance that helps bring the body into a state of harmony with its environment by introducing chemical, cellular, and

systematic balance. This harmonizing function reduces the effects of unfavorable conditions and stimulates the body's own immune and healing functions. These adaptogenic substances help the body to adapt to various stressful challenges presented by the environment and reduce the damage inflicted on the body. They tend to promote the body's own ability to cope successfully with stress, thus prolonging well-being", the effects of adaptogen could relate to some biological activities including antioxidative, anti-cholinesterase, anti-inflammatory and anti-anxiety, which associated with the excessive activity of stress system (Chrousos and Gold, 1992, Panossian, 2003). Several parts of *Acanthopanax trifoliatus*, a Thai traditional herb promoted *in vitro* antioxidant and anti-cholinesterase activities. Extracts from the leaves of this plant could also exhibit *in vivo* adaptogenic related biological effects including anti-inflammatory and anti-anxiety activities. Polyphenolics and flavonoid, phytochemicals that play important role in antioxidation, anti-inflammatory and adaptogenic actions in plants (Hoorn, 2003, Panossian and Wagner, 2005, Wagner et al., 1994) were found to available in high amount in *A. trifoliatus*, especially in the leaves and the roots. Active polyphenolics and flavonoid compounds were structurally identified. *A. trifoliatus* could then be considered as the potential plant that promote adaptogenic effects which conduced to the chemical, biological and physical standardization of the leaf extract of this plant. However, at present there is no *A. trifoliatus* product used for health or medicinal purposes in the marketplace, especially in Thailand. Since young leaves of *A. trifoliatus* have been traditionally consumed as vegetables, and the leaf extract of this plant showed various biological activities related to adaptogenic properties with no acute toxicity was found, therefore, it would be great opportunity to develop the effective, high quality and standardized health supplement or herbal medicine for adaptogenic related purposes in the near future.

### 13. Acknowledgement

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# Hulless Barley

## – A Rediscovered Source for Functional Foods

### Phytochemical Profile and Soluble Dietary Fibre Content in Naked Barley Varieties and Their Antioxidant Properties

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### 1. Introduction

#### 1.1 The history of the use of barley

The beginning of agriculture is dated back to 10,000 years ago in the Pre-Pottery Neolithic Near East. Barley (*Hordeum vulgare* L.) is one of the cereal founder crops and it is believed that first plant domestication took place within the Fertile Crescent (Lev-Yadun et al., 2000). *Hordeum vulgare* L. subsp. *spontaneum* (wild barley) is said to be the ancestor of today's barley. The spread of barley most likely started in present-day Israel, northern Syria, southern Turkey, eastern Iraq and western Iran. With the movement of civilizations accompanied by the establishment of trade routes the use and cultivation of barley reached Europe. Barley was a popular food in ancient Greece and Italy and used as an ingredient for preparing porridge or unleavened bread. Greek and Roman scholars such as Hippocrates or Pliney the Elder, respectively, considered barley as a healthy and nourishing food and barley gained as well recognition for medical treatments. In the ancient Rome, gladiators believed that barley could increase strength and stamina and thus preferred it to other cereals. Barley reached Spain around 5,000 BC and spread then over today's Germany and France. Indications of domestication of barley on the British Isles date back until 3,000 BC and one millennium later, barley was introduced to Northern Europe. Probably due to the nourishing properties and the ruggedness of the crop, barley became a major food especially for poor people throughout history (Newman & Newman, 2005).

In 2009, barley was the twelfth most important agricultural commodity of the world in terms of production. After maize, wheat and paddy rice it was the fourth most important

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cereal crop (FAO, 2011). The annual production was about 152 million tons, of which the Russian Federation produced around 17.9 million tons followed by France, Germany and Ukraine with 12.9, 12.3 and 11.8 million tons, respectively. In the European Union, barley still attains distinction as the second cereal crop after wheat.

Barley falls botanically into the family of the grasses (*Poaceae=Gramineae*), tribe *Triticeae*, genus *Hordeum*. It is a diploid ( $2n=2x=14$ ) mainly self-pollinating crop. In contrast to domesticated variations wild barley has a brittle spike where the ripe spikelets separate from each other easily; this favours the spread of the seed but complicates harvest. Non-brittle variations can be harvested unproblematically but survive only under domestication. A barley head consists of triplets of spikelets that are alternatively arranged on the rachis. Due to the morphology of the spikelets one can distinguish between two- and six-rowed barleys. Two-rowed barleys have two lateral unfertile spikelets in each triplet and thus only two rows of fertile spikelets. In six-rowed barley all six spikelets are fertile (Takahashi, 1955; Zohary & Hopf, 2000).

Cultivated barley can as well be classified by the form of their caryopsis. In hulless (syn. hull-less or naked) barley varieties the expression of the recessive naked caryopsis gene (*nud*, *nudum*) prevents the intergrowth of husks and caryopsis. Consequently, the kernels thresh free and lemma and palea do not adhere to the caryopsis at maturity. This is why hulless barley (*Hordeum vulgare* var. *nudum*) requires no further dehulling for the production of food. Cultivation of hulless barley is as old as that of hulled barley but is less common worldwide due to significantly lower yields and only minor breeding activities (Atanassov et al., 2001; Pandey et al., 2006). Compared to hulled barley, the free-threshing character of hulless barley proportionally increases contents of protein and the limiting amino acids lysine and threonine, respectively, (Baidoo & Liu, 1998; Bhatty, 1999) and as well levels of  $\beta$ -glucan but lowers contents of insoluble dietary fibre components (Xue et al., 1997; Baidoo & Liu, 1998).

## 1.2 Importance of barley as food ingredient

Starch is the major component in barley kernels amounting 60 – 70% of the dry matter. Starch itself is composed of two types of glucose polymers namely the highly branched amylopectin and the linear amylose. According to the proportions of amylose and amylopectin barley can further be classified: normal naked barley contains 25 – 30% amylose, waxy varieties less than 15% whereas high-amylase cultivars more than 35% amylose (Ajithkumar et al., 2005; Bhatty, 1986; 1999). Among the waxy cultivars there are also zero-amylase waxy barleys that contain no amylose (Izydorczyk et al., 2000). Both polymers strongly influence the quality of the obtained products, where amylopectin is responsible for the crystallinity of starch and excellent water absorbing properties (Bhatty, 1997).

Hulless barley is a good source of dietary fibre providing soluble and insoluble dietary fibre fractions (Bhatty, 1999; Izydorczyk et al., 2000). Mixed-linkage (1 $\rightarrow$ 3),(1 $\rightarrow$ 4)- $\beta$ -D-glucans (hereafter termed as  $\beta$ -glucan) are a major part of the soluble dietary fibre (SDF) in barley. Nevertheless, its content generally underlies a natural fluctuation depending on the variety and conditions before and after harvest (Ehrenbergerová et al., 2008). Together with

arabinoxylans, a fraction of partly soluble nonstarch polysaccharides (NSP) occurring in the cell walls,  $\beta$ -glucan has a great impact on cereal processing and product properties. In Europe, the European Food Safety Authority (EFSA) has come to the decision that a claim like “regular consumption of  $\beta$ -glucans contributes to maintenance of normal blood cholesterol concentrations” will be allowed (EFSA, 2009). Until then, the interest of food producers and consumers in using hulless barley for food purposes may increase, although up to now, barley flour is hardly used for human consumption. It is assumed that barley  $\beta$ -glucans have the same effects than oat  $\beta$ -glucans. In order to bear the claim, EFSA demands a quantity in food of at least 3 g/day of  $\beta$ -glucans from oats, oat bran, barley, barley bran, or from mixtures of non-processed or minimally processed  $\beta$ -glucans in one or more servings.

On the other side of the coin, the higher the  $\beta$ -glucan content in barley flours the worsen are the baking qualities, due to the high water binding capacity and thus decreasing the water availability for the gluten network (Gill et al., 2002). Furthermore, high  $\beta$ -glucan contents must not necessarily go along with increased health enhancing properties unless the extractability and/or digestibility are not considered (Xue et al., 1997).

### 1.3 Phytochemicals in barley

Over the last decade, fruits, vegetables and cereals have been studied for various bioactive compounds such as phenolics and flavonoids as health promoting and disease preventing effects were found in both *in vitro* and *in vivo* studies. Phytochemicals, per definition, are substances that are likely to contribute to health or are essential for the maintenance of health (Cooke et al., 2002). In contrary to vitamins or minerals, there is no evidence that they are essential or even required to sustain life. It is hypothesised that phytochemicals that are located in the fibre matrix, in addition to or instead of the fibre itself, are responsible for the reduced risk of various diseases associated with oxidative stress, such as cancer, cardiovascular and neurodegenerative diseases (Jacobs & Steffen, 2003). Thus, the consumption of whole grains is seen as health-promoting (Slavin, 2003), because whole-grains and products thereof present a good source of natural antioxidants (Decker et al., 2002). Polyphenols constitute together with carotenoids the only dietary antioxidants present in the colon in valuable concentrations (Scalbert & Williamson, 2000; Manach et al., 2004). The release of phenolics in the colon may explain the inverse association between whole-grain consumption and incidence of certain chronic diseases (Andreasen et al., 2001; Kern et al., 2003). Moreover, an apparent increase of antioxidant release during enzymatic incubation was found in *in vitro* studies (Pérez-Jiménez & Saura-Calixto, 2005; Nagah & Seal, 2005; Menga et al., 2010).

Barley grains contain a wide range of phenolic acids, which are either derivatives of benzoic acid ( $C_6-C_1$ ) or cinnamic acid ( $C_6-C_3$ ). In general, higher levels of phenolic compounds were reported for barley and oat compared to wheat and rye (Zielinski & Kozlowska, 2000). Ferulic acid is the most abundant phenolic acid present in cereals, representing up to 90% of total polyphenols (Sosulski et al., 1982). Also Hernanz et al. (2001) and Naczk & Shahidi (2006) reported that ferulic acid is the dominant free phenolic acid in barley seeds. The composition of phenolic acids in 30 barley varieties was investigated by Yu et al. (2002) where they found varying levels of benzoic and cinnamic acids. Phenolic compounds are

said to exhibit one or more of the following roles: free radical scavenger, reducing agent, potential producer of prooxidant metals and quencher of singlet oxygen formation.

Anthocyanins in wheat and barley are found either in the pericarp or the aleurone layer causing purple and blue hues of kernel colour, respectively. The black pigmentation of the lemma and pericarp of barley, however, is described to be due to melanin-like pigment (Woodward, 1941; Lundqvist et al., 1996) which may overlap other pigments. Black kernel colour due to melanin-like pigment is unknown to wheat species.

Lutein and zeaxanthin are the two main carotenoids identified in barley (Goupy et al., 1999; Panfili et al., 2004). The electron rich chain makes them effective radical scavengers (Cooke et al., 2002) and inhibit free radical propagation reactions such as lipid peroxidation. Lutein and zeaxanthin are responsible for the coloration of the macula lutea ('yellow spot') of the retina, the area of maximal visual acuity. Hence, dietary lutein and zeaxanthin are supposed to protect against age-related macular degeneration and cataract (Seddon et al., 1994; Beatty et al., 2000). Furthermore, lutein and zeaxanthin possibly act together with other bioactive compounds against cancer, cardiovascular risk and other diseases (Wahlqvist & Wattanpenpiaboon, 1998; Mares-Perlman et al., 2002; Calvo, 2005). Vitamin E or tocopherols are also present in markedly concentrations (Cavallero et al., 2004; Andersson et al., 2008).

## 2. Materials and methods

### 2.1 Barley samples

Twenty-nine barley varieties were grown at the experimental station Groß-Enzersdorf (16°35' E; 48°13' N) in 2007, 2008 and 2009. The varieties were sown at the beginning of March and harvested mid July. Monthly precipitation and mean temperature of the growing seasons at the growing site for the three years are shown in Fig. 1. Post-harvest treatment included sifting via air classification. Barley samples were stored at 4 °C until use. Samples were milled directly before analyses using a laboratory mill (MF10 basic with a 1 mm sieve, IKA, Austria) to obtain wholemeal samples.

Twelve coloured naked barley varieties were further selected to study the distribution of the compounds of interest within the different milling fractions. These varieties were milled with a MLU 202 roller mill (Bühler, Switzerland). The grain was not conditioned before milling and the feed rate for milling was approximately 5 kg h<sup>-1</sup>. Six flour fractions (B1-B3, C1-C3) from the starchy endosperm were collected and merged to give a straight-run white flour. Brans and shorts were collected separately. All millstreams were stored at 4 °C until use.

### 2.2 Analyses

#### 2.2.1 Agronomic traits

Data were collected for thousand kernel weight (TKW, g), hectolitre weight (HLW, kg hL<sup>-1</sup>) and seed plumpness (SP25, %). HLW was measured by a ¼ L chondrometer. Counting of seeds was done by a Contador machine (Pfeuffer GmbH, Kitzingen, Germany) and weighed. SP25 was determined by sieving 100 g of grain with a Sortimat laboratory machine (Pfeuffer GmbH, Kitzingen, Germany). Grains remaining on the 2.5 mm sieve were considered as plump.

### **2.2.2 Basic chemical composition**

Moisture and ash content of the barley flour was determined according to AOAC approved standard methods 940.56 and 920.153, respectively (AOAC, 1995). Crude protein (PROT) was determined according to the ICC standard method 105/2 using the factor 5.83×N for conversion, whereas total starch content was determined according to the ICC standard method 168 (ICC, 2001).

### **2.2.3 Dietary fibre**

Mixed linkage  $\beta$ -glucan (BG) (AOAC method 995.16) content was determined in barley wholemeal samples using a commercially available test kit (Megazyme, Bray, Ireland).

Total pentosanes (PENT) and water-extractable pentosanes (we-PENT) were analysed according to the method described by Douglas (1981). Briefly, for determination of we-PENT, 25 mg sample were suspended in 10 mL water and extracted for 2 h on a rotary shaker. One millilitre of the aqueous phase was mixed with 5 mL of the reaction solution (consisting of 110 mL glacial acid, 2 mL HCl conc., 5 mL phloroglucinol solution (20% (w/v) in ethanol) and 1 mL 1.75% (w/v) glucose solution) and incubated for 25 min in a boiling water bath and shaken from time to time. After cooling, the we-PENT content was calculated from the difference of the absorbances at 510 and 552 nm according to a xylose calibration curve (2 g L<sup>-1</sup>). To determine total pentosanes (PENT) content, 5 mg of the sample was suspended in 2 - 4 mL water and incubated with the reaction solution.

### **2.2.4 Secondary plant metabolites**

#### **Extraction of free and bound phenolic compounds**

Free and bound phenolic acids were extracted according to the methods of Adom et al. (2003) and Mattila et al. (2005) with minor modifications. Briefly, 0.15 g of barley flour was extracted twice with 80% aqueous methanol for 30 min. Supernatants were pooled after centrifugation at 2500g for 10 min and represented the crude extracts of free phenolics. Samples were stored at -20 °C until HPLC analysis and filtered prior injection into the HPLC.

To extract the bound phenolic compounds, the above obtained residues were blended with sodium hydroxide to reach a final concentration of 2 M NaOH. Samples were vortex mixed and incubated in the dark on a shaker (Rotator, VWR, Austria) overnight (16 h). The mixture was brought to pH 3 with hydrochloric acid. After centrifugation, supernatants and residues were extracted separately three times with ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness and stored at -20 °C until analysis. Before quantification, bound phenolic compounds were dissolved in 50% methanol.

#### **Anthocyanin content**

Anthocyanins were extracted with acidified methanol (methanol and 1 M HCl, 85:15, v/v) with a solvent to sample ratio of 1:10 for 30 min on a magnetic stirrer and then separated by centrifugation. The supernatants were collected and kept in the dark and cold (+4 °C) until procedure. The residues were twice re-extracted under the same conditions, and supernatants

of all three cycles were combined. Total anthocyanin content (TAC) was calculated using a calibration curve of cyanidin-3-O-glucoside (C-3-glc) as reference. Extracts were measured at 525 nm in a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). Contents were expressed as mg C-3-glc equ. per 100 g, dm (Abdel-Aal & Hucl, 1999).

Extracts were further proceeded to quantify different anthocyanidins. Therefore, samples were kept at -30 °C overnight and the precipitates were separated via centrifugation at 2500g thereafter. The solvent was removed at 40 °C using a rotary evaporator and the resulting anthocyanin concentrates were re-suspended in methanol prior to HPLC analysis.

### Carotenoid content

Five gram of barley flour and 0.02 g butylated hydroxytoluene (BHT) were weighed into amber glass flasks. After addition of 20 mL of the extraction solvent (MeOH:ethyl acetate:petroleum ether, 1:1:1, v/v/v), samples were extracted on a magnetic stirrer for 2 h and then filtrated to recover the extract and the residue. The extraction procedure was repeated twice using the residue. The collected combined supernatants were transferred into a separation funnel and washed three times with a saturated NaCl-solution (37.5% NaCl in water). Remaining water in the organic phase was thereafter removed with sodium sulphate and the extract was evaporated until dryness at 35 °C. Samples were further kept under a stream of nitrogen to avoid any oxidation, sealed and immediately stored at -20 °C until analysis. Prior analysis, samples were thawed, solubilised in tetrahydrofuran (THF) and transferred into amber glass HPLC vials and kept under nitrogen-stream before closing.

### HPLC analysis

The profile of anthocyanidins and their corresponding glycosides as well as phenolic compounds and carotenoids were determined using a HPLC system (Shimadzu, Korneuburg, Austria) consisting of a SPD-M10AVP photodiodearray detector, chromatogram integrator, LC-10ADVP pump and online degasser. Data signals were processed on a PC running the LC solution Multi software (Shimadzu, Korneuburg, Austria). Analytical separation of anthocyanidins and phenolic acids was carried out using a Phenomenex Luna 250×4.6 mm, 5 µm (HPLC Services, Breitenfurt, Austria) column, whereas carotenoids were separated on a Vydac 201TP54, C18 5µm, 250×4.6 mm (MZ-Analysentechnik GmbH, Mainz, Germany) column.

For the determination of anthocyanidins, dissolved anthocyanidin concentrates were passed through a 0.45 µm PTFE-filter and a 20 µL aliquot of the sample solution was injected. Elution for anthocyanidins was executed under gradient conditions with (A) 4.5% formic acid in water and (B) acetonitrile. The solvent gradient was programmed as follows: 10% B at 0 min, increasing to 12% within 9 min, to 13% within the next 7.5 min, 25% within the next 13.5 min, 90% within the next 15 min, holding at 90% for 5 min, followed by a decrease to 10% within the next 5 min, before equilibration at 10%. The solvent flow rate was set at 0.8 mL min<sup>-1</sup> and the chromatogram was recorded at 520 nm (anthocyanidins and glycosides) at 35 °C. Peak areas were used for all calculations. Identification of compounds was done by comparing the retention time and the UV spectra with those of pure substances. DAD response was linear for all anthocyanidins and anthocyanins within the

calibration range of 0.05 – 40.0 µg mL<sup>-1</sup>, with correlation coefficients exceeding 0.999. Coefficients of variation for sample replicates were consistently below 10%.

Phenolic acids were determined as follows: Twenty microliter of sample were injected into the column and eluted under gradient conditions performed with 0.05% trifluoroacetic acid in water (A) and 0.05% trifluoroacetic acid in acetonitrile (B). The solvent gradient was programmed as follows: at 0 min, 10% B; increasing from 3 to 15 min to 15% B; 25 min, 20% B; 30 min, 40% B; 36 – 40 min, 80% B; decreasing thereafter to 10% B within the next 4 min and equilibrating before the next injection. The flow rate was 1.0 mL min<sup>-1</sup>. Analyte detection was at 260 nm for 4-OH-benzoic and vanillic acid and at 280 nm for caffeic, *p*-coumaric and *trans*-ferulic acid. DAD response was linear for all phenolic acids within the calibration range of 0.06 – 125.0 µg mL<sup>-1</sup>, with correlation coefficients exceeding 0.999. Phenolic acids in the samples were identified by comparing their relative retention times and UV spectra with authentic compounds. Coefficients of variation for sample replicates were consistently below 10%.

Carotenoids were separated under isocratic conditions with methanol:acetonitrile (9:1, v/v) and 1 mL min<sup>-1</sup> flow rate. The temperature was set at 25 °C, and lutein (LUT) and zeaxanthin (ZEA) were detected at 450 nm. Peak areas were used for all calculations. Identification of compounds was done by comparing the retention time and the UV spectra with those of pure substances.

## 2.2.5 Determination of the antioxidant capacity

Extracts containing the free and bound phenolic compounds were analysed for their total antioxidant capacity by the ABTS radical cation scavenging assay (Trolox equivalent antioxidant capacity (TEAC)) and the ferric reducing antioxidant power (FRAP) assay. To distinguish between the two extracts, the terms TEACf and FRAPf were used for the extract containing the free phenolic acids, whereas TEACb and FRAPb refer to the extract containing the bound phenolic acids.

The total phenolic content (TPC) of extracts was determined using the Folin-Ciocalteu reagent (Singleton et al., 1999) and was termed thereafter as TPCf or TPCb for free and bound phenolics, respectively. An appropriate dilution of extracts (120 µL) was added to 600 µL of freshly diluted 10-fold Folin-Ciocalteu reagent. 960 µL of sodium carbonate solution (75 g L<sup>-1</sup>) was added to the mixture after 2 min reaction time. The absorbance of the resulting blue colour was measured at 760 nm against a blank after 5 min of reaction at 50 °C. Ferulic acid was used as standard, and TPC was expressed as mg ferulic acid (FA) equ. per 100 g dm.

The TEAC assay was analysed following a modified method of Pellegrini et al. (2003) and Moore et al. (2005). A stable stock solution of ABTS radical cation was produced by reacting a 7 mmol L<sup>-1</sup> aqueous solution of ABTS with 2.45 mmol L<sup>-1</sup> potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12 – 16 h before use. On the day of analysis, an ABTS radical cation working solution was obtained by diluting the stock solution in ethanol to an absorbance of 0.70 ± 0.02 AU at 734 nm. Hundred microliters of extract were mixed with 1.25 mL of the ABTS working solution and

absorbance was read at 734 nm after a 1 min reaction time. Results were expressed as TEAC in mmol of Trolox per kg, dm.

The FRAP assay is based on the reduction of the Fe<sup>3+</sup>-TPTZ complex to the ferrous form at low pH. This reduction is monitored by measuring the absorption change at 595 nm (Benzie & Strain, 1999). Briefly, 0.2 mL of sample extract was mixed with 1.3 mL of the FRAP reagent. Absorption was measured at 595 nm in a spectrophotometer (U-1100, Hitachi, Japan) after 30 min of incubation at 37 °C. The FRAP reagent was prepared daily and consisted of 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl<sub>3</sub> in a ratio of 10:1:1 (v/v/v). FRAP values were obtained by comparing the absorption change in the test mixture with doses obtained from increasing concentrations of Fe(III) and expressed as mmol of Fe(II) equ. per kg, dm.

### 2.2.6 Statistical analysis

Data were reported as mean ± standard deviation for at least duplicate extracts. Statistical analysis were carried out using STATGRAPHICS Centurion® XV (StatPoint Technologies Inc., Warrenton, VA), SAS® 9.2 (SAS Institute Inc., Cary, NC) and/or GenStat® 13<sup>th</sup> Ed. (VSN International Ltd., Hemel Hempstead, UK) software packages. Linear mixed model analysis of variance was carried out for the multi-year data (2007-2009) with genotypes as fixed effects and year and genotype by year interaction (G×Y) as random effects. Model selection was based on the corrected Akaike information criterion (AIC). Significance of random effects was tested by the log-likelihood ratio test. Multivariate analyses were performed with collected data from 2008, i.e. principal component analysis (PCA) and canonical discriminant analysis. Multiple mean comparisons were carried out at the 5% significance level using the Duncan's multiple range test.

## 3. Results

### 3.1 Description of investigated hulless barley genotypes

Twenty-nine spring barley genotypes with different countries of origin, two- or six-rowed types as well as different seed colours were selected for this study. HB803 and Pronghorn (Washonubet) are white waxy genotypes, whereas all others were of normal starch type. Pronghorn was produced by crossing Wanubet and Shonubet and is currently filed as patent (Patent No. 20090064357).

The seed colour of barley genotypes is dependent on different pigments which are located in different seed layers. A great diversity from yellowish-white to blue, purple and black is reported in the literature. The interest in re-introducing coloured varieties is closely associated with their possible health promoting effects. Most of black and blue genotypes came from Ethiopia, a country rich in coloured barley germplasm. Apart from Purple Nudum, all purple classified genotypes are 6-rowed and bear the term "black" in their name. As they originated in majority from China, it is anticipated that the investigated genotypes have been selected from Chinese landraces. After their first propagations in 2006, all genotypes have been classed according to their seed colour. As the colour appeared dark-purple to black, they were finally carried on as purple (Table 1).

Id	Variety name	Accession No.	Origin <sup>1</sup>	Rows	Colour	TKW <sup>2</sup>	HLW	SP25	PROT	BG	TPC	TAC	LUT	ZEa
1	SNC04		AT	2	Black	38.3	76.4	40.44	11.57	4.84	244.6	1.99	0.728	0.871
2	Ae 13 dunkel	BVAL355117	ET	6	Black	37.2	77.0	46.49	11.78	4.63	286.3	0.92	0.583	0.456
3	Naked Black	BVAL355163	ET	2	Black	31.6	73.5	20.34	10.35	4.78	233.3	1.50	0.580	0.711
4	Violaceum 2	C661	CN	6	Black	30.6	78.2	44.25	12.28	5.00	306.3	2.13	0.751	0.824
5	Ethiopia 179	E360 (CILho9978)	ET	2	Black	37.8	77.6	44.02	14.16	4.45	258.8	1.39	0.271	0.485
6	Ethiopia 12	E604 (CILho1967, PI24901)	ET	2	Black	38.2	74.1	24.28	15.11	4.74	328.3	1.56	0.482	0.641
7	Black Naked	SY	2	Black	26.3	76.6	11.99	4.33	289.8	1.36	0.709	0.806		
8	Murasaki Hadaka	J307	JP	6	Black	40.0	76.0	41.73	12.86	5.11	489.6	2.30	0.690	0.937
9	Digersano		IT	2	White	37.2	77.9	30.97	12.20	3.88	367.6	0.96	0.283	0.398
10	HB803 2		CA	2	White	45.7	67.9	63.62	12.53	5.05	403.8	1.29	0.472	0.710
11	Hora	BVAL350010	DE	2	White	37.2	69.9	50.37	12.14	3.86	253.4	0.44	0.579	0.658
12	Lawina		DE	2	White	38.7	75.8	44.66	14.41	4.43	389.9	0.51	0.386	0.474
13	Taiga	BVAL350017	DE	2	White	37.6	75.4	43.63	12.77	4.92	368.0	0.93	0.289	0.465
14	Pronghorn (Washonubet) <sup>2</sup>		US	2	White	31.0	71.8	13.61	11.51	5.97	371.4	0.82	0.476	0.676
15	Himalayense Type 5	C651	CN	6	Blue	34.7	76.6	38.42	13.31	4.84	381.2	1.96	0.346	0.633
16	Debre Zeit AFS 2 (12-20-3)	E515	ET	2	Blue	38.9	75.2	22.15	13.13	5.81	402.6	1.34	0.337	0.476
17	Addis Ababa 56 (3-10-1b)	E550	ET	6	Blue	30.1	73.9	13.79	14.30	4.93	391.6	1.29	0.494	0.581
18	Ethiopia 96	E632	ET	6	Blue	25.8	75.3	13.02	13.43	4.73	336.8	0.73	0.310	0.382
19	Edenre Sel. Blau	GE040	AT	6	Blue	28.7	74.1	18.46	14.71	4.23	407.3	1.20	0.244	0.473
20	Indian 3	I311	IN	6	Blue	30.7	75.4	12.92	14.06	4.78	388.2	1.09	0.331	0.463
21	Gho 1 (1392)	N308	NP	6	Blue	28.5	77.9	37.97	10.77	4.50	279.1	1.24	0.185	0.423
22	Sama 9 (1462)	N624	NP	6	Blue	30.1	75.0	26.40	12.41	4.59	375.3	1.13	0.378	0.510
23	Black Hull-Less	HOR11402	CN	6	Purple	33.4	73.8	37.33	15.06	5.45	245.3	6.62	0.368	0.570
24	Schwarze Nackte Kraftborn	HOR21199	DE	6	Purple	32.7	78.5	46.69	13.88	5.33	272.8	6.45	0.254	0.479
25	Black Hull-less	HOR2593 (P124849)	CN	6	Purple	36.4	73.7	40.78	14.81	5.10	285.0	6.21	0.261	0.412
26	Schwarze Chinesische	HOR3727	CN	6	Purple	32.9	78.9	38.13	12.40	4.50	230.5	9.81	0.306	0.554
27	Purple Nucleum	HOR4024 (CILho2250)	PK	2	Purple	33.7	81.3	29.29	13.52	5.71	322.5	14.65	0.343	0.550
28	Black Hulless	HOR4940 (Taastrup c25)	US	6	Purple	36.0	76.1	43.05	14.02	4.98	248.1	7.59	0.285	0.454
29	Lih Dhamta Gal (1459)	N023	NP	6	Purple	33.6	78.2	58.23	12.32	5.65	305.0	1.38	0.412	0.643
	Average S.E.D.					2.6	2.8	11.53	14.49	0.50	93.0	3.82	0.121	0.161

<sup>1</sup> AT, Austria; CN, China; DE, Germany; ET, Ethiopia; IN, India; NP, Nepal; PK, Pakistan; SY, Syria; US, United States of America. <sup>2</sup> TKW, 1000 kernel weight (g); HLW, hectolitre weight (kg hL<sup>-1</sup>); SP25, seed plumpness (% of seeds that remain on a 2.5 mm slotted sieve); PROT, crude protein content (%); BG, beta-glucan content (%); TPC, total phenolic content (mg FA equ. 100 g<sup>-1</sup> dm); TAC, total anthocyanin content (mg C-3-O-glc equ. 100 g<sup>-1</sup> dm); LUT, lutein content (ppm); ZEA, zeaxanthin content (ppm). <sup>3</sup> waxy genotypes.

Table 1. Description of the investigated spring hulless barley genotypes and their least square means for agronomic and quality traits (2007-2009)

### 3.2 Analyses of three consecutive harvests

In 2007, sowing of spring cereals was followed by a six weeks period without rainfall resulting in reduced plant emergence and development of stands with the consequence of lower performance of spring barley in regard to agronomic traits. The establishment of stands was also limited in 2008 due to the absence of sufficient rainfall in March and April. At anthesis (end of May), during the soft dough stage of grain filling and at harvest (mid July), the rainfalls were higher than the average. The climatic conditions of 2009 were comparable with 2007 in spring, whereas sufficient rainfall was available in June and July (Fig. 1). In a previous study, no correlation was found for the precipitation between 2006 and 2007, whereas the mean temperatures showed a strong association. Therefore, the limiting factor for a sustainable spring crop production in eastern Austria is the unequal distribution of rainfall from year to year (Bokore, 2008).

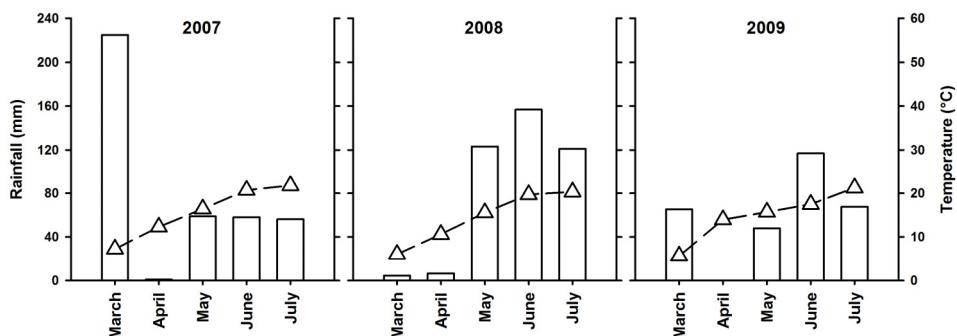


Fig. 1. Precipitation (illustrated as bars) and mean temperature (displayed as triangles) at the experimental site over the investigation period

Mixed model ANOVA revealed significant differences among genotypes (Table 1). Mean TKW and HLW of three consecutive harvests varied within genotypes and ranged from 26 – 46 g and 68 – 81 kg hL<sup>-1</sup>, respectively. Highest TKW on a three years average was found for the white waxy genotype HB803 followed by the black Murasaki Hadaka and the white Lawina. Only a small range within HLW was observed, where highest values were found in the group of purple genotypes (Schwarze Nackte Kraftborn, Schwarze Chinesische and Purple Nudum). Highest seed plumpness (SP25) was recorded for HB803, Hora and the purple Lih Dhanra Gal. Regarding the investigated quality traits, highest protein values were found in the group of purple genotypes (Black Hull-less) and the blue Ederle Sel. Blau.  $\beta$ -Glucan content ranged from 4 to 6% in average, where the highest value belong to Pronghorn, a white waxy genotype followed by Debre Zeit AES 2 (5.8% dm) and Purple Nudum (5.7% dm). TPCf differed between the sample set widely, where the lowest levels were recorded for black genotypes and the highest for the white and blue group. By far the highest TAC was found for the purple genotypes, followed by the blue and black genotypes. LUT and ZEA were quantified using HPLC, and highest levels were found for the black genotypes SNG04, Violaceum 2 and Murasaki Hadaka.

Eticha et al. (2011) recently published a comparison of agronomic and quality traits of 13 diverse anthocyanin pigmented wheat genotypes. Concerning random effects, they found

significant greater variance components for G×Y than that of the year effect for all traits with the exception of heading date.

An excerpt of the gross composition of whole meal samples of the 29 barley genotypes, harvested in 2008 is presented in Table 2. Available starch was the major constituent (43 - 65% dm), whereas the protein content ranged from 8.7 to 13.3% dm.

Earlier studies indicated that hulless barley genotypes are richer in  $\beta$ -glucan compared to their hulled counterparts. In this study, the  $\beta$ -glucan content of the 29 hulless barley genotypes ranged from 3.2 (Hora) to 6.1%. With 4.8 and 5.6%  $\beta$ -glucan the waxy genotypes HB308 and Pronghorn were significantly higher than the average of this sample set, but lower as the purple variety Purple Nudum (Table 2). There seems to be no relationship between kernel size and the content. The variation in the  $\beta$ -glucan content might underlie other genetic variations and is strongly determinated by the variety. Generally, high-amylase and waxy hulless barleys tend to contain more  $\beta$ -glucan than zero-amylase and regular hulless barleys. More precisely, waxy barley cultivars possess higher levels of water and acid extractable  $\beta$ -glucans than regular barleys (Ajithkumar et al., 2005; Izzydorczyk et al., 2000). Our results are consistent with previously published studies (Andersson et al., 2008; Gao et al., 2009).

Total pentosanes (PENT) in barley wholemeal flours varied greatly between 4.1 and 9.2% dm (Table 2). With that wide range, barley genotypes are between the ranges of wheat and rye. Holtekjølen et al. (2006) found generally higher values for hulless genotypes and this observation was later on confirmed within the HEALTHGRAIN diversity screen (Andersson et al., 2008). Water-extractable pentosanes (we-PENT) were a minor constituent ranging from 0.1% (Taiga) to 1.1% (Purple Nudum).

Wholemeal samples were as well extracted for free and bound phenolic acids and the Folin Ciocalteu reagent was used to measure the total phenolic content (TPC). Antioxidant properties were determined from both extracts by estimating the radical scavenging abilities using the stable ABTS radical (thereafter termed TEACf and TEACb) and the reducing abilities of Fe(III). The latter results were expressed as FRAPf and FRAPb (Table 2). Highest TPCf and TPCb were found for the blue genotypes Debre Zeit AES 2 and Addis Ababa 56, followed by the white genotype Lawina. Interestingly, both TPC of the free and the bound fraction ranged between 200 and 500 mg FA equ. 100 g<sup>-1</sup> and thus are at the first glance in contrast with findings from Verardo et al. (2011) and Madhujith & Shahidi (2009) who observed higher phenolic contents in the bound fraction. However, similar observations were found with samples of 2009, when twelve genotypes were milled and the three millstreams were analysed for their content of phytochemicals. Mean average of TPCf was higher than TPCb for the flour (grouped according to seed colour) (Fig. 3 & 4) but not for the shorts and bran fraction, whereas the content of quantified phenolic acids determined by HPLC showed clearly higher contents in the bound fraction for almost all detected phenolic acids. Thus, components co-extracted with the phenolic compounds may have interfered with the test reagent. Similarly, no differences between free and bound phenolics were found in terms of reducing abilities of Fe(III), but with ABTS<sup>+</sup> scavenging capacities.

None of the attributes correlated with TPCf, whereas highly significant ( $p<0.0001$ ) correlations were obtained for the antioxidant properties and the TPC of the bound phenolic fraction, i.e. TEACb correlated with TPCb ( $r=0.74$ ) and with FRAPb ( $r=0.82$ ). Correlation was as well found between FRAPb and TEACb ( $r=0.81$ ). Comparable correlation coefficients for phenolic

ID <sup>1</sup>	PROT <sup>2</sup>	ASH	STARCH	BG	PENT	we-PENT	TPCf	TPCb	FRAPf	FRAPb	TEACf	TEACb	TAC	YP
1	12.0	0.82	54.6	4.5	6.0	0.54	243.3	211.4	22.9	18.8	4.19	25.1	2.06	1.89
2	9.7	1.39	52.8	4.5	5.3	0.54	284.2	261.4	31.2	22.7	4.96	29.8	1.70	0.97
3	8.3	1.36	48.3	4.7	4.1	0.27	216.9	219.2	23.1	18.0	4.30	25.8	1.76	2.08
4	9.6	0.85	54.8	4.4	8.3	0.71	365.5	427.6	40.6	36.3	13.70	41.1	1.21	2.59
5	11.5	0.91	55.2	4.3	5.0	0.44	246.5	210.5	25.7	19.0	4.28	25.7	2.02	1.11
6	13.4	1.01	50.3	4.4	5.8	0.27	306.0	271.4	35.8	28.3	5.56	33.4	1.59	1.92
7	10.7	1.68	49.7	4.1	6.5	0.56	294.5	222.4	35.1	25.1	5.11	30.7	1.46	2.41
8	11.5	0.94	53.8	4.3	5.7	0.43	472.8	231.6	27.4	20.2	3.60	10.8	1.17	2.98
9	8.8	0.83	64.8	3.2	4.5	0.50	249.9	204.5	37.9	19.8	4.76	28.5	0.46	1.74
10	10.6	0.68	59.5	4.6	4.1	0.24	459.3	278.3	39.8	19.4	4.12	24.7	0.93	0.93
11	9.2	0.82	59.1	3.2	4.1	0.24	460.0	241.1	44.2	21.0	3.67	22.0	0.96	0.70
12	10.4	0.93	51.1	4.8	4.7	0.39	514.2	262.9	40.5	28.6	8.30	24.9	1.28	1.91
13	12.3	0.91	49.7	4.7	4.5	0.12	482.6	311.4	45.1	23.6	11.30	33.8	0.46	1.07
14	8.7	0.99	54.8	5.6	4.8	0.49	314.2	233.4	49.2	22.1	9.07	27.2	0.82	2.16
15	10.8	0.90	51.3	4.8	7.8	0.75	490.5	247.9	32.1	31.4	5.53	33.2	2.36	1.49
16	10.8	0.83	54.0	5.3	5.3	0.26	526.7	224.9	35.4	25.4	4.69	28.1	1.04	1.29
17	11.8	4.74	52.3	4.2	5.7	0.30	534.1	251.1	34.7	25.5	4.84	29.1	1.62	1.87
18	10.9	2.57	55.3	4.1	6.2	0.57	512.5	292.2	32.3	26.7	4.89	29.3	0.89	0.81
19	13.6	1.06	52.3	3.8	8.4	0.88	495.4	349.5	27.6	32.3	5.83	35.0	1.04	1.18
20	12.3	1.03	50.1	4.4	8.2	0.42	515.6	254.8	33.8	24.1	4.56	27.4	0.99	1.14
21	9.1	3.73	55.7	4.5	8.5	0.68	364.0	317.9	26.7	31.0	5.82	34.9	1.17	0.89
22	9.5	0.81	44.9	4.4	7.4	0.86	465.4	344.9	28.0	33.7	5.99	36.0	1.25	1.28
23	11.1	0.92	56.3	5.1	6.0	0.52	239.8	261.3	27.5	28.5	5.35	32.1	2.59	1.28
24	12.2	2.00	51.8	4.4	9.2	0.94	311.2	299.8	34.7	34.0	6.09	36.5	3.32	0.93
25	10.6	1.11	55.0	4.8	5.4	0.71	266.1	294.1	27.9	30.4	5.62	33.7	3.57	1.28
26	13.5	1.10	46.8	6.1	9.1	0.42	323.4	367.9	35.3	38.0	13.62	40.9	6.54	1.40
27	10.9	0.84	55.7	4.5	6.9	1.13	262.8	304.3	27.6	30.7	5.52	33.1	3.14	1.19
28	12.4	0.92	42.7	4.5	8.2	0.92	276.4	289.3	27.7	31.5	5.65	33.9	2.30	1.51
29	11.1	1.17	48.7	4.6	8.6	0.78	438.0	363.8	47.3	40.7	12.51	37.5	1.24	1.60
	10.9	1.31	52.8	4.5	6.0	0.52	376.9	277.6	33.7	27.1	6.3	30.5	1.76	1.50
	8.2	0.68	47.7	3.2	4.3	0.10	216.9	204.5	22.9	18.0	3.6	10.8	0.46	0.70
	13.6	4.74	64.8	6.1	7.6	1.10	534.1	427.6	49.2	40.7	13.7	41.1	6.54	2.98
	13.30	70.03	8.52	12.85	16.96	46.73	29.18	19.75	21.24	22.87	45.73	20.23	69.60	37.56

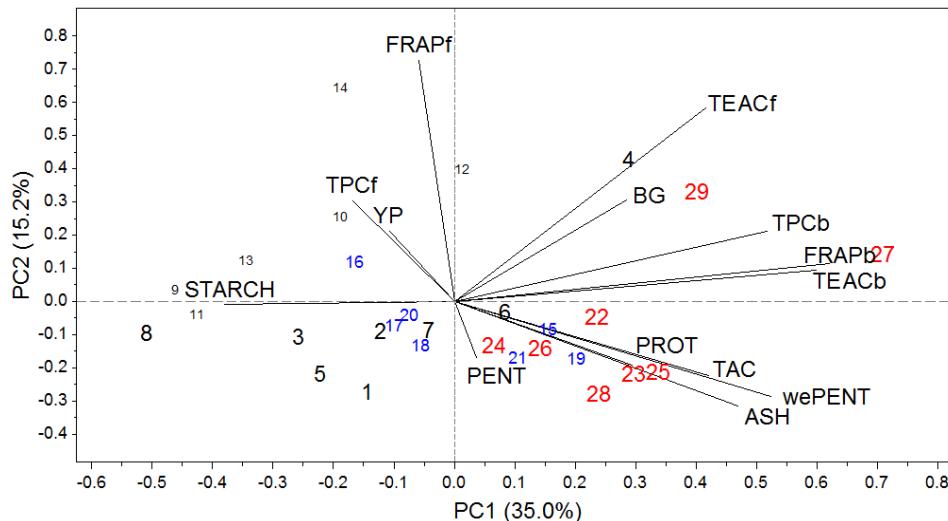
<sup>1</sup>Identification of varieties according to Table 1. <sup>2</sup>PROT, crude protein content (%); ASH, ash content (%); STARCH, total starch content (%); BG, beta-glucan content (%); PENT, total pentosan content (%); we-PENT, water-extractable pentosanes (%); TPCf, total phenolic content of free phenolics (mg FA equ. 100 g<sup>-1</sup> dm); TPCh, total phenolic content of bound phenolics (mg FA equ. 100 g<sup>-1</sup> dm); FRAPf, ferric reducing antioxidant power of free phenolics (mmol Fe(II) kg<sup>-1</sup>); FRAPb, ferric reducing antioxidant power of bound phenolics (mmol Fe(II) kg<sup>-1</sup>); TEACf, Trolox equivalent antioxidant capacity of free phenolics (mmol TAA kg<sup>-1</sup>); TEACb, Trolox equivalent antioxidant capacity of bound phenolics (mmol TAA kg<sup>-1</sup>); TAC, total anthocyanin content (mg C-3-O-glc equ. 100 g<sup>-1</sup> dm); YP, sum of lutein and zeaxanthin content (mg kg<sup>-1</sup>)

Table 2. Chemical composition of the different hulless barley varieties (harvest 2008). All data are expressed on dry weight.

compounds and antioxidant activity were obtained for durum and common wheat (Adom et al., 2003; Siebenhandl et al., 2007), whereas an earlier study with three common wheats reports no correlation between TPC and the radical scavenging capacity (Yu et al., 2002).

### 3.3 Multivariate analysis

Principal components (PCs) analysis revealed four principal components with eigenvalues >1. Variation explained by these four PCs was 35.1, 16.0, 12.2 and 9.1%. The biplot of PC1 and PC2 is demonstrated in Figure 2. From the biplot it can be seen that on the one hand TPC<sub>b</sub>, FRAP<sub>b</sub> and TEAC<sub>b</sub> are highly correlated (acute angles between the respective vectors of similar size), whereas TPC<sub>f</sub>, FRAP<sub>f</sub> and TEAC<sub>f</sub> show no to small correlations. The well-known negative correlation between STARCH and PROT can be seen by the opposite direction of the variable vectors. BG shows no correlation to PROT, STARCH or PENT. The biplot also reveals a certain grouping of genotypes according to their seed colour. White seeded genotypes are mainly located in the upper left part of the plot, black seeded types in the lower left part (with the exception of Violaceum 2), blue aleurone types are located in the lower central part and purple types are located in the lower right part of the plot.

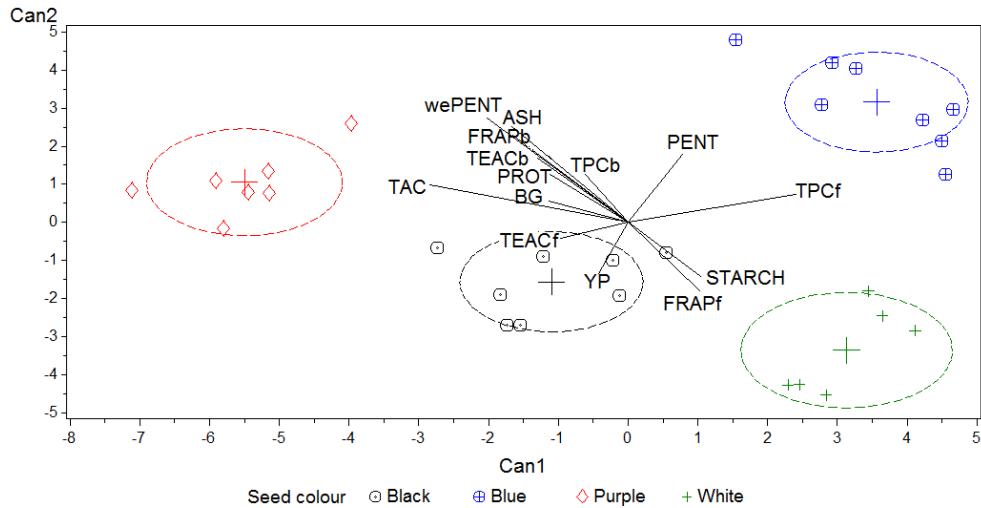


The length of each variable vector is proportional to its contribution to separating the genotypes, and the direction of the vector indicates its relative contribution to PC1 and PC2. Genotypes are presented with their ID as indicated in Table 1, where black letters were used for black genotypes, blue letters for blue, red letters for purple and small black letters for the white ones. Abbreviations used for traits are according to Table 2.

Fig. 2. Biplot of spring barley genotypes and variables

According to the canonical discriminant analysis the seed colour groups can be significantly separated by the data from the chemical analysis. Highly significant first (Can1) and second (Can2) discriminant functions were observed. The plot of discriminant scores is shown in Figure 3. It is obvious that along Can1 three colour groups can be separated, i.e. purple, black and white together with blue. Can1 is mainly determined by TAC and TPC<sub>f</sub>. While

white and blue seeded genotypes are overlapping along Can1 they can be significantly separated along Can2, which is mainly influenced by we-PENT, ASH and FRAPb. Thereby, discriminant analysis confirmed impressively the separation of seed colour groups which was already suggested by PCA.



The length of each variable is proportional to its contribution to separating the seed colour classes, and the direction of the vector indicates its relative contribution to the Can1 and Can2 linear combinations. Abbreviations for variables see Table 2.

Fig. 3. Plot of canonical discriminant scores with 95% confidence regions for the mean (big cross) of each seed colour class

### 3.4 Comparison of milling fractions of different genotypes

The milling process was done using a roller mill. Six flour fractions (B1-B3, C1-C3) from the starchy endosperm were collected and merged to give a straight-run white flour. Brans and shorts were collected separately. The investigated twelve genotypes showed differences in the flour yield. Generally, the flour yields were very low (in average 29%) with higher yields of shorts (65%) and bran (6.6%) (Table 3). White coloured hulless barley varieties had the highest flour yield, whereas the proportion of bran was smallest in the group of black genotypes.

#### 3.4.1 Distribution of phenolic acids

As shown in Fig. 4, all colour groups had in common that the flour fraction showed the lowest content of phenolic compounds and antioxidant properties, followed by the shorts and bran fraction. It is visible from the shapes of the boxes that all colour groups contained genotypes with higher and lower attribute levels. In general, bran levels were approximately three to five times higher than the corresponding flour and all millstreams differed significantly from each other. Within the different milling fractions and using the seed colour as criterion, a few significant differences were found in respect to phenolic

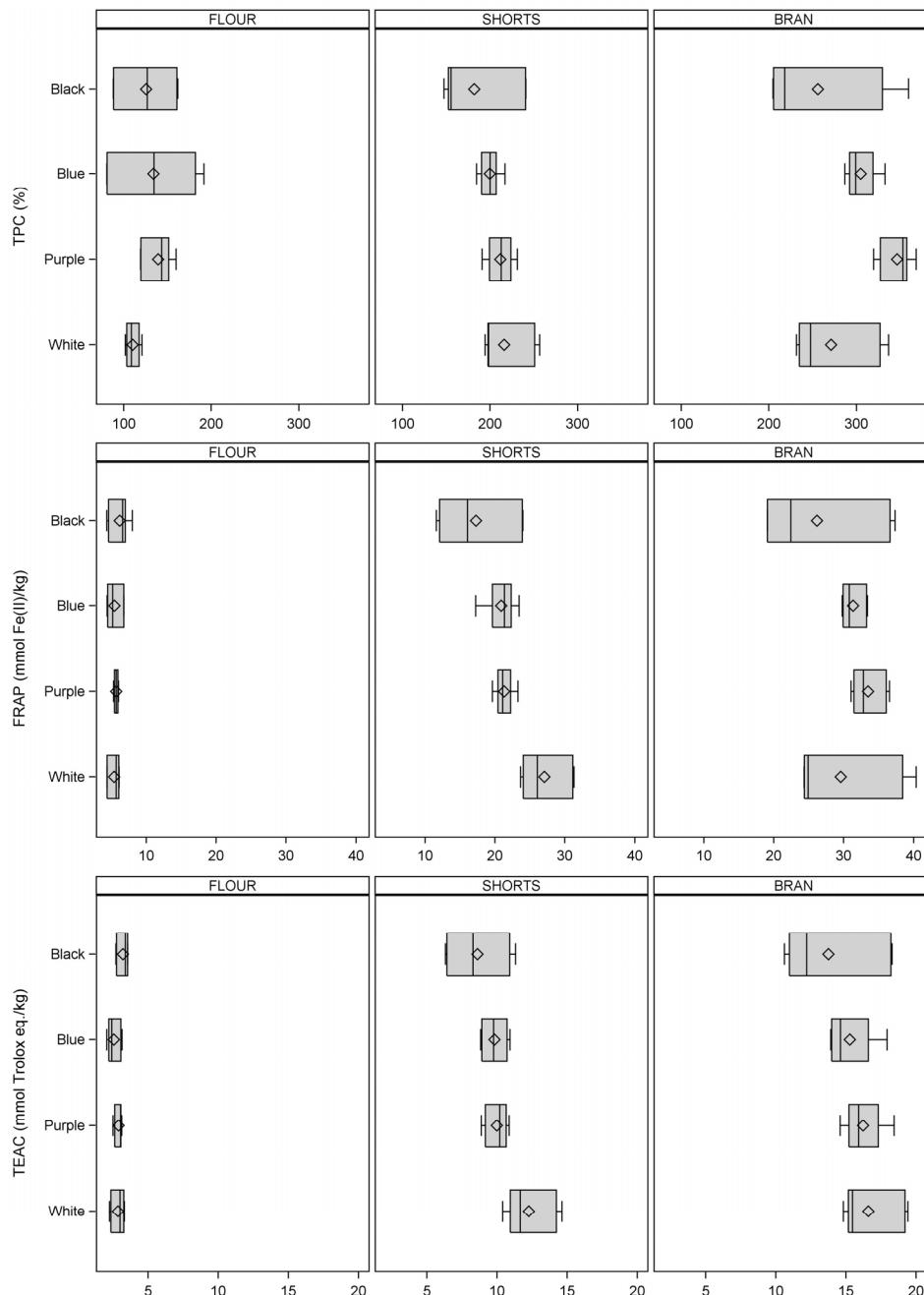


Fig. 4. Variation of free phenolic acids and their antioxidant capacities in regard to grain colour and milling fraction

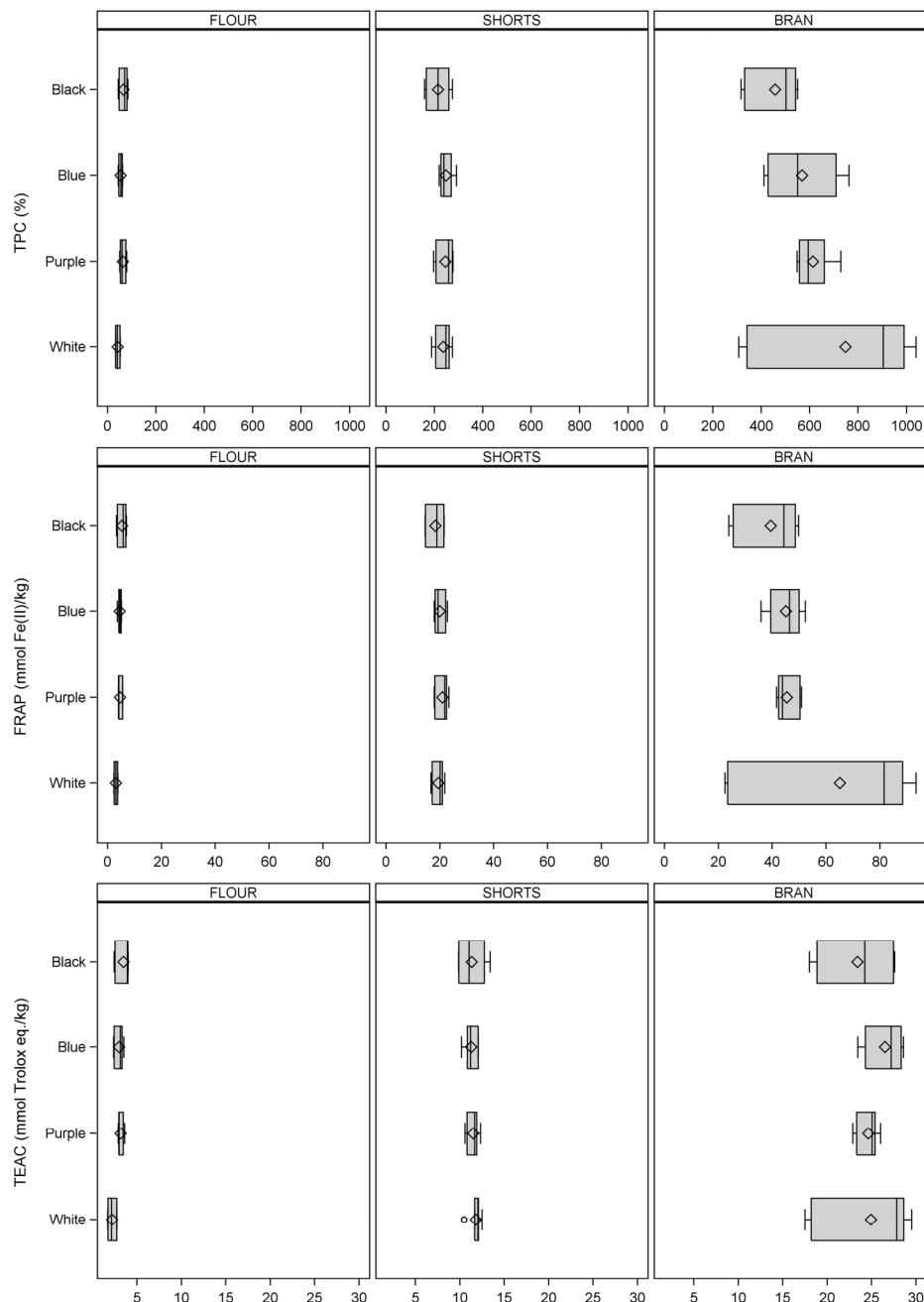


Fig. 5. Variation of bound phenolic acids and their antioxidant capacities in regard to grain colour and milling fraction

ID	Genotype	Flour (%)	Shorts (%)	Bran (%)
4	Violaceum 2	23.5	72.1	4.4
5	Ethiopia 179	26.8	68.3	4.9
7	Black Naked	25.4	72.1	2.5
10	HB803	36.2	54.1	9.7
11	Hora	41.8	54.0	4.2
12	Lawina	36.3	55.7	8.0
15	Himalayense Type 5	25.0	61.0	14.1
17	Addis Ababa 56	24.4	72.6	2.9
21	GHO 1	26.4	67.3	6.3
25	Black Hulless	36.3	59.1	4.6
27	Purple Nudum	21.1	68.8	10.1
29	Lih Dhanra Gal	23.1	68.8	8.1

Table 3. Flour yield of twelve different hulless barley genotypes

content (TPC) and antioxidant properties (FRAP, TEAC), although the ranking varied with the analysis: within the flour fraction, black genotypes differed significantly from the others in respect to their ability to scavenge the ABTS radical ( $p=0.0086$ ). Regarding the bran fractions, a significant difference was found for TPC, where the purple ones yielded in the highest content and differed significantly from the others. Differences between the genotypes were found within each milling fraction with different orders of rankings but following the same trend. Flours with high antioxidative properties or high yield on phenolic substances were genotypes like Black Naked (ID 7) and Addis Ababa 56, whereas flours of the genotypes Himalayense Type 5 and HB803 had the lowest values. Lawina and Black Naked were highest in shorts and bran fraction, and the purple Lih Dhanra Gal in the bran fraction. Low values were found for the black Ethiopia 179 and Violaceum 2.

It is difficult to compare the phenolic acid profile and the antioxidant properties compiled by different researchers, as each group uses a different methodology to extract and measure the effect. Throughout the literature, the phenolic content correlated significantly with the antioxidant capacity. Thus, there is no doubt that phenolic compounds are mainly responsible for the antioxidant properties in cereals (Perez-Jimenez & Saura-Calixto, 2005). Furthermore, a high correlation was as well found for the total phenolic content, determined with the Folin-Ciocalteu reagent and test systems using DPPH- and ABTS radicals as well as the reducing ability as in the FRAP assay.

Sharma & Gujral (2010) estimated the phenolic content in barley ranging from 2.14 to 2.36  $\mu\text{g FA g}^{-1}$  flour and 3.57 – 5.02  $\mu\text{g g}^{-1}$  in bran. Madhujith et al. (2006) reported a range of 0.17 to 6.26 mg FA  $\text{g}^{-1}$ , depending on genotype and fraction, and Menga et al. (2010) found varying levels of TPC and TEAC, when comparing a methanolic extract with an extract after enzymatic digestion.

Residues after the extraction of the free phenolic compounds were used to isolate the bound phenolic compounds after an alkaline hydrolysis. As it was observed for the free phenolic compounds, differences were found between the milling fractions, where the flour fractions were lowest in the phenolic content and the antioxidant properties (Fig. 5). Comparing the four colour groups, significant differences were only found for the flour fraction, whereas the

shorts and the bran fraction did not differ in any of the investigated attributes. It is the group of white genotypes which was lowest, followed by the blue, purple and black ones. The more detailed examination of differences between the genotypes revealed a similar picture as was already observed for the free phenolic compounds. Flours with high phenolic content and antioxidant properties derive from the genotypes Violaceum 2, Black Naked (ID 7) and Purple Nudum with a three-fold difference to genotypes with lower expression of these attributes, e.g. Lawina and HB803. In compliance with the flour fractions, the genotype Purple Nudum showed high levels of phenolic compounds and antioxidant properties in the shorts fractions. A different picture was observed when genotypes were classed by the bran fraction. Here, the white genotypes Hora and HB803 exceeded the investigated set of genotypes two- to threefold in terms of FRAP and TPC levels. The occurrence of higher levels of bound phenolic compounds is in agreement with Nordkvist et al. (1984), Verardo et al. (2008) and Madhujith & Shahidi (2009).

In addition to the determination of the TPC with the spectrophotometric method, extracts were as well analysed by HPLC in order to quantify individual phenolic acids. The phenolic acids quantified in this study included 4-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid and ferulic acid (Table 4). Generally, free phenolic acids comprised a very small proportion of the quantified phenolic acids. Highest content was found for the blue genotype Himalayense Type 5. Bound phenolic acid content made up more than two thirds of the total content. Ferulic acid was by far the abundant phenolic acid and the difference between the flour and bran fraction was in average 10fold. Highest content was found in the waxy HB803 and Purple Nudum, whereas Lawina and Ethiopia 179 contained fewest of all. *p*-Coumaric acid was the second phenolic acid and was significantly higher in HB803 and Hora.

### 3.4.2 Distribution of anthocyanins

Bran, shorts and flour fractions of the black, purple and blue genotypes were screened and compared for their total content of anthocyanins (TAC) and their individual anthocyanins. In general, a sharp decline in the content of anthocyanins was observed with the increase in endosperm in the millstream. Black Hull-less (ID 25) and Purple Nudum were the only genotypes which contained malvidin-3-O-glucoside and apigeninidin. Sorghum is the only plant known to contain significant quantities of luteolinidin and apigeninidin (both 3-deoxyanthocyanidins), which are not commonly found in higher plants. First indications by their existence were given by Nip & Burns (1969; 1971) who isolated apigeninidin and luteolinidin from the seeds of red and white sorghum varieties. Both 3-deoxyanthocyanidins are also reported as the major anthocyanidins from black sorghum varieties (Awika et al., 2004). High contents of cyanidin-3-O-glucoside were found as well in Purple Nudum. Delphinidin was dominant in the black genotype Violaceum 2 (Table 5) but also present in purple genotypes like Black Hull-less (ID 25), Purple Nudum and Lih Dhanra Gal. Kim et al. (2007) reported that cyanindin 3-glucoside was predominant in the purple group, followed by peonidin-3-glucoside and pelargonidin 3-glucoside, whereas delphinidin 3-glucoside was highest in the blue and black group. Thus, our results basically display the same observations. The black 6-rowed genotype Violaceum 2 was salient in respect to the position in the biplot (Fig. 2), as it was displayed in the upper right corner together with the purple representatives Lih Dhanra Gal and Purple Nudum. It is therefore an apposite argument to question the taken classification as the stem of the name contains as well an indication for

purple colour. Probably, the kernels appeared black due to the high content of anthocyanins or the latter have been overlaid by some other darker pigments. On the other hand, the tested Violaceum 2 line could be the result of outcrossing between a purple and a black genotype during a cycle of multiplication.

		ID <sup>1</sup>		Free/Bound		Fraction <sup>2</sup>				ID		Free/Bound		Fraction						
		B	n.d.	4-OH-benzoic acid	vanillic acid	p-coumaric acid	ferulic acid	cafeic acid	B	30.3	2.6	0.8	5.8	8.2	S	29.6	2.5	0.4	8.6	14.1
4	5	B	n.d.	1.4	n.d.	5.0	n.d.		12	15	17	25	27	B	30.3	2.6	0.8	5.8	8.2	
		S	n.d.	1.1	0.1	4.0	n.d.							S	29.6	2.5	0.4	8.6	14.1	
		F	5.2	2.0	0.1	3.3	6.6							F	15.2	1.1	0.2	5.4	7.1	
		B	6.6	25.6	74.7	1854.1	10.6							B	11.9	15.9	82.3	887.1	17.4	
		S	4.8	11.2	10.7	852.3	3.9							S	5.0	8.2	17.9	639.5	2.5	
		F	0.6	3.4	3.2	212.3	1.3							F	1.2	1.0	2.9	67.8	0.4	
5	7	B	1.6	1.5	n.d.	5.9	n.d.							B	12.9	3.4	0.3	5.8	3.9	
		S	20.4	3.4	5.2	5.4	19.5							S	9.6	1.6	0.3	8.3	9.2	
		F	10.4	0.1	2.1	1.4	6.9							F	5.9	0.6	0.4	3.7	6.1	
		B	6.8	14.0	27.9	936.8	8.1							B	4.7	11.3	39.0	1342.3	23.2	
		S	4.1	8.2	7.9	510.3	3.6							S	5.4	9.0	11.4	707.5	3.0	
		F	0.6	1.7	1.8	110.2	0.7							F	n.d.	1.0	1.5	92.7	1.1	
7	10	B	4.0	9.3	1.8	20.7	n.d.							B	21.6	2.1	0.7	7.3	5.8	
		S	1.2	8.0	0.9	4.1	n.d.							S	22.0	0.7	0.1	7.0	9.8	
		F	1.3	1.2	0.5	4.6	4.4							F	14.3	0.2	0.4	4.3	10.2	
		B	10.1	28.1	353.4	1534.5	14.0							B	12.1	27.6	170.3	1587.7	25.5	
		S	6.2	12.9	22.2	790.3	14.3							S	3.1	5.3	14.0	563.7	12.5	
		F	0.6	2.2	3.9	184.7	0.17							F	0.3	1.5	3.6	135.6	0.7	
10	11	B	1.1	3.8	4.7	6.2	2.3							B	1.3	8.2	4.2	15.9	n.d.	
		S	3.2	8.8	1.7	3.4	4.0							S	0.6	11.5	1.2	10.1	n.d.	
		F	n.d.	2.7	0.7	1.9	3.9							F	n.d.	1.4	0.5	3.9	n.d.	
		B	14.1	43.7	1733.6	2311.2	26.3							B	13.8	22.7	38.8	1618.6	7.6	
		S	5.8	11.2	167.4	759.7	4.9							S	5.8	10.2	14.9	867.1	4.4	
		F	1.1	1.1	17.7	74.0	0.7							F	0.5	2.0	2.8	142.2	0.9	
11	bound	B	5.3	14.2	11.2	18.0	6.3							B	4.3	6.6	6.0	16.6	n.d.	
		S	2.0	5.8	2.1	10.9	19.9							S	2.1	6.9	1.5	11.7	n.d.	
		F	0.2	1.9	0.7	2.4	10.7							F	0.1	2.0	0.7	5.2	n.d.	
		B	11.3	44.7	1427.7	1996.1	20.9							B	13.2	26.2	52.2	2025.2	6.5	
		S	4.2	11.1	87.3	805.9	6.2							S	6.8	13.8	11.0	997.6	2.8	
		F	0.7	2.0	15.2	102.6	0.7							F	1.4	3.9	2.5	220.2	1.0	

<sup>1</sup> ID number according to Table 1. <sup>2</sup>F, flour fraction; S, shorts; B, bran fraction.

Table 4. Content and composition of free and bound phenolic acids (in ppm) in the different millstreams of barley genotypes

ID <sup>1</sup>	Fraction <sup>2</sup>	Delph-3-O-glc <sup>3</sup>	Cyan-3-O-glc	Delph-3-O-rham	Pel-3-O-glc	Mal-3-O-glc	Luteo	Api	Cyan	Delph	Peo	Mal
4	B	+ <sup>4</sup>	+		•		+	+	•	+++	•	
	S	+	•	•	•		+	+	•	++	•	•
	F	•	•	•	+		•	•	+	+	•	•
5	B	•	•		•		•		•	•	•	•
	S	•	•		•		•		•	•	•	•
	F	•	•		+				•	•	•	•
15	B	+	+	•	•		+	•	•	++		
	S	•	•	•	•		+	•	•	+		
	F	•	•		+		+	•	+	•	•	•
17	B	+	•	•	•		+	•	•	++	•	•
	S	+	•	•	•			•	•	+	•	•
	F	•	•		•			•	+	•	•	•
21	B	+	•	•	•			+	+	++	•	
	S	•	•	•	•			•	•	+	•	
	F	•	•		•			•	•	•	•	
25	B	+	+	•	+	+	++	+	+	+++	•	
	S	•	+	•	•	+	+	+	+	++	•	
	F	•	•		+		•	+++	+	•	•	•
27	B	•	+++		++	++	+	+	+	++	+	•
	S	•	++		+	+	+	+	•	+	+	•
	F	•	+		+		•	+++	•	•	•	•
29	B	+	•	•	•		•	+	•	++	•	
	S	•	•	•	•		•	•	•	+	•	
	F	•	•	+	•			•	+	•	•	•

<sup>1</sup> ID number according to Table 1. <sup>2</sup>F, flour fraction; S, shorts; B, bran fraction. <sup>3</sup>Delph, Delphinidin; Cyan, Cyanidin; Pel, Pelargonidin; Mal, Malvidin; Luteo, Luteolinidin; Api, Apigeninidin; Peo, Peonidin. <sup>4</sup>• = in traces, + = 1.0 – 3.0 ppm, ++ = 3.1 – 5.0 ppm, +++ = > 5.1 ppm.

Table 5. Distribution of various anthocyanins (ppm) within the three milling fractions of barley genotypes

#### 4. Conclusion

The basic function of food to satiate and to provide macro- and micronutrients has become in the Western industrialised countries less and less important. With increasing prosperity and/or educational knowledge additional attributes are demanded by consumers who search for sensorial sensations or tie their consumption of food with social prestige or ethical reasons. Among these supplementary functions of food the health-awareness of consumers

is used to a high degree by the food and marketing industry for the promotion of existing and newly developed food products.

Thus, scientists work together interdisciplinary to study the biochemical mechanisms of food in order to quantify possible health benefits. Since the late 1980s, most studies have mainly focused on the polysaccharide moiety, while the potential role of whole grain antioxidants was considered less up to now. The newly introduced term "dietary fibre-antioxidants" assumes that the beneficial effects attributed to the cereal dietary fibre are not only due to the polysaccharide moiety, but also to the associated polyphenolic compounds (Vitaglione et al., 2008).

These phytochemicals as well as the insoluble fraction of dietary fibres are located in the outer parts of the kernel and thus are removed within the milling process when gaining refined white flour. Barley grains differ considerably from those of other cereals in regard to their chemical constitution, as a large proportion of the soluble dietary fibre is located in the endosperm. Hence, this valuable fraction will not be lost by using normal milling practise for the production of flour. A recent experiment showed that the baking quality of hulless barley flour is sufficient to bake pure barley bread. Thus, hulless barley is an interesting alternative for commonly used grains and could contribute to a higher diversity in human nutrition (Kinner et al., 2011).

In this context, the phytochemical content and total antioxidant capacity of 29 hulless barley genotypes with different seed colours were investigated. Significant differences in the content of anthocyanins and carotenoids, and in reducing power were observed between genotypes, and between the different millstreams of the respective plant material. From the results of this study, and from other studies previously carried out (Siebenhandl et al., 2007; Bokore, 2008; Menga et al., 2010) it can be concluded that on the one hand genetic and environmental factors have a major impact on the phytochemical profile and the antioxidant activity, but on the other hand also postharvest treatments such as fractionation of the raw material can have a major influence. Thus, it is anticipated that the development of barley cultivars for human consumption should focus on breeding for specific end uses (Hang et al., 2007). Due to significant environmental influences on many phytochemicals genotypes with a stable performance across a wide range of environments are necessary to assure a continuous quality for food processors. Before a specific breeding programme can be started, the already existing germplasm has to be evaluated to identify possible genotypes which could serve as donors of valuable traits in cross breeding programmes.

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## **Part 4**

### **Antioxidative Properties**



# Herbal Antioxidants as Rejuvenators in Alternative Medicine

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## 1. Introduction

Life expectancy has nearly doubled during the 20th century. This dramatic increase in the aging population presents with an enormous challenge to maintain a healthy old age. A comprehensive solution to address this important problem is compounded by a multitude of factors that contribute to the accumulation of damage to macromolecules, cells, tissues and organs during aging. The rejuvenation strategies, currently being employed with a targeted approach, to reverse or repair the damage associated with aging have only yielded partial benefits. The complexity of the aging process highlights the need for a holistic approach, as recommended by the alternative systems of medicine, to prevent the progressive deterioration of the aging cells. Herbal antioxidants have been successfully employed as rejuvenators, for several centuries in the Indian systems of alternative medicine. This chapter describes the concepts behind the application of herbal antioxidants to promote longevity and scientifically validate their potential by highlighting the molecular mechanisms underlying the biological activity of the phytochemicals in these herbs.

## 2. Oxidative and nitroxidative stress – major etiological factors in aging phenomenon

Oxidative stress is widely recognized to play a causal role in the aging phenomenon. Every day metabolic activities including breathing could lead to the exposure of cells to biochemical substances such as free radicals and the gradual accumulation of free radicals can induce cellular damage. According to the free radical theory of aging, which is based on Harman's hypothesis, continued creation of free radicals influenced by a multitude of factors associated with routine life style is responsible for the aging process (Harman, 1972). Normally, the antioxidant mechanism fails either due to overproduction of free radicals or insufficient activities of scavenging enzymes, or both causing lipid peroxidation. Since lipid peroxidation is a self-propagating chain reaction the initial oxidation of only a few lipid molecules can result in significant tissue damage and disease.

Moreover, it has already been established that superoxide radicals that are generated during normal cellular respiration could eventually lead to nitration of proteins (Figure 1). Nitration of tyrosine, a posttranslational modification of immense functional importance, occurs by the substitution of a nitro group in place of hydrogen on carbon 3 in the phenolic

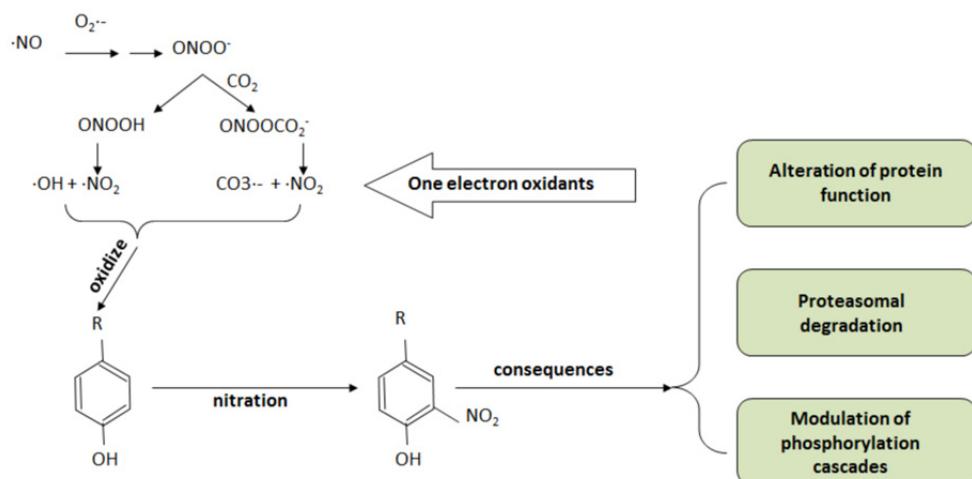


Fig. 1. Protein nitration and its consequences

ring of a tyrosine residue (Radi, 2004). In vivo nitration mostly depends on superoxide (O<sub>2</sub><sup>-</sup>) and nitric oxide (NO) radicals, generated during routine cellular activity, as they react to form peroxynitrite (ONOO<sup>-</sup>). Peroxynitrite eventually leads to the formation of one-electron oxidants (CO<sub>3</sub><sup>-·</sup>, OH, NO<sub>2</sub>) which can oxidize the phenolic ring of tyrosine to yield the tyrosyl radical (Tyr<sup>·</sup>). The addition of NO<sub>2</sub> to Tyr<sup>·</sup> in a radical–radical termination reaction results in tyrosine nitration.

Although the nitration of tyrosine occurs at relatively low levels compared to tyrosine phosphorylation, protein nitration can result in serious biological consequences as it can modulate phosphorylation cascades. Nitrated proteins are often targets of proteasomal degradation. Age-related increase in nitrotyrosine has been reported in the hippocampus, which is responsible for spatial memory, in humans with mild cognitive impairment (Butterfield et al., 2007). As the metabolic activity of brain is relatively high, the aberrant electron transfers result in additional free radical formation and subsequent protein nitration in aged brain tissue. Therefore, protein nitration is generally considered as a biomarker for age-related neurodegeneration (Butterfield et al., 2006; Chung and David, 2010). Moreover, nitroxidative damage of neurons can also contribute to age-related deficits in memory function. Thus, oxidative and nitroxidative stress are crucial factors responsible for the aging process.

### 3. Alternative medicine concepts in promoting longevity

Herbal antioxidants are widely used as dietary supplements for promoting longevity in alternative medicine systems of Ayurveda and Siddha, which originate from India (Table 1). These traditional systems of medicine are based on the concept of tridosha theory, according to which vatha, pitha and kapha are the three humors that govern the physiological function of the body. When the normal balance of the three humors is deranged they are known as doshas which ultimately results in pathological consequences during the aging process. A set of applications known as “Kaya Karpam”, which literally means transforming the body

from its fragile state to a stable stone like state, are used to attenuate vatha, pitha or kapha rationalizing their extensive use in complementary and alternative medicines for promoting health and wellness.

S. No.	Common name	Botanical name	Part used
1.	Aloe	<i>Aloe vera</i>	Succulent leaf
2.	Ashwagandha	<i>Withania somnifera</i>	Root
3.	Black nightshade	<i>Solanum nigrum</i>	Fruits and leaves
4.	Cardamom	<i>Elateria cardamom</i>	Fruit
5.	Chaste tree	<i>Vitex negundo</i>	Leaves
6.	Clearing nut	<i>Strychnos potatorum</i>	Seed
7.	Country green	<i>Amaranthus gangeticus</i>	Leaves
8.	Cumin	<i>Cuminum cyminum</i>	Seed
9.	False daisy	<i>Eclipta alba</i>	Leaves
10.	Ginger	<i>Zingiber officinale</i>	Rhizome
11.	Indian acalypha	<i>Acalypha indica</i>	Leaves
12.	Indian Sarsaparilla	<i>Hemidesmus indicus</i>	Root
13.	Indian sorrel	<i>Oxalis corniculata</i>	Leaves
14.	Lemon	<i>Citrus medica</i>	Fruit
15.	Neem	<i>Azadirachta indica</i>	Leaves
16.	Nut grass	<i>Cyperus rotundus</i>	Tuberous root
17.	Red spiderling	<i>Boerhaavia diffusa</i>	Whole plant
18.	Sessile joyweed	<i>Alternanthera sessilis</i>	Leaves
19.	Stonebreaker	<i>Phyllanthus niruri</i>	Whole plant
20.	Triphala	<i>Terminalia chebula</i> <i>Terminalia bellerica</i> <i>Emblica officinalis</i>	Dried fruit Dried fruit Dried fruit
21.	Tulsi / Holy basil	<i>Ocimum sanctum</i>	Leaves

Table 1. Medicinal herbs used as rejuvenators in Indian system of alternative medicine

This concept is similar to one of the central theories of aging which suggests that every day metabolic activities including breathing could lead to the exposure of cells to biochemical substances such as free radicals and antioxidants can be employed to prevent the gradual accumulation of free radical induced cellular damage during aging. Several studies have shown that the life span of an organism can be extended by diminishing oxidative stress (Finkel and Holbrook, 2000). Consistent with this notion, the "Kaya Karpam" herbs extensively used to promote longevity in alternative medicine systems have been found to have significant antioxidant properties. Some of the current research findings also substantiate the use of medicinal herbs, because phytochemicals such as resveratrol, sulforaphanes and curcumin, are considered to possess neurohormetic property because of their ability to protect neurons against injury and disease by stimulating the production of antioxidant enzymes, neurotrophic factors, protein chaperones and other proteins that help cells to withstand stress (Mattson and Cheng, 2006). The subsequent sections describes the mechanism underlying the prophylactic as well as therapeutic effects of some common herbal antioxidants employed in Indian systems of alternative medicine to promote longevity.

#### 4. Common herbal antioxidants used in Indian system of alternative medicine

Triphala, ashwagandha, tulsi, ginger and neem are extensively used in Indian systems of alternative medicine, as they are considered to possess significant rejuvenating properties.

##### 4.1 Triphala

Triphala is an herbal formula consisting of dried fruits from A) *Terminalia chebula*, B) *Terminalia bellirica* and C) *Emblica officinalis*, the three myrobalans, in equal parts (Figure 2). The chebulic and belleric myrobalans belong to the family Combretaceae, while the emblic myrobalan, commonly known as amlaki, belongs to the family Phyllanthaceae. Triphala is widely used as a dietary supplement for promoting longevity in alternative medicine systems of Ayurveda and Siddha (Jagetia et al., 2002). Triphala is classified as a kaya karpam and has already been reported for its significant antioxidant activity (Naik et al., 2005; Hazra et al., 2010). The constituents of triphala were reported to enhance the antioxidant status in aging animal models (Yokozawa et al., 2007; Mahesh et al., 2009). In addition, triphala has been reported to possess several other important medicinal properties (Srikumar et al., 2005; Rasool and Sabina, 2007; Shi et al., 2008) that strengthen its potential to deal with multiple problems associated with aging. Such a multi-faceted activity of triphala is consistent with the complementary and alternative medicine concepts of a holistic approach to restore the normal balance of the three humors and supports its potential to rejuvenate the aging cells.



Fig. 2. Constituents of triphala

##### 4.2 Ashwagandha

Ashwagandha (*Withania somnifera*, Figure 3) belongs to Solanaceae family and has been used for centuries in Indian systems of alternative medicine to treat various ailments. It is commonly known as Indian Ginseng because of its comparable medicinal value to ginseng which is acclaimed for its adaptogenic activity in alleviating stress induced illness. The medicinal properties of ashwagandha are mostly attributed to its tuberous roots whose extracts are widely marketed as an over the counter herbal supplement. Adaptogens, like ashwagandha, are believed to facilitate the maintenance of homeostasis by normalizing physiological as well as biochemical changes induced by stress. The anti-inflammatory (Maitra et al., 2009), anti-cancer (Ichikawa et al., 2006) and immunomodulatory (Bhattacharya and Muruganandam, 2003) activities of ashwagandha rationalize its extensive use in promoting longevity. Ashwagandha's antioxidant activity (Udayakumar et al., 2010) suggests that a common molecular mechanism may be responsible for its diverse biological effects.



Fig. 3. Ashwagandha herb

#### 4.3 Tulsi

Tulsi (*Ocimum sanctum*, Figure 4), a medicinal herb classified as a kaaya karpam, is cultivated in India for medicinal as well as religious purposes. It is commonly known as "holy basil". It is an aromatic plant which belongs to the family Lamiaceae. Due to its immense medicinal value it is extensively used in Indian systems of alternative medicines to heal a number of ailments. Several studies have reported the significant antioxidant activity of tulsi (Kelm et al., 2000; Yanpallewar et al., 2004; Samson et al., 2007). Tulsi treatment enhanced the antioxidant status in discrete regions of brain (Samson et al., 2007) and the phytochemical contents of tulsi have been suggested to be responsible for this effect. The stress alleviating potential of *Ocimum sanctum* extract is also reflected by its efficacy in attenuating the immunological changes induced by noise exposure (Archana and Namasivayam, 2000). In addition, tulsi has been reported to possess several other important medicinal properties (Singh et al., 1996; Mediratta et al., 2002; Adhvaryu et al., 2007; Kim et al., 2010) that strengthen its potential to deal with aging and oxidative stress. Thus, Tulsi is capable of promoting a healthy aging due to its multiple but complimentary and synergistic medicinal properties which can combat a diverse spectrum of disorders associated with aging.



Fig. 4. Fresh and shade dried tulsi

#### 4.4 Ginger

Ginger (Figure 5) is a widely used herbal supplement, often used in a number of culinary preparations all around the world. It is a rhizome of the herb *Zingiber officinale*, which belongs to the family Zingiberaceae. Due to its diverse healing properties it is extensively used in alternative medicines such as Chinese medicine, Ayurveda, Siddha and Unani. The Indian systems of medicines recommend the use of ginger as a kaya karpam or rejuvenator. It is used both in fresh and dried form to treat nausea and vomiting (Jewell, 2003; Borrelli et al., 2005; Chaiyakunapruk et al., 2006), osteo and rheumatoid arthritis (Srivastava and Mustafa, 1992; Bliddal et al., 2000; Altman and Marcussen, 2001), diabetes mellitus (Sekiya et al., 2004; Goyal and Kadnur, 2006; Heimes et al., 2009), indigestion and some cardiovascular disorders (Jiang et al., 2005; Nicoll and Henein, 2009). Various studies have demonstrated the anti-oxidant (Kabuto et al., 2005; Ahmed et al., 2008; Pan et al., 2008; Dugasani et al., 2010), anti-inflammatory (Young et al., 2005), anti-cancer (Jiang et al., 2005; Rhode et al., 2007; Shukla and Singh, 2007) and anti-microbial (Park et al., 2008; Singh et al., 2008b) properties of ginger. These multiple biological properties of ginger support its clinical application as an herbal rejuvenator.

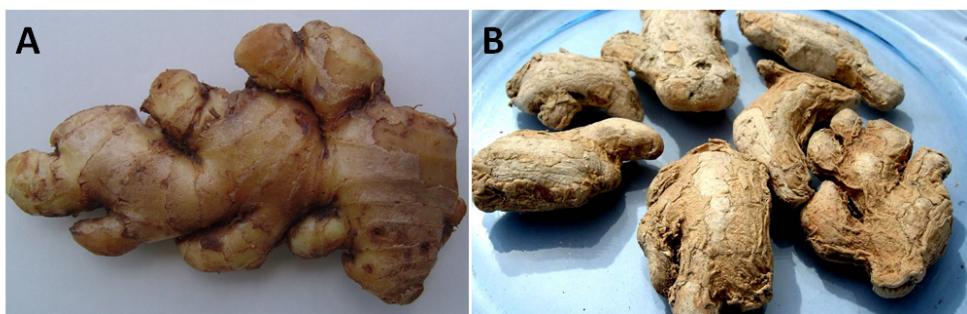


Fig. 5. Fresh and dried ginger

#### 4.5 Neem

*Azadirachta indica*, commonly known as neem, belongs to the family Meliaceae, is a large ever green tree with immense medicinal applications. Various parts of the neem tree such as leaves (Figure 6), flowers, seeds, roots and bark are used as traditional remedies for a number of ailments in the Indian systems of alternative medicine. However, the wide ranging medicinal value of the neem leaves stands out in comparison with other parts of the tree. Various studies have indicated that the neem leaves have anti-microbial (Wolinsky et al., 1996; Thakurta et al., 2007; Zhang et al., 2010), anti-inflammatory (Okpanyi and Ezeukwu, 1981; Thoh et al., 2010), analgesic (Khattak et al., 1985), anti-diabetic (van der Nat et al., 1991), immunomodulatory (Ray et al., 1996), anti-oxidant (Arivazhagan et al., 2000) and anti-cancer (Miller et al., 1992; Akudugu et al., 2001) properties. Due to its numerous pharmacological activities neem leaves are used as a kaya karpam to promote longevity.



Fig. 6. Neem leaves

## 5. Phytochemicals responsible for the therapeutic effects of herbal antioxidants

Triphala has been used for centuries in Indian systems of alternative medicine as a dietary supplement for rejuvenation due to its prophylactic and therapeutic properties (Jagetia et al., 2002). The three constituents of triphala have distinct as well as common phytochemicals that are considered to complement each other and act in a synergistic manner. *Terminalia chebula* is reported to contain ascorbic acid, gallic acid, tannic acid, syringic acid and epicatechin while *Terminalia bellerica* has gallic acid, tannic acid and ascorbic acid (Singh et al., 2008a). *Embllica officinalis* is a rich source of vitamin C and also has flavonoids, kaempferol, ellagic acid and gallic acid (Singh et al., 2008a).

Several active compounds of ashwagandha, especially steroidal lactones, have been isolated, in the quest to identify the constituent(s) responsible for its wide ranging clinical application in alternative medicine. A HPLC analysis demonstrated the presence of Withanolide-A, withanone, withaferine-A, withastramonolide, 27-hydroxywithanone, withanoside, physagulin (Dhar et al., 2006). Although active compounds like withaferin A and withanisoide D are considered as major players, it is still not clear whether any one of these compounds are responsible for the biological activities of ashwagandha or whether they exert a synergistic action when administered in their natural form.

Basils contain a wide range of essential oils rich in phenolic compounds (Phippen and Simon, 2000) and a wide array of other natural products including polyphenols such as flavonoids and anthocyanins. Epidemiological studies have suggested positive associations between the consumption of phenolic-rich foods or beverages and the prevention of disease (Scalbert and Williamson, 2000). These effects have been attributed to antioxidant components such as plant phenolics, including flavonoids and phenylpropanoids among others. One of the widely used medicinal herb among the basils is *Ocimum sanctum* Linn, which is commonly known as holy basil or "Tulsi". *Ocimum sanctum* has been reported to exhibit several medicinal properties and the active principles present in *Ocimum* species such as rosmarinic acid, lithospermic acid, eugenol, methyleugenol, urosolic acid, h-caryophyllene, methylchavicol, linalool, 1,8-cineole phenolics and flavonoids such as orientin, vicenin etc have been attributed to be responsible for its diverse medicinal activities (Hase et al., 1997; Kelm et al., 2000).

Ginger has a rich array of useful bio-active compounds. The antioxidant property of ginger can be attributed to the phytochemicals present in ginger which includes volatile compounds such as sesquiterpenes (curcumene  $\beta$ -phellandrene, geraniol, 1,8-cineole, citral, terpineol, borneol, linalool, nerol) and monoterpenoid hydrocarbons ( $\alpha$ -zingiberene,  $\beta$ -sesquiphellandrene,  $\alpha$ -farnesene,  $\beta$ -bisabolene,  $\alpha$ -curcumene) and non-volatile compounds like gingerols, shogaols, paradols, and zingerone (Gong et al., 2004; Jolad et al., 2004).

A large number of medicinally useful phytochemicals have been isolated and identified from the neem leaves. They include azadirachtin, azadirachtin-A,  $\beta$ -sitosterol, hyperoside, isoazadirolide, nimbaflavone, nimbadiol, nimbinene, nimbolide, quercetin, quercitrin, rutin, vilasanin, 3-acetyl-7-tigloyl-lactone-vilasinin (Akhila and Rani, 1999; Siddiqui et al., 2004). These wide ranging bio-active compounds of neem leaves can be considered to be responsible for its multiple biological / medicinal applications.

## 6. Signaling molecules that mediate the biological effects of herbal antioxidants

Recent studies have identified some of the molecular targets of triphala. Activation of ERK and p53 were found to mediate the growth inhibitory effects of triphala in pancreatic cancer (Shi et al., 2008). Chebulagic acid isolated from *Terminalia chebula* has been reported to inhibit COX-2/5-LOX (Reddy et al., 2010). Apart from this, triphala has been shown to possess significant antioxidant (Naik et al., 2005; Mahesh et al., 2009; Hazra et al., 2010) and nitric oxide scavenging activity (Jagetia et al., 2004). In addition, the anti-cancer (Deep et al., 2005; Sandhya and Mishra, 2006; Shi et al., 2008), immunomodulatory (Srikumar et al., 2005) and anti-inflammatory (Rasool and Sabina, 2007) activity of triphala directly targets the common health problems associated with aging. The selective cytotoxic and apoptotic activity of triphala on cancer cells while sparing the adjacent normal cells (Shi et al., 2008) further strengthens its compelling role as a dietary supplement to promote longevity.

The biological effects of ashwagandha have been shown to be mediated by several signaling molecules. The anti-cancer activity of ashwagandha has been attributed to suppression of NF- $\kappa$ B (Kaileh et al., 2007) and inhibition of notch-1 signaling (Koduru et al., 2010). Withaferin A, an active principle in ashwagandha, has been reported to cause FOXO3a- and Bim-dependent apoptosis in breast cancer cells (Stan et al., 2008) and inhibit Hsp90 chaperone activity in pancreatic cancer cells (Yu et al., 2010). Ashwagandha's anti-inflammatory activity has been attributed to the inhibition of nitric oxide production and iNOS expression and subsequent downregulation of NF- $\kappa$ B (Oh et al., 2008). Alkaloids of ashwagandha have been reported to enhance neurite outgrowth and spatial memory (Tohda and Joyashiki, 2009). Deceleration of senescence has been reported in fibroblasts (Widodo et al., 2009). Such a broad spectrum of biological effects suggests that a common systemic mechanism regulated by the antioxidant property of ashwagandha underlies its adaptogenic activity in promoting a healthy aging.

The antioxidant activity of tulsi has been demonstrated by its attenuation of stress-induced changes in antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase and endogenous antioxidants such as reduced glutathione (Samson et al., 2007). Moreover, *Ocimum sanctum* was effective in scavenging the DPPH, superoxide, nitric oxide, hydroxyl and ABTS radicals in a dose dependent manner (Samson et al., 2007). The

interruption of the free-radical chain of oxidation by the hydrogen donated from the phenolic compound's hydroxyl groups, thereby forming stable free radicals, which do not initiate or propagate further oxidation of lipids, is probably responsible for the antioxidant property of Tulsi. The anti-oxidant property of *Ocimum sanctum* has also been confirmed by other reports (Devi and Ganasoundari, 1999). Furthermore, flavonoids are useful exogenous agents in protecting the aging brain, other organs and tissues of the body against free-radical induced damage. It appears that the phenolic and flavonoid contents of *Ocimum sanctum* are responsible for the attenuation of oxidative damage. *Ocimum sanctum* treatment attenuated noise-induced changes in levels of neurotransmitters such as dopamine and serotonin in brain (Samson et al., 2006). *Ocimum sanctum* has also been reported to normalize the stress induced membrane changes in the hippocampus and sensorimotor cortex (Sen et al., 1992). These reports indicate that *Ocimum sanctum* is a non-specific anti-stressor. Apart from these, treatment with *Ocimum sanctum* extract decreased the expression of PCNA, GST-*pi*, Bcl-2, CK and VEGF, and increased the expression of Bax, cytochrome C, and caspase 3 in gastric carcinoma (Manikandan et al., 2007), suggesting that these molecules may be potential targets that mediate the pharmacological effects of *Ocimum sanctum*.

The active principles of ginger such as 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol demonstrated significant free radical scavenging activity particularly against DPPH radical, superoxide radical and hydroxyl radical in in-vitro assays (Dugasani et al., 2010). Moreover, the efficacy of ginger in protecting the cells from oxidative stress was indicated by the attenuation of lindane-induced lipid peroxidation, and modulation of reduced glutathione (GSH), glutathione peroxidase (Gpx), glutathione reductase (GR), and glutathione-S-transferase (GST) after dietary treatment with ginger (Ahmed et al., 2008). The active compound 6-shogaol was found to downregulate iNOS and COX-2 gene expression by inhibiting the activation of NF- $\kappa$ B (Pan et al., 2008). Zingerone, another major active compound of ginger, has been reported to modulate age-related NF- $\kappa$ B activation through the MAPK signaling pathway (Kim et al., 2010). Another study indicated that 6-gingerol has been reported to inhibit NO synthesis and protect against peroxynitrite-mediated damage (Ippoushi et al., 2003). Apart from these, several studies investigating the anti-cancer potential of ginger have reported the involvement of signaling molecules such as NF- $\kappa$ B, TNF- $\alpha$ , AKT, ERK1/2, p38 MAPK, MMP-2 and MMP-9 in mediating the cellular responses to various phytochemicals of ginger (Rhode et al., 2007; Habib et al., 2008; Hung et al., 2009; Yodkeeree et al., 2009; Kim et al., 2010).

The biological effects of the active principles present in neem are regulated through various signaling molecules. Increase in expression levels of HMOX1, AKR1C2, AKR1C3, and AKR1B10 was observed in prostate cancer cells after treatment with an ethanolic extract of neem leaves (Mahapatra et al., 2011). Inhibition of PC-3 cell proliferation and Bcl-2 expression was observed after neem treatment (Priyadarsini et al., 2009). A methanolic extract of neem leaves inhibited NF- $\kappa$ B activity in cultured human leukemia cells (Schumacher et al., 2011). Azadirachtin, an active principle of neem, has been reported to activate transcription factors like CREB, Sp1, NF- $\kappa$ B (Thoh et al., 2011). The viability of human cervical cancer HeLa cells were suppressed by azadirachtin and nimbolide by p53-dependent p21 accumulation and down-regulation of cell cycle regulatory proteins cyclin B, cyclin D1 and PCNA (Priyadarsini et al., 2010). Apart from this, the antioxidant potential of neem leaves were demonstrated by the reduction of DPPH, ABTS, superoxide, hydroxyl, and nitric oxide radicals by different extracts of neem leaves (Manikandan et al., 2009).

## 7. Conclusion

Emerging studies on herbs employed as rejuvenators, for centuries in Indian system of alternative medicine, validates their clinical application by highlighting the mechanistic principle underlying the biological effects of these herbs. Analytical reports on triphala, ashwagandha, tulsi, ginger and neem suggest that these medicinal herbs have a rich array of a diverse spectrum of bio-active compounds. The abundance of phytochemicals with antioxidant properties, such as phenolics, flavonoids and carotenoids, may be held responsible for the rejuvenating activity of these medicinal herbs. The molecular targets of major phytochemicals identified in each of the herbal antioxidants discussed in this chapter suggest that NF- $\kappa$ B signaling pathway plays an important role in regulating the biological activity of the herbal rejuvenators. In addition, it is also evident that several other signaling pathways, which differ for each of these herbs, also mediate their biological effects. Thus, the medicinal herbs employed to promote longevity in Indian systems of alternative medicine have multiple pharmacological effects. However, the antioxidant property of these herbs seems to have a major role in determining their rejuvenating potential.

## 8. Acknowledgements

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# **Assessment of the Antidiabetic Potential of an Aqueous Extract of Honeybush (*Cyclopia intermedia*) in Streptozotocin and Obese Insulin Resistant Wistar Rats**

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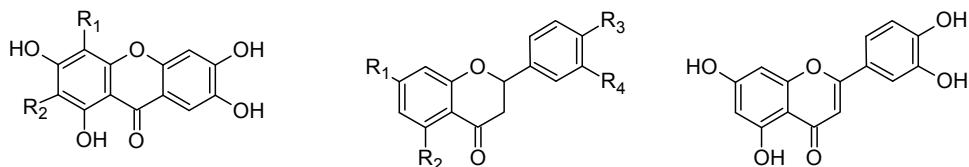
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## **1. Introduction**

It has been estimated that diabetes will affect 439 million adults by 2030, with the major increase occurring in developing countries (Shaw et al., 2010). It is projected that it will rank as the 9th leading cause of death in low-income countries (Mathers & Loncar, 2006). There are two major types of diabetes, i.e. type 1 (T1D) or insulin dependent diabetes and type 2 (T2D) or non-insulin dependent diabetes. The incidence of T2D is reaching epidemic proportions and has been associated with an increase in obesity (Venables & Jeukendrup, 2009). According to the World Health Organisation (WHO, 2011) the main complications associated with diabetes are cardiovascular disease and renal failure. Although genetic factors may play a role, life-style factors, such as reduced exercise and poor diet, specifically a high carbohydrate, high fat diet devoid or low in fruit and vegetables, have been shown to increase the risk of diabetes (Astrup, 2001).

Medicinal plants have been used in folk medicine and traditional healing systems such as Ayurveda and Traditional Chinese Medicine (TCM) for the treatment of diabetes (T.S.C. Li, 2003; Modak et al., 2007; Singh et al., 2009; Yen et al., 2003). On the African continent as many as 90% of the populations of some countries relies on plants as the principal source of medicine for the treatment of different diseases, including diabetes (Hostettman et al., 2000), as they provide an affordable alternative to drugs. In South Africa a large number of plants, belonging to plant families such as the Asteraceae and Lamiaceae, amongst others, have been traditionally used for the treatment of diabetes (Deutschländer et al., 2009; Erasto et al., 2005; Thring & Weitz, 2006).

Globally, there is a movement towards alternatives to single chemical entities as favoured by the pharmaceutical industry. These alternatives are rationally selected, carefully standardised, synergistic traditional herbal formulations and botanical drug products which are supported by robust scientific evidence (Patwardhan & Mashelkar, 2009). In many instances the value of herbal and medicinal plant extracts lies not in a single compound, but in their complex phytochemical nature. These complex mixtures of often unspecified compounds are able to modulate multiple targets (Y. Li et al., 2008). Antidiabetic phenolic compounds in the extracts also have the ability to ameliorate oxidative stress (Han et al., 2007), an underlying mechanism to the pathogenesis of diabetes (Ceriello & Motz, 2004). One such compound is the xanthone C-glucoside, mangiferin (1,3,6,7-tetrahydroxy-xanthone-C2- $\beta$ -D-glucoside), demonstrating antihyperlipidaemic, antihyperglycaemic and antioxidant properties (Wauthoz et al., 2007). Mangiferin (Fig. 1) was shown to protect against streptozotocin (STZ)-induced oxidative damage to cardiac and renal tissues in Wistar rats (Muruganandan et al., 2002). Its presence in the endemic South African *Cyclopia* spp. (family Fabaceae; tribe Podalyrieae) suggested the potential use of these plant species as antidiabetic nutraceuticals or even phytopharmaceuticals. Hesperidin (Fig. 1), another antioxidant and compound demonstrating hypoglycaemic properties in rodents (Akiyama et al., 2010; Jung et al., 2004), is also one of the major monomeric polyphenols present in *Cyclopia* spp. (Joubert et al., 2003). A decoction of *Cyclopia* spp. was used in the past as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis. However, it is as the herbal tea, honeybush, that *Cyclopia* spp. are increasingly appreciated by consumers world-wide. The tea is primarily exported to the Netherlands, Germany, United Kingdom and United States of America. It is even exported to traditional tea-drinking countries such as Sri Lanka, India, Japan and China. Commercial herbal tea production comprised three main species, viz. *C. genistoides*, *C. intermedia* and *C. subternata*. Of these, *C. intermedia*, harvested almost exclusively from the wild, provides the bulk of honeybush production.



**2, mangiferin**  
(R<sub>1</sub> = H; R<sub>2</sub> = C- $\beta$ -D-glucoside)

**4, eriodictyol-glucoside**  
(R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub> = OH; O- or  
C- $\beta$ -D-glucosyl in unknown position)

**7, luteolin**

**3, isomangiferin**  
(R<sub>1</sub> = C- $\beta$ -D-glucoside; R<sub>2</sub> = H)

**5, eriocitrin**  
(R<sub>1</sub> = O- $\beta$ -D-glucosyl; R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub> = OH)

**6, hesperidin**  
(R<sub>1</sub> = O- $\beta$ -D-glucosyl; R<sub>2</sub>, R<sub>4</sub> = OH;  
R<sub>3</sub> = OCH<sub>3</sub>)

**8, hesperetin**  
(R<sub>1</sub>, R<sub>2</sub>, R<sub>4</sub> = OH; R<sub>3</sub> = OCH<sub>3</sub>)

Fig. 1. Structures of phenolic compounds of *C. intermedia* extract.

A contributing factor to its growing popularity is the body of scientific evidence that honeybush has potential health benefits, including antioxidant, anticancer and phytoestrogenic properties (Joubert et al., 2008a). The greatest demand is for the traditional product, which is 'fermented' (oxidised) to form the characteristic dark-brown colour and sweet flavour. However, this is accompanied by a substantial reduction in the phenolic content of the plant material and extract, as well as a decrease in antioxidant activity (Joubert et al., 2008b), justifying investigating the benefits of unfermented *C. intermedia* for human health.

With T2D being the most common form of diabetes, representing more than 90% of all cases (WHO, 2011), the antidiabetic potential of unfermented *C. intermedia* was investigated using a diet-induced obese insulin resistant (OBIR) rat model. Feeding rats a high fat diet induces a state of insulin resistance associated with impaired insulin-stimulated glycolysis and glycogen synthesis (Kim et al., 2000). The STZ-induced diabetic rat model, following pancreatic  $\beta$ -cell destruction and resulting in insulin deficiency rather than insulin resistance (OBIR model), was used to establish the optimal acute glucose lowering dose of a *C. intermedia* extract. The investigation focused on a hot water extract of *C. intermedia* as it represents normal preparation of the herbal tea, albeit under more severe extraction conditions, but without introducing qualitative changes to composition as is the case for organic solvent extraction.

## 2. Material and methods

### 2.1 Chemicals

General analytical grade laboratory reagents were purchased from Sigma-Aldrich (St. Louis, USA) and Merck (Darmstadt, Germany). Authentic reference standards were obtained from Sigma-Aldrich (mangiferin, hesperidin) and Extrasynthese (Genay, France; eriocitrin, luteolin). Isomangiferin was isolated from *C. subternata* (De Beer et al., 2009). Acetonitrile for HPLC analysis was gradient grade for liquid chromatography (Merck, Darmstadt, Germany). HPLC grade water was prepared by purifying laboratory grade water (Continental Water Systems Corp., San Antonio, USA) with a Milli-Q 185 Académic Plus water purification system (Millipore, Bedford, USA).

STZ, fluothane, 50% dextrose solution, metformin hydrochloride and rosiglitazone maleate (Avandia®) were obtained from Sigma-Aldrich, AstraZeneca Pharmaceuticals (Johannesburg, South Africa), Intramed (Johannesburg, South Africa), Rolab (Johannesburg, South Africa) and GlaxoSmithKline (Bryanston, South Africa), respectively.

### 2.2 Plant material and extract preparation

*Cyclopia intermedia* shoots (ca 220 kg) were harvested according to normal practice by cutting the shoots sprouting from the rootstock directly above soil level. All the plant material was harvested from a natural stand on Nootgedacht Farm in the Langkloof area, South Africa. The part of the stem without leaves or with only a few leaves (Fig. 2) was removed before further processing, entailing cutting of the shoots into small pieces (<4 mm) and mechanical drying at 40 °C to less than 10% moisture content (Joubert et al., 2008b), giving ca 50 kg dried plant material. The plant material was pulverised with a Retsch rotary mill before pilot plant extraction. Water heated to >95 °C was added to the pulverised plant material in a 1:10 (m/v)

ratio and continuously stirred for 30 min. (final extract temperature was 70 °C), whereafter the extract was pumped to an in-line continuous centrifuge for removal of the insoluble matter. Following centrifugation, the extract, containing 2.69 g soluble matter/100 mL, was cooled to ca 20 °C using a tubular heat exchanger and aliquots bottled for daily feeding of OBIR rats. The aliquots were stored at -20 °C until use. An aliquot (ca 1000 mL) was freeze-dried in an Edwards Modulyo bench-top freeze-drier (Edwards High Vacuum Ltd, Crowley, UK) for acute dosing of STZ-induced diabetic rats and for phenolic content analysis.



Fig. 2. (a) *Cyclopia intermedia* plant showing aerial parts (top section of a typical shoot with thin stems and leaves). (b) Harvested shoots before removal of the stem parts without leaves.

### 2.3 HPLC analysis of extract

HPLC analysis was performed using an Agilent 1200 series HPLC system consisting of a quaternary pump, autosampler, in-line degasser, column oven and diode-array detector (Agilent Technologies Inc., Santa Clara, USA) controlled with Chemstation 3D LC software. Separation was performed on a Zorbax Eclipse XDB-C18 column (150 x 4.6 mm, 5 µm particle size, 80 Å pore size) from Agilent Technologies protected by a guard column with the same stationary phase. The separation was achieved with mobile phases consisting of 0.1% formic acid and acetonitrile using the solvent gradient reported previously (De Beer & Joubert, 2010). The flow-rate and column temperature were maintained at 1 mL/min and 30 °C, respectively. The freeze-dried extract was dissolved in 16% DMSO (ca 5 mg/mL) and filtered using a 33 mm Millex-HV PVDF syringe filter unit with 0.45 µm pore size (Millipore) before injection (20 µL). Compound identification was based on retention times and UV-Vis spectra of authentic standards where available (mangiferin, isomangiferin, eriocitrin, hesperidin, luteolin and hesperetin). An additional peak was identified as an

eriodictyol-glucoside based on similarity of its retention time and UV-Vis spectrum with a compound identified previously using liquid chromatography with mass spectrometric detection (De Beer & Joubert, 2010). An unidentified compound was also observed, which had retention time and UV-Vis characteristics similar to a compound previously detected in several *Cyclopia* spp. (De Beer & Joubert, 2010). Mangiferin, isomangiferin and luteolin were quantified using the peak areas at 320 nm, while the eriodictyol-glucoside, eriocitrin, hesperidin, hesperetin and the unidentified compound were quantified using the peak areas at 288 nm. A calibration series consisting of mangiferin (0.05-2.5 µg injected), isomangiferin (0.05-2.5 µg injected), eriocitrin (0.01-0.8 µg injected), hesperidin (0.01-1.5 µg injected), hesperetin (0.003-0.25 µg injected) and luteolin (0.05-0.35 µg injected) was used for external calibration. The eriodictyol-glucoside was quantified in terms of eriocitrin equivalents, while the unidentified compound was quantified in terms of hesperidin equivalents.

## 2.4 Animal study

Ethical approval was obtained from the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council of South Africa.

Male Wistar rats, obtained from the Primate Unit of the Medical Research Council (Tygerberg, South Africa), were used throughout the study. The rats were housed individually in wired top and bottom cages, fitted with Perspex™ houses and kept in a controlled environment of 23–24 °C, 50% humidity and a 12 h light/dark cycle.

### 2.4.1 STZ-induced diabetic rats

Adult male Wistar rats (200–250 g) were injected intramuscularly with freshly prepared STZ [35 mg/kg body weight (BW)] in 0.1 M citrate buffer (pH 4.5) to induce stable non-ketoacidotic diabetic rats. Blood samples were taken from the tail tip 72 hrs after STZ injection and the plasma glucose concentrations determined using a glucometer (Precision Q.I.D, Abbott Laboratories, Johannesburg, South Africa). Rats with fasting blood glucose levels of >15.0 mmol/L were considered diabetic and were selected for the acute dose finding study of the extract.

### 2.4.2 Acute dosing of diabetic STZ rats with honeybush extract

The efficacy of a single dose of honeybush extract was determined by administering different doses of freeze-dried extract to STZ diabetic rats. Following a 3 hr fast, baseline plasma glucose concentrations of diabetic STZ rats were determined. The freeze-dried honeybush extract (reconstituted in a fixed volume of distilled water yielding e.g. 5 mg/mL for the 5 mg/kg BW dose, etc.) was administered by oral gavage under light anaesthesia (by inhalation of 2% fluothane with 98% oxygen) to four experimental groups of five STZ rats each at doses of 0 (vehicle control), 5, 25 and 50 mg/kg BW. Plasma glucose concentrations were determined hourly over a 6 hr period.

## 2.5 Chronic treatment of OBIR rats with honeybush extract

The efficacy of honeybush extract to ameliorate diet-induced insulin resistance, characterised by hyperglycaemia, hyperinsulinaemia, hyperglucagonaemia and dyslipidaemia was investigated using OBIR Wistar rats chronically exposed to the extract for 12 wks (described below).

### 2.5.1 Inducing insulin resistance in Wistar rats

Three-week old weanling Wistar rats (male) were fed a 40% high fat diet (Table 1) and 30% sucrose in their drinking water *ad libitum* for 9 wks. The high fat diet in combination with sucrose induces insulin resistance and obesity with slightly elevated fasting glucose concentrations (Hallfrisch et al., 1981; Krygsman et al., 2010). After 9 wks on the high fat and sucrose diet, blood was collected for baseline glucose and insulin determination. Thereafter the rats were allocated into experimental groups and maintained on the high fat and sucrose diet during the subsequent 12-wk treatment.

### 2.5.2 Experimental groups

The untreated control consisted of six 12-wk old OBIR rats that were randomly assigned to the control group.

Groups E1 – E5 (honeybush extract treated groups) consisted of five groups of ten OBIR rats each, receiving 538, 1075, 1792, 2150 or 2688 mg/100 mL honeybush extract (hot water soluble solids), respectively, as their drinking fluid, which also contained 30% sucrose (Table 2). The daily fluid intake was measured for each rat and the average amount of liquid

Nutrients	% Energy
Protein	15.09
Fat	40.17
Saturated fatty acids	18.27
Monounsaturated fatty acids	11.45
Polyunsaturated fatty acids	5.75
Carbohydrate	44.73
Kcal/g of food	2.06
Kcal/g of sucrose	0.60
<b>Total energy of diet (Kcal/g)</b>	<b>2.66</b>

Table 1. High fat diet (HFD) macronutrient and calorific composition.

Group	Weight	Treatment concentration	Fluid intake	Treatment intake/day	Mangiferin intake/day	Hesperidin intake/day
<b>Control</b>	476 ± 20	-	30 ± 2	-	-	-
E1	441 ± 12	538	33 ± 4	77.2 ± 9.6	4.47 ± 0.55	0.27 ± 0.03
E2	456 ± 20	1075	42 ± 5	206.9 ± 27.5	11.99 ± 1.60	0.73 ± 0.10
E3	442 ± 25	1792	31 ± 4	255.1 ± 64.6	14.79 ± 3.74	0.65 ± 0.23
E4	438 ± 21	2150	32 ± 2	299.1 ± 23.5	17.33 ± 1.36	1.06 ± 0.08
E5	457 ± 36	2688	43 ± 2	531.3 ± 61.1	30.79 ± 3.54	1.88 ± 0.22
Met	489 ± 22	-	31 ± 4	22.0	-	-
Rosi	479 ± 26	-	29 ± 2	4.0	-	-

Table 2. OBIR rat body weight (g), extract concentration (mg/100 mL), fluid intake (mL), treatment intake (mg/kg BW), i.e. *C. intermedia* extract (E1-5), metformin (Met) or rosiglitazone (Rosi), as well as equivalent intake of mangiferin (mg/kg BW) and hesperidin (mg/kg BW), for chronic treatment experiment.

consumed per week for each rat was calculated (Table 2). Aliquots of the aqueous honeybush extract were defrosted daily. In the case of E1 to E4 the extract was diluted with water to give the required concentration of hot water soluble solids, while E5 represented the undiluted extract.

The metformin and rosiglitazone treated groups consisted of three OBIR rats each that received metformin hydrochloride or rosiglitazone maleate at dosages of 22 and 4 mg/kg BW, respectively, in distilled water.

### 2.5.3 Blood parameters

*Determination of fasting plasma glucose.* The STZ-induced diabetic and OBIR rats were fasted for 3 hrs and overnight, respectively, prior to determining their fasting plasma glucose concentrations. A drop of blood was collected from the tail tip and the plasma glucose concentration determined.

*Determination of fasting serum insulin.* Rats were anaesthetised by 2% fluothane inhalation with 98% oxygen. Blood was collected from the tail tip into Eppendorf tubes and stored on ice until centrifuged at 2500 rpm for 15 min. at 4 °C. Following centrifugation, the serum samples were stored at -20 °C until analysis. The serum insulin concentration was determined by radioimmunoassay using a rat insulin measurement kit from Linco® Research (St. Charles, USA).

*Intravenous glucose tolerance test (IVGTT).* Rats fasted overnight were anaesthetised as described above and a drop of blood obtained from the tail tips. This was used for measuring baseline glucose. Glucose (50% dextrose solution), at a dose of 0.5 mg/kg BW, was injected intravenously over 20 sec and glucose measurements taken at 5, 10, 20, 30, 40, 50 and 60 min.

*Determination of fasting plasma cholesterol.* Rats were anaesthetised as described above and blood, collected from the tail tips, was prepared and stored at -20 °C for analysis. Total cholesterol concentrations were determined by Pathcare Laboratories (Cape Town, South Africa), using a Bayer-Technicon RA 1000 auto-analyser.

### 2.5.4 Immunocytochemistry and image analysis of the pancreata

*Harvesting of pancreata.* After 12 wks of treatment the rats were euthanised by exsanguination under sodium barbital anaesthesia and pancreata harvested. The whole pancreas was removed, fixed overnight in 4% buffered formaldehyde (pH 7.5) and processed into paraffin wax by standard histological methods. Serial 4 µm thick sections were cut for immunocytochemistry.

*Immunocytochemistry.* Serial wax sections attached onto silane coated slides were de-waxed with xylene and hydrated through descending grades of ethanol into water. Slides were rinsed in 50 mM Tris-buffered-saline (pH 7.4) and double immuno-stained, using anti-insulin and anti-glucagon primary antibodies. Primary antibody binding to insulin or glucagon was detected by avidin D-biotinylated horseradish peroxidase or streptavidin-biotin-complex/alkaline phosphatase conjugated link antibodies. Insulin positive labeling ( $\beta$ -cells) was visualised with fuchsin red and  $\alpha$ -cells with diaminobenzidine tetrahydrochloride. Method controls involved omission of the primary antibody (anti-insulin or anti-glucagon).

**Image analysis.** Both  $\beta$ - and  $\alpha$ -cell areas were measured on each section (minimum of ten sections per group). Computer-assisted measurements were taken with a Canon Powershot S40 digital camera (Tochigi, Japan) mounted on an Olympus BX60 light microscope (Tokyo, Japan), attached to a personal computer to capture images. The acquired images were transferred to the computer using remote capture software from Canon. Image analysis was performed with Leica Qwin Plus Software (Cambridge, UK). The ratio of either the  $\beta$ -cell positive or the  $\alpha$ -cell positive area to the total pancreas area was calculated.  $\beta$ -Cell and  $\alpha$ -cell sizes were calculated by dividing the total area of each of the cell types by the number of nuclei counted.

## 2.6. Statistical analysis

Results were entered into an Excel spreadsheet and statistically analysed. For the acute STZ rat experiment hourly ( $t = 1\text{--}6$ ) glucose concentrations were compared against baseline ( $t = 0$ ) and the control for each corresponding time point. For the chronic OBIR rat experiments each data point was compared to the corresponding control value using ANOVA with a Dunnet post-hoc test (Prism version 5, Graphpad software®). The values presented are the mean  $\pm$  SE.

## 3. Results

### 3.1 Extract composition

The phenolic compound structures for the major compounds of the *C. intermedia* extract are shown in Fig. 1, the plant shoots in Fig. 2 and the extract HPLC profile and quantitative data in Fig. 3. The major compounds detected included the xanthone isomers, mangiferin and isomangiferin, the flavanone glycoside, hesperidin (hesperetin-7-O-rutinoside), and an unidentified compound previously detected in several *Cyclopia* spp. (De Beer & Joubert, 2010). Additionally, a flavone, luteolin, and three flavanones, namely an eriodictyol-

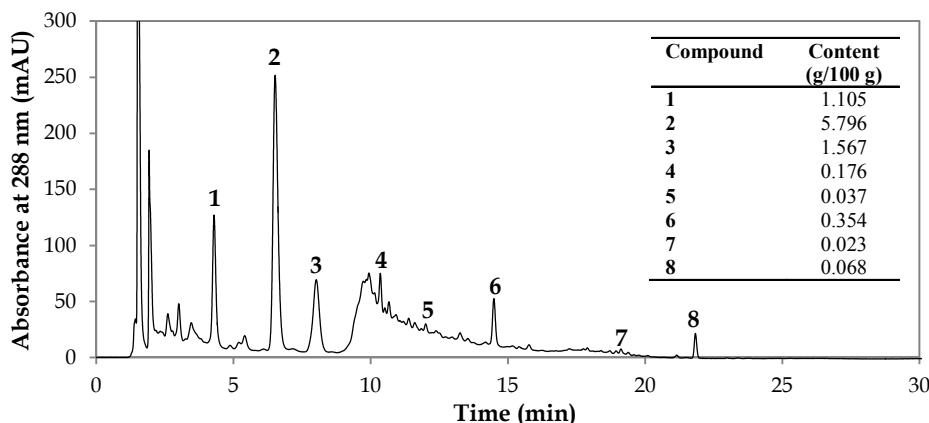


Fig. 3. Phenolic profile and phenolic composition (g/100 g) of *C. intermedia* extract (1, unidentified compound; 2, mangiferin; 3, isomangiferin; 4, eriodictyol-glucoside; 5, eriocitrin; 6, hesperidin; 7, luteolin; 8, hesperetin).

glucoside, eriocitrin (eriodictyol-7-O-rutinoside) and hesperetin, the aglycone of hesperidin, were also detected in small quantities.

### 3.2 Animal study

Results of the intake data are summarised in Table 2. The average intake of honeybush extract by the various treatment groups varied from 77 to 531 mg/kg BW. This equals 4.5 to 30.8 mg/kg BW of the major xanthone, mangiferin, and 0.3 to 1.9 mg/kg BW of the major flavanone, hesperidin.

Intramuscular injection of Wistar rats with STZ (35 mg/kg BW) induced diabetes in the rats at an average fasting plasma glucose concentration of  $27.8 \pm 1.0$  mmol/L (data not shown). The acute effects of administering the honeybush extract by oral gavage under light anaesthesia at doses of 0 (vehicle control), 5, 25 and 50 mg/kg BW are shown in Fig 4. The optimal acute oral glucose lowering dose for the honeybush extract in STZ-induced diabetic rats for the dose range tested was 50 mg/kg BW. This was the only acute dose of the extract that significantly reduced the mean blood glucose concentrations relative to the baseline fasting blood glucose concentration. Reductions of  $33.5 \pm 1.7\%$  ( $p<0.05$ ),  $34.3 \pm 3.6\%$  ( $p<0.05$ ) and  $35.6 \pm 3.5\%$  ( $p<0.01$ ) were observed after 4, 5, and 6 hrs, respectively (Fig. 4).

After a chronic 3-month treatment of OBIR rats with aqueous honeybush extract their hyperglycaemic fasting blood glucose concentrations were reduced to normoglycaemic values. In other words, the honeybush extract reduced the fasting blood glucose levels from 12.2 mmol/L of the control to 4.8 – 5.4 mmol/L ( $p<0.001$ ). All extract concentrations were effective. Metformin and rosiglitazone had very similar effects and reduced the fasting glucose to 6.3 mmol/L ( $p<0.001$ ) and 5.6 mmol/L ( $p<0.001$ ), respectively (Fig. 5).

Untreated control OBIR rats had IVGTT peak glucose concentrations of  $18.2 \pm 1.74$  mmol/L. Treatment with the honeybush extract reduced this value to  $14.9 \pm 0.7$  mmol/L (treatment E2,  $p<0.05$ ),  $14.8 \pm 0.9$  mmol/L (treatment E3,  $p<0.05$ ) and  $13.5 \pm 1.2$  mmol/L (treatment E4,

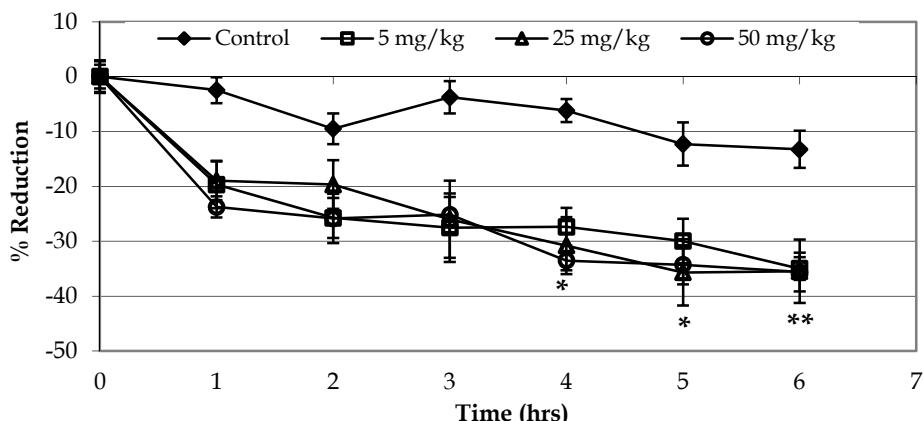


Fig. 4. Acute glucose lowering effect of *C. intermedia* extract in STZ diabetic rats ( $n=5$ ). Significant differences from baseline indicated with \* ( $p<0.05$ ) or \*\* ( $p<0.01$ ).

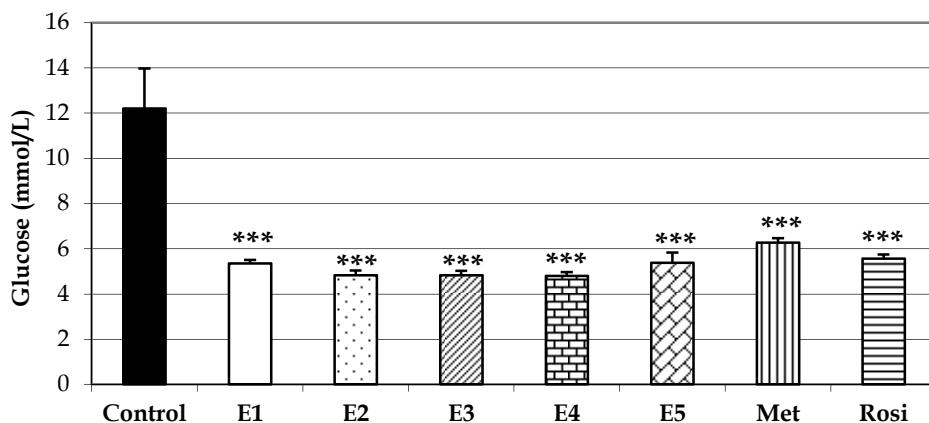


Fig. 5. The effect of chronic treatment with *C. intermedia* extract, metformin and rosiglitazone (see Table 2 for treatment codes and dosage) on fasting plasma glucose concentration of OBIR rats ( $n=10$ ). Significant differences from control indicated with \*\*\* ( $p<0.001$ ).

$p<0.05$ ), respectively (only E3 is shown for clarity; Fig. 6). The IVGTT area under the curve values were reduced from  $670 \pm 9$  for the control to  $474 \pm 15$  ( $p<0.05$ ) and  $496 \pm 13$  ( $p<0.05$ ) for E2 and E5, respectively (data not shown). E1 (the lowest dose), as well as the known drugs, metformin and rosiglitazone, had no significant effect ( $p\geq 0.05$ ) (Fig. 6).

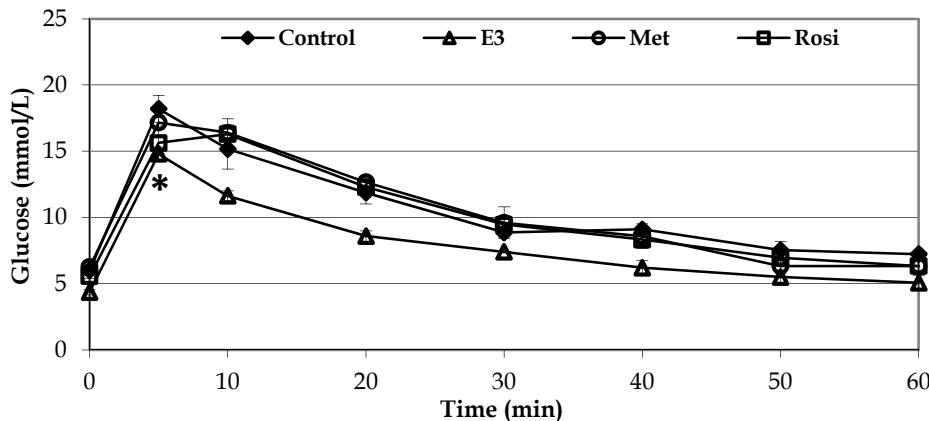


Fig. 6. The effect of *C. intermedia* extract, metformin (Met) and rosiglitazone (Rosi) treatment on intravenous glucose tolerance in OBIR rats ( $n=10$ ) (see Table 2 for treatment codes and dosage). E3 differs significantly from the control as indicated with \* ( $p<0.05$ ).

Treatment of OBIR rats with honeybush extracts, E1 to E5, for 3 months lowered the total plasma cholesterol concentration compared to the untreated rats ( $2.9 \pm 0.3$  mmol/L) by 31.6 - 39.1% (Fig. 7). The effect of metformin and rosiglitazone on total cholesterol was not determined.

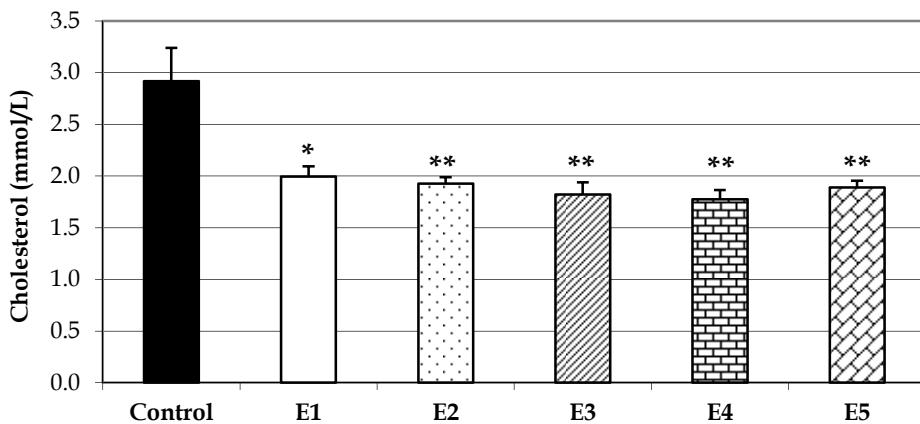


Fig. 7. The effect of chronic treatment with *C. intermedia* extract (see Table 2 for treatment codes and dosage) on fasting total cholesterol concentrations in OBIR rats (n=10). Significant differences from control indicated with \* (p<0.05) or \*\* (p<0.01).

The average  $\alpha$ -cell size in untreated OBIR rats was  $106 \pm 13.6 \mu\text{m}^2$ . Treatment with the honeybush extracts, E1 to E4, reduced the  $\alpha$ -cell size to about half, i.e. to  $48.4 - 54.9 \mu\text{m}^2$  (p<0.01). Metformin and rosiglitazone had similar effects and also reduced the average  $\alpha$ -cell size to  $41.2 \pm 1.9 \mu\text{m}^2$  and  $44.0 \pm 1.9 \mu\text{m}^2$ , respectively (p<0.01) (Fig. 8). No data were available for E5. These changes in the  $\alpha$ -cell size were also reflected in the decreased  $\alpha$ -cell to  $\beta$ -cell ratio for all treatments (Fig. 9).

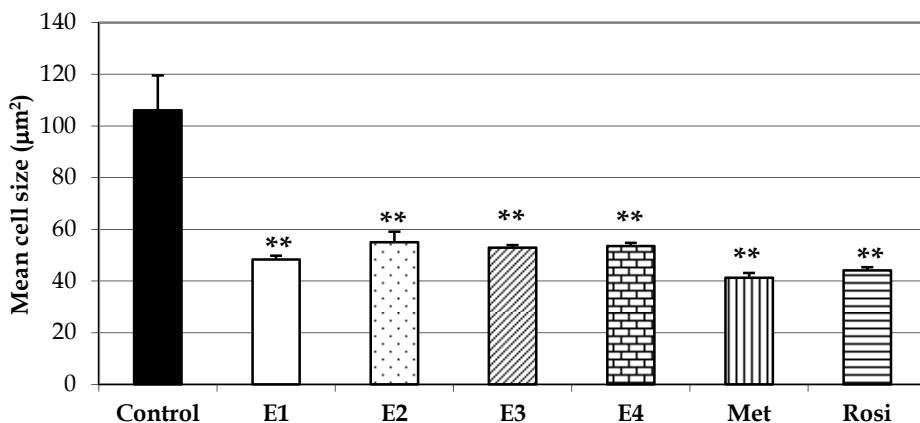


Fig. 8. The effect of chronic treatment with *C. intermedia* extract, metformin and rosiglitazone (see Table 2 for treatment codes and dosage) on mean  $\alpha$ -cell size in the pancreata of OBIR rats (n=10). Significant differences from control indicated with \*\* (p<0.01).

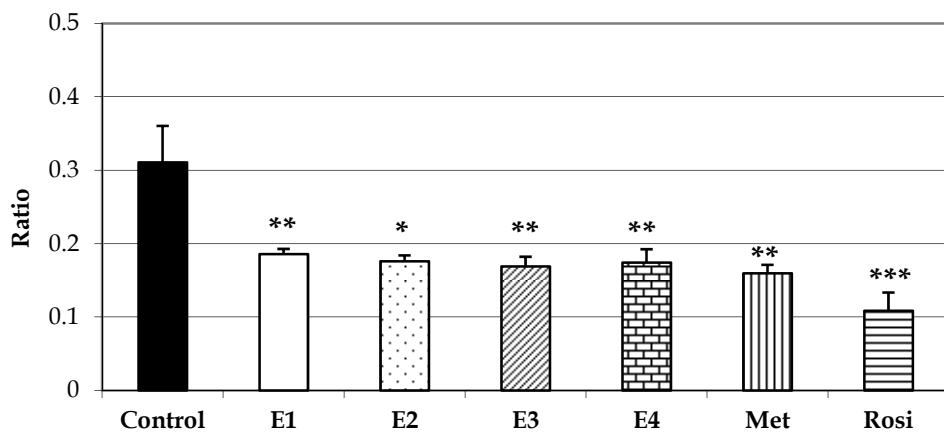


Fig. 9. The effect of chronic treatment with *C. intermedia* extract, metformin and rosiglitazone (see Table 2 for treatment codes and dosage) on  $\alpha$ - to  $\beta$ -cell ratio in the pancreata of OBIR rats ( $n=10$ ). Significant differences from control indicated with \* ( $p<0.05$ ), \*\* ( $p<0.01$ ) or \*\*\* ( $p<0.001$ ).

#### 4. Discussion

The content of mangiferin, isomangiferin and the unidentified compound was higher than the average for unfermented *C. intermedia* extracts previously analysed (De Beer & Joubert, 2010; Joubert et al., 2008b), while the hesperidin and eriocitrin contents of the present extract were lower. De Beer & Joubert (2010) detected luteolin only in trace amounts, while no hesperetin was detected. Joubert et al. (2003) found that the plant material (dry mass basis) contain 1.69% mangiferin, while isomangiferin and hesperidin, respectively, comprised 0.22 and 1.76% of the dry plant material. Other phenolic compounds of *C. intermedia* include flavones, isoflavones, other flavanones and coumestans (Ferreira et al., 1998; Kamara et al., 2003). Quantitative differences between the present extract and aqueous hot water extracts analysed previously (De Beer & Joubert, 2010; Joubert et al., 2008b) could be attributed to natural variation and/or selective use of the upper part of the shoot. This has implications for standardisation and efficacy. Testing of more *C. intermedia* extracts, specifically for their efficacy as antidiabetic extracts, is required before more comprehensive claims can be made.

STZ was originally developed as an antibiotic derived from *Streptomyces achromogenes* but it is toxic to pancreatic  $\beta$ -cells. It selectively enters insulin producing  $\beta$ -cells via their GLUT2 glucose transporter proteins, inducing irreparable DNA damage and death of  $\beta$ -cells in a dose dependent manner (Lenzen, 2008). In the Wistar rat, intramuscular injection of STZ (35 mg/kg BW) increased fasting plasma glucose concentrations by ca 500% from the average normoglycaemic concentration of 5.3 mmol/L, resulting in stable non-ketoacidotic T1D diabetic rats. An acute 50 mg/kg BW dose of the aqueous honeybush extract induced a sustained glucose lowering effect from 3 to at least 6 hrs in these STZ-induced diabetic rats. These results are comparable to that of Miura et al. (2001) who assessed the acute antidiabetogenic effect of an extract of *Anemarrhena asphloides* in a hyperglycaemic KK-Ay

diabetic mouse model. This plant, which contains the xanthones, mangiferin and a 7-glucoside of mangiferin, is used as an Oriental medicine for the treatment of diabetes. After oral treatment the maximal glucose lowering effect of the aqueous *Anemarrhena aspholoides* extract was achieved after 7 hrs. Mangiferin and its glucoside showed similar activity at a dose of 90 mg/kg BW. Mangiferin administered intraperitoneally for 30 days to mildly hyperglycaemic STZ-induced rats at doses of 10 and 20 mg/kg BW ameliorated the diabetic effects including weight loss, hyperglycaemia and hypercholesterolaemia (Dineshkumar et al., 2010). Prior to the latter study, the antidiabetic effects of mangiferin in STZ-induced diabetic Wistar rats were shown at the same doses after chronic treatment for 14 and 28 days (Muruganandan et al., 2005).

In the present study 50 mg/kg BW of honeybush extract, equalling a dose of 2.90 mg mangiferin, was effective at reducing plasma glucose concentrations in our STZ-induced diabetic rat model. Other compounds in the extract could also contribute to the observed hypoglycaemic effect through synergistic or additive effects. Hesperidin, in particular, comprising 0.35% of the honeybush extract, was shown to have a hypoglycaemic effect in marginally hyperglycaemic Wistar rats normalising their blood glucose concentrations after 16 days (Akiyama et al., 2010). Jung et al. (2004), using a spontaneously diabetic C57BL/KsJ-db/db mouse model, showed that a 5-wk supplementation of the diet with 0.02% hesperidin ameliorated the development of hyperglycaemia in these mice. Both Akiyama et al. (2010) and Jung et al. (2004) attributed the hypoglycaemic effect of hesperidin to upregulation of glucose regulating enzymes, in particular glucokinase, which enhances glycolysis and increases glycogen synthesis.

Confirmation of the antidiabetic potential of *C. intermedia* extract in the STZ-induced diabetic rat model was followed up with a chronic study in the high fat diet-induced OBIR rat model. Feeding non-predisposed Wistar rats a high fat (Table 1) and sucrose diet from weanling for three months induces obesity and glucose intolerance. Although these obese rats maintain near normal fasting glucose levels they present with hyperinsulinaemia, hyperglucagonaemia and dyslipidaemia (Buettner et al., 2006; Chalkley et al., 2002; Kamgang et al., 2005). The addition of refined sugars, i.e. sucrose, exacerbates the metabolic aberrations by the disruption of fatty acid metabolism resulting in hepatic and subsequent muscle insulin resistance (Fukuchi et al., 2004). The chronic hyperinsulinaemia associated with insulin resistance increases hepatic lipogenesis due to increased expression of the major lipogenesis gene sterol response element-binding protein 1c (SREBP1c). In these obese rats increasing levels of glucagon fail to regulate the insulin action, leading to increases in hepatic fat accumulation and hypertriglyceridaemia (Buettner et al., 2006). Interestingly, whereas the insulin sensitivity of the SREBP1c pathway is retained, the forkhead box protein O1 (FOXO1) pathway becomes insulin resistant, resulting in decreased hepatic glucose uptake and increased gluconeogenesis. Together the increase in fatty acid synthesis and hepatic glucose release worsens muscle and pancreatic islet insulin resistance (Liu et al., 2011). Ultimately, high fat feeding is directly associated with pancreatic endocrine dysfunction and subsequent morphological changes of the pancreatic islets' endocrine cell ratios and cell sizes. The compensatory response to the increased demand for insulin results in pancreatic  $\beta$ -cell hypertrophy and an increase in  $\alpha$ -cell number and volume (Buettner et al. (2007). As the diabetic pathogenesis worsens the  $\beta$ -cell mass declines and the  $\alpha$ - to  $\beta$ -cell ratio increases (Liu et al., 2011). Feeding the same 40% high fat diet to pregnant Wistar dams during gestation resulted in offspring with similar increases in  $\alpha$ -cell volume, while  $\beta$ -cell

volume decreased (Cerf et al., 2005). In human T2D pancreatic islets, larger islets with an increased proportion of pancreatic  $\alpha$ -cells have been shown to have impaired function (Deng et al., 2004). Despite the diet-induced metabolic and morphological aberrations these rats only develop slight hyperglycaemia over time without progressing to overt T2D. The similarities of the diet-induced OBIR rat to the pathophysiology of human obesity and the metabolic syndrome are well established (Buettner et al., 2007) and this animal model was therefore selected to test the possible ameliorating effects of honeybush extract on these metabolic aberrations.

Inclusion of the honeybush extract in an otherwise diabetogenic diet for OBIR rats resulted in normalisation of the pre-existing hyperglycaemia over a wide range of dosages. This confirms that the extract has glucose lowering potential without causing hypoglycaemia. The latter is a potentially undesirable side effect of some T2D agents, specifically the sulfonylureas and meglitinides (Bennett et al., 2011). In addition, the normoglycaemic effect of the honeybush extract was achieved without dietary intervention. The extract proved to be as effective as metformin and rosiglitazone, which are regarded as the gold standards for treating human T2D. The efficacy of the extract at all doses and the lack of a clear dose response could be contributed to the length of the treatment (12 wks) and relative high doses even at the lowest dosage level. The reduction in IVGTT peak values and the area under the curve values in OBIR rats treated with the honeybush extract clearly indicated improved glucose homeostasis. Further, the improvement in glucose control was not associated with significantly increased fasting insulin concentrations. This may suggest that the mechanism whereby the extract elicits its effect on glucose metabolism is independent of insulin, reflecting adaptive mechanisms in the fasted state. This would imply that, due to the low fasting glucose concentrations,  $\beta$ -cells need to release less insulin to maintain glucose homeostasis.

Plasma cholesterol levels of honeybush extract treated OBIR rats were significantly reduced when compared with the plasma cholesterol levels of the untreated control rats. Mangiferin and hesperidin have been shown to lower plasma cholesterol levels of diabetic rats (Akiyama et al., 2010; Dineshkumar et al., 2010; Muruganandan et al., 2005). Mangiferin, apart from lowering total cholesterol, also increases high-density lipoprotein-cholesterol levels and therefore decreases the atherogenic index in diabetic rats (Muruganandan et al., 2005). The hypolipidaemic effect of hesperidin has been attributed to the inhibition of hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA:cholesterolacyl transferase, key enzymes in cholesterol synthesis and cholesterol esterification, resulting in the reduction of plasma cholesterol (Bok et al., 1999; Jung et al., 2006). Finally, a potential role of some minor compounds such as the inositol, (+)-pinitol, shown to be present in *C. intermedia* plant material (Ferreira et al., 1998), in the glucose- and cholesterol-lowering effects (Bates et al., 2000; Choi et al., 2009) elicited by the honeybush extract cannot be excluded.

World-wide over 18 million people have died of cardiovascular disease in 2005. WHO has identified high total cholesterol as a major contributing risk factor which is modifiable and thereby could potentially reduce the incidence of cardiovascular disease (Rodgers et al., 2004; Roth et al., 2011). Polyphenols have anti-atherosclerotic properties and are protective against cardiovascular disease. Apart from their plasma cholesterol lowering effects, polyphenols are associated with improved endothelial function and oxidative status

(Badimon et al., 2010). The protective effects of dietary flavonol intake against coronary heart disease mortality in humans was highlighted in a meta-analysis (Huxley & Neil, 2003), indicating a 20% lower risk in individuals with the highest consumption of dietary flavonols. In another study, Peters et al. (2001) suggested drinking three cups of tea (*Camellia sinensis*) a day could reduce the risk of myocardial infarction by 11%. In human trials coronary artery disease patients have shown improved endothelial function and coronary microcirculation following polyphenolic treatment (Hozumi et al., 2006).

The main morphometrical finding following honeybush treatment for 12 wks was a decrease in the  $\alpha$ -cell area, and subsequently by inference, total pancreas  $\alpha$ -cell volume. The role of glucagon in the pathogenesis of T2D has been largely neglected by researchers. In mice, persistent hyperglucagonaemia induced by glucagon-producing glucagonomas results in T2D (Y. Li et al., 2008). Glucagon secretion by the  $\alpha$ -cell is tightly regulated by insulin via insulin receptors at the surface of  $\alpha$ -cells. This ensures that normoglycaemia is maintained. However, as insulin resistance increases, as is the case with the OBIR rat model, dysregulation of glucagon secretion by insulin occurs, resulting in persistent hyperglucagonaemia. Morphologically, this results in increased  $\alpha$ -cell numbers,  $\alpha$ -cell volume and glucagon secretion that worsen the insulin resistance of the liver causing more hyperglycaemia and T2D (Liu et al., 2011). Honeybush extract treatment reduced the average  $\alpha$ -cell size and subsequently the total  $\alpha$ -cell area leading to an improved  $\alpha$ - to  $\beta$ -cell ratio in OBIR rats. The reduction of  $\alpha$ -cell size and therefore glucagon secretion can have profound effects on glucagon induced insulin secretion and thereby alleviate the increased stress of hypersecretion on the  $\beta$ -cells (Liu et al., 2011). Similar to our findings, treatment of a high fat diet/STZ T2D mouse model with sitagliptin, a dipeptidyl peptidase-4 inhibitor and a new generation of antidiabetic drug, reversed the increased proportion and distribution of pancreatic  $\alpha$ -cells and thereby restored the  $\alpha$ - to  $\beta$ -cell ratio following chronic 11 week treatment. As with our study the reduction of the  $\alpha$ - to  $\beta$ -cell ratio reflected the overall improvement of the glucose and lipid metabolism (Mu et al., 2006). The fact that metformin and rosiglitazone had a similar beneficial effect on the pancreatic  $\alpha$ - to  $\beta$ -cell ratio as the honeybush extract is not surprising. Both metformin and rosiglitazone are established oral drugs for the treatment of T2D. Metformin, an activator of AMP-activated protein kinase (AMPK), not only ameliorates hyperglycaemia but has been shown to have additional beneficial effects on lipid metabolism. Activation of AMPK by metformin increases fatty acid oxidation by inactivation of acetyl-CoA carboxylase and suppression of lipogenesis by inhibiting SREBP-1 expression (Zhou et al., 2001). Rosiglitazone, a peroxisome proliferator activated receptors- $\gamma$  agonist, has been shown to improve insulin resistance and to have a positive effect on lipid metabolism in pre-diabetic animals by inhibiting malonyl-CoA and thereby increasing fatty acid oxidation in skeletal muscle and liver (Zhao et al., 2009). In addition, rosiglitazone has also been demonstrated to activate AMPK but via a different pathway from metformin (Fryer et al., 2002). Taken together, the positive morphological changes observed support the improved metabolic changes induced by chronic ingestion of aqueous honeybush extract.

## 5. Conclusion

The efficacy of an aqueous hot water honeybush extract in reducing hyperglycaemia was demonstrated in two different diabetic rodent models (STZ-induced T1D and diet-induced

T2D OBIR rat models). Furthermore, the extract promoted normoglycaemia in the diet-induced T2D OBIR rat model and improved other metabolic aberrations associated with T2D. The high concentration of mangiferin and hesperidin in the extract could be partially responsible for the observed effects.

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## Antihyperglycaemic Activity of *Bauhinia megalandra*

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### 1. Introduction

Glycaemia is a homeostatic parameter that is very efficiently controlled thanks to the equilibrium that exists between the mechanisms involved in supply and removal of blood glucose (Fig. 1).

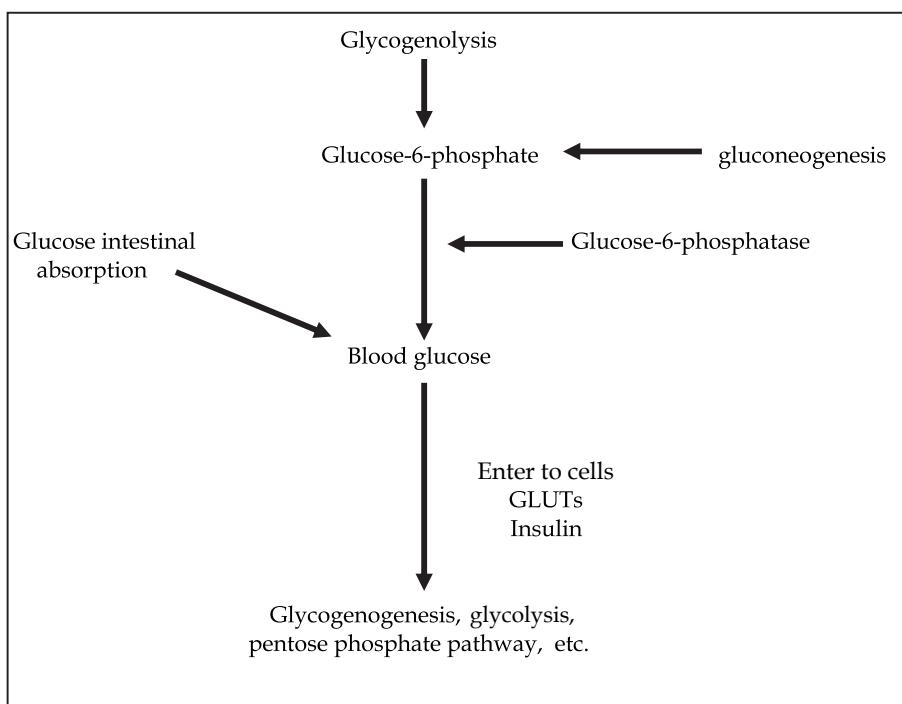


Fig. 1. Control of glycaemia. Plasma glucose concentration is closely controlled thanks to equilibrium between the mechanisms that supply glucose to the blood and remove it.

The intestinal absorption is one of the mechanisms that supplies glucose to the blood and occurs in two steps (Fig. 2): the first is mediated by the  $\text{Na}^+$ -glucose transporter (SGLT1)

located at the apical membrane of the enterocyte and it translates 2 Na<sup>+</sup> ions in favour of its concentration gradient per molecule of glucose moved in favour or against its concentration gradient into the cell, depending on the amount of the carbohydrate ingested and the time of digestion. The second step is carried out by the glucose transporter 2 (GLUT2) situated at the basolateral membrane of the enterocyte and this is able to transport glucose by a facilitated diffusion in or out of the cell (Nelson & Cox, 2005).

During a short fasting period, hepatic glycogen is degraded (glycogenolysis) in order to supply glucose to the blood. If the fasting period is prolonged a new synthesis of glucose occurs (gluconeogenesis) especially in liver and kidney. The final product of both processes is glucose-6-phosphate which is hydrolyzed to glucose and phosphate by the enzyme glucose-6-phosphatase (EC 3.1.3.9), allowing the glucose to exit the cell and enter the blood. In consequence this enzyme plays an important role in controlling the glycaemia (Ashmore and Weber 1959). The glucose-6-phosphatase is located in the endoplasmic reticulum (80-90 %) and nuclear envelope (10-20 %) mainly in the liver, kidney, enterocyte and β cells of pancreatic islets. Based on kinetic, genetic and molecular studies, Arion *et al.*, (1975) and Burchell & Waddell (1991) postulated that the glucose-6-phosphatase is constituted by (Fig. 3): A transmembrane protein, the catalytic subunit, whose active centre faces the cistern of the endoplasmic reticulum and shows a low specificity, being able to hydrolyze several phosphoric esters and pyrophosphate.

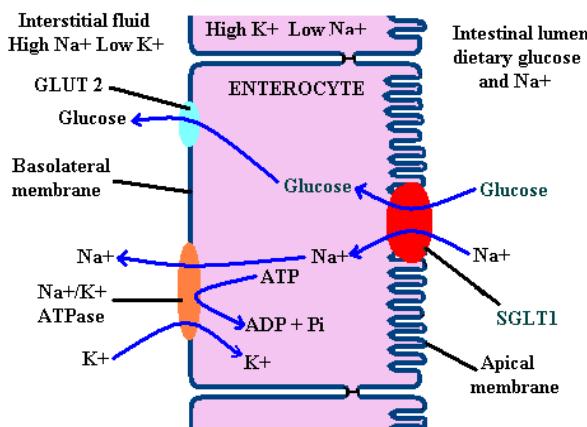
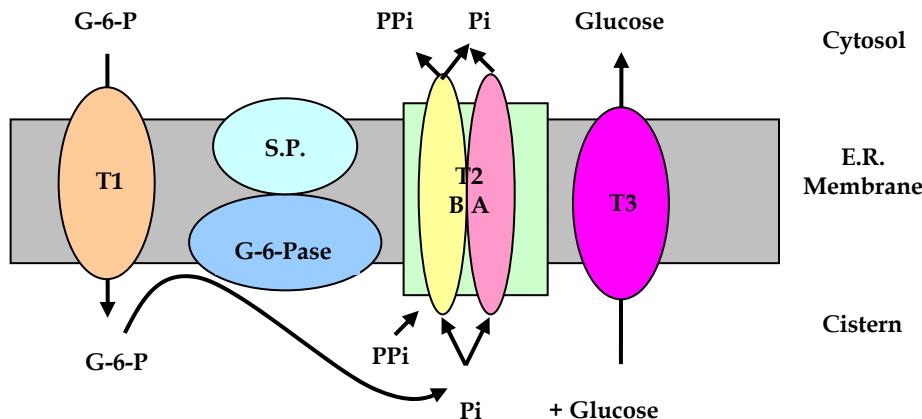


Fig. 2. Glucose intestinal absorption. It is a process that occurs in two steps, the first is mediated by SGLT1, which transports one molecule of glucose and 2 Na<sup>+</sup> and is located at the apical membrane of the enterocyte. The second is carried out by the GLUT2 which transports glucose in favour of its gradient and is placed at the basolateral membrane of the cell. The participation of the Na<sup>+</sup>-K<sup>+</sup> ATPase located on the basolateral membrane is also required to maintain the ionic gradients.

T1 is a highly specific transporter for glucose-6-phosphate, the substrate of the enzyme. The T2 is able to transport phosphate, pyrophosphate and carbamoylphosphate, and it has been suggested that it comprises 2 subunits. The glucose produced by the enzyme is transported by T3 also called GLUT 7. Finally it is thought there exists a stabilizing protein for the catalytic subunit. The glucose-6-phosphatase in addition to its hydrolytic activity, in conditions of high

glucose concentration and a phosphate donor such as pyrophosphate or carbamoylphosphate is able to synthesize glucose-6-phosphate (Foster & Nordlie, 2002).



1. Glucose-6-phosphate + H<sub>2</sub>O → glucose + phosphate
2. Mannose-6-phosphate + H<sub>2</sub>O → mannose + phosphate
3. Pyrophosphate + H<sub>2</sub>O → 2 phosphates
4. Glucose + pyrophosphate → glucose6-phosphate + phosphate
5. Glucose + carbamoylphosphate → glucose-6-phosphate + NH<sub>3</sub> + CO<sub>2</sub>

Fig. 3. Glucose-6-phosphatase model. Arion *et al.*, (1975) and Burchell & Waddell (1991) postulated the model of the catalytic subunit and transporters for the microsomal glucose-6-phosphatase. G-6-Pase: catalytic subunit; S.P.: stabilizing protein; PPi: pyrophosphate; Pi: phosphate and E.R.: endoplasmic reticulum. The glucose-6-phosphatase catalytic subunit is able to catalyze reactions 1-5.

The glucose transporters, GLUTs, (Joost & Thorens, 2001) mediate the facilitated transport of the glucose in and out of the different cells; of particular interest is the participation of the GLUT 4 which is located in skeletal and heart muscle and adipose tissue. The GLUT 4 is held in intracellular vesicles and by insulin action is translated to the plasma membrane and in consequence entry of glucose into those tissues increases thus lowering the glycaemia.

Drugs that inhibit the mechanism supplying glucose to the blood are considered antihyperglycaemic agents, on the other hand those that increase, directly or indirectly, the glucose entry to the tissues are considered hypoglycaemic agents. McCormack *et al.*, (2001) suggest that the enzyme glucose-6-phosphatase could be a potential target for antihyperglycaemic agents.

The microsomal fraction, which is enriched in endoplasmic reticulum, obtained by differential centrifugation is composed of intact and disrupted vesicles; the proportions of each can be measured assaying the glucose-6-phosphatase using mannose-6-phosphate as substrate, a molecule that is not translated by the T1 transporter (Arion *et al.*, 1976) in consequence all the mannose-6-phosphate hydrolyzed, by the microsomal fraction, is due to the activity of the catalytic subunit of the disrupted vesicles. The microsomal fraction

prepared without further treatment is called non-treated; the microsomes can be disrupted by the use of detergents, nitrogen cavitation, sonic disruption and more recently by the use of histones (Benedetti *et al.*, 2002). The term intact microsome is theoretical, and is estimated by subtracting from the enzyme activity of the non-treated microsomes that of the enzyme exhibited by the disrupted vesicles present in such preparations. Latency is a common characteristic of membranes bound enzymes, corresponding to the activity expressed only when the membrane are disrupted and is not present in the non-treated system. Generally it is calculated as the percentage of increase in activity due to the disruption of the vesicles.

Since time immemorial humans have been using plants for the empirical treatment of different illnesses, and in particular diabetes. In Venezuela, like many other tropical countries, the leaves of the *Bauhinia* species have been used by folk medicine in the empirical treatment of diabetes. The plants, belonging to the *Fabaceae* family (Hoyos 1978) are trees that can reach 10 m in height, with large well-developed evergreen leaves divided from the apex to 1/3 of its length and are hermaphrodite. The specie *Bauhinia megalandra* is characterized by having white flowers either single or in raceme with long and thin petals; the fruit is brown located in a compact pod. It is fast growing, long-lived and with deep roots (Figure 4).

The *Bauhinia megalandra* used on this work was always collected during the rainy season in the campus of the Universidad Central de Venezuela at Caracas and identified by Dr. Stefen Tillett of the Ovalles Herbarium of the Pharmacy Faculty of the Universidad Central de Venezuela.

## **2. Effects of *Bauhinia megalandra* leaf aqueous extract on gluconeogenesis and glucose-6-phosphatase**

Due to good results reported for metabolic studies using precision-cut liver slices (McKee *et al.*, 1988; Dogterom, 1993), we studied the gluconeogenesis capacity of 200 µm thick liver slices prepared from 48 h fasted rats using a Krundieck Tissue Slicer (Alabama Research and Development, Alabama, USA), and incubated as described elsewhere (Krebs *et al.*, 1963), in Krebs-Ringer bicarbonate buffer supplemented with bovine serum albumin saturated with oleic acid and using lactate or fructose as gluconeogenic substrate (Gonzalez-Mujica *et al.*, 1998) in the absence (control) or presence an aqueous extract of *Bauhinia megalandra* leaves. As shown in Table 1, the gluconeogenic activity of the control liver slices was almost linear during 90 minutes and of the same magnitude with both substrates.

In the presence of the plant extract there was a drastic decrease in the gluconeogenic capacity of the liver slices, this being of higher magnitude when lactate was the gluconeogenic substrate in comparison with fructose, these results suggest that in the *Bauhinia megalandra* leaf extract compounds are present that inhibit the gluconeogenesis at a point further on than the entrance of both substrates.

The microsomal fraction used as source of the enzyme glucose-6-phosphatase was prepared as described elsewhere (Marcuci *et al.*, 1983), briefly: livers from overnight fasted rat were homogenized in 3 volumes of 0.32 M sucrose, 3 mM MgCl<sub>2</sub>, centrifuged at 20000 g for 20 min at 4° C, the supernatant was centrifuged at 105000 g for 1h at 4° C, the pellet constituting the microsomal fractions and was resuspended in 0.25 M sucrose 1 mM MgCl<sub>2</sub> 5 mM HEPES pH 6.5 at a final protein concentration of 20 mg/mL and kept at -80° C until use. The entire microsomal fraction used in this work was at least constituted by 95 % intact vesicles.



Fig. 4. *Bauhinia megalandra*, flower and leaves.

Incubation Time (min)	Control		<i>Bauhinia megalandra</i>	
	Lactate or Fructose		Lactate	Fructose
30	31.1 ± 9.3		15.9 ± 7.3	18.1 ± 3.8
60	54.5 ± 17.2		11.6 ± 6.5	38.0 ± 6.0
90	90.2 ± 18.5		16.9 ± 8.8	34.0 ± 9.0

Table 1. Effects of *Bauhinia megalandra* leaf aqueous extract on hepatic gluconeogenesis. Liver slices from 48 hours fasted rats were incubated in Krebs-Ringer bicarbonate, supplemented with oleic acid saturated bovine serum albumin and in the presence of lactate or fructose as gluconeogenic substrate in the absence (control) or in the presence of 1 mg/mL of the *Bauhinia megalandra* leaf extract. At the indicated times, samples of the medium were withdrawn in order to measure glucose by the glucose oxidase-peroxidase method (Trinder 1969). The results are expressed in nmol of glucose produced/mg of liver dry weight and correspond to the means of 8-13 experiments ± standard deviation. The differences between control and plant extract treated were statistically significant at  $p<0.05$  for fructose and  $p<0.005$  for lactate.

The activity of the enzyme glucose-6-phosphatase was carried out following the method described by Burchell *et al.*, (1988) using glucose-6-phosphate or pyrophosphate as substrate in intact and disrupted (histone treated) hepatic microsomes. In brief, in a volume of 0.1 mL, 1-30 mM glucose-6-phosphate, 2 mM EDTA, 16 mM HEPES, pH: 6.5 and approximately 20 µg of microsomal proteins were present. In order to disrupt microsomes, 80 µg of histones were present in the incubation medium. The reaction was carried out at 30° C for 10 min without shaking and stopped by the addition of 0.9 mL of 0.28 % ammonium molybdate, 1.11 % SDS and 1.11 % ascorbic acid in 0.33 M sulphuric acid. The colour was developed at 47° C for 20 min and the absorbance was read at 820 nm. The assay was similar when pyrophosphate was

the substrate of the enzyme with the following differences: 0.5-5 mM pyrophosphate, 16 mM cacodilate, pH: 6.5, and the colour was developed at 30° C during 10 min.

Glucose-6-phosphate as substrate			
	Intact	Disrupted	
	V <sub>MAX</sub>	K <sub>M</sub>	V <sub>MAX</sub>
Control	7.4 ± 0.9*	5.4 ± 1.1	9.8 ± 0.8
10 µg/mL	6.2 ± 1.4	5.6 ± 0.7	9.8 ± 0.7
20 µg/mL	5.1 ± 1.0*	7.5 ± 0.6	9.5 ± 0.3

Pyrophosphate as substrate			
	Intact	Disrupted	
	V <sub>MAX</sub>	K <sub>M</sub>	V <sub>MAX</sub>
Control	6.6 ± 1.4	1.3 ± 0.5	7.0 ± 0.9
10 µg/mL	5.7 ± 1.6	1.0 ± 0.6	7.4 ± 0.8
20 µg/mL	6.0 ± 1.5	1.2 ± 0.4	6.6 ± 1.3

Table 2. Kinetic parameters of hepatic microsomal glucose-6-phosphatase in the absence or presence of the aqueous extract of *Bauhinia megalandra* leaves. The activity of the hepatic microsomal glucose-6-phosphatase was assayed using glucose-6-phosphate or pyrophosphate as substrate in the absence (control) or in the presence of the indicated concentration of the aqueous extract of *Bauhinia megalandra* leaves. The kinetic parameters were calculated from the Michaelis and Menten graphs using the Enzfitter program (Leatherbarrow 1987) and the V<sub>MAX</sub> for glucose-6-phosphatase activity is expressed in µmol of phosphate liberated/h × mg of microsomal proteins and the V<sub>MAX</sub> for the pyrophosphatase activity is expressed in µmol of pyrophosphate hydrolyzed/h × mg of microsomal proteins; for both the K<sub>M</sub> is expressed in mM. The experiments were performed using the liver of 3-5 fasted (24 h) rats, and each point represents the mean of 3-4 experiments ± standard deviation. \* Means differences statistically significant at p < 0.05.

As shown in Table 2, when glucose-6-phosphate was the substrate of the glucose-6-phosphatase of intact microsomes, the plant extract decreased in a statistically significant way the V<sub>MAX</sub> with a moderate increase of the K<sub>M</sub> without affecting the enzyme of disrupted microsomes.

The plant extract was without effect on the glucose-6-phosphatase activity in intact or disrupted microsomes when pyrophosphate was the substrate (Table 2). These results suggest that the compounds present in the *Bauhinia megalandra* aqueous extract inhibit one of the transporters of the glucose-6-phosphatase system, probably T1 (Gonzalez-Mujica et al., 1998). The inhibition of the hepatic gluconeogenesis could be a consequence of the inhibition of the glucose-6-phosphate transporter (T1).

The glucose-6-phosphatase activity of non-treated microsomes, when assayed with 15 mM glucose-6-phosphate as substrate, was linear during 30 min. (Figure 5); in the other hand, in the presence of 20 µg/mL of the plant extract the enzyme activity was linear just up to 20 min, but all the values were lower than the control, after that an inflection occurred and the slope of the line declined (Figure 5), suggesting that some compound(s) present in the *Bauhinia megalandra* leaf extract inhibit other transporter of the glucose-6-phosphatase

system, different than T1, and in consequence the enzyme products accumulated in the microsomal cistern leading to inhibition of the catalytic subunit. Due to the fact that the plant extract does not affect the kinetic parameters of the glucose-6-phosphatase using pyrophosphate as substrate, there is no effect on the pyrophosphate transporter (T2), it is possible to suggest that the inhibition occurs at the glucose transporter (T3).

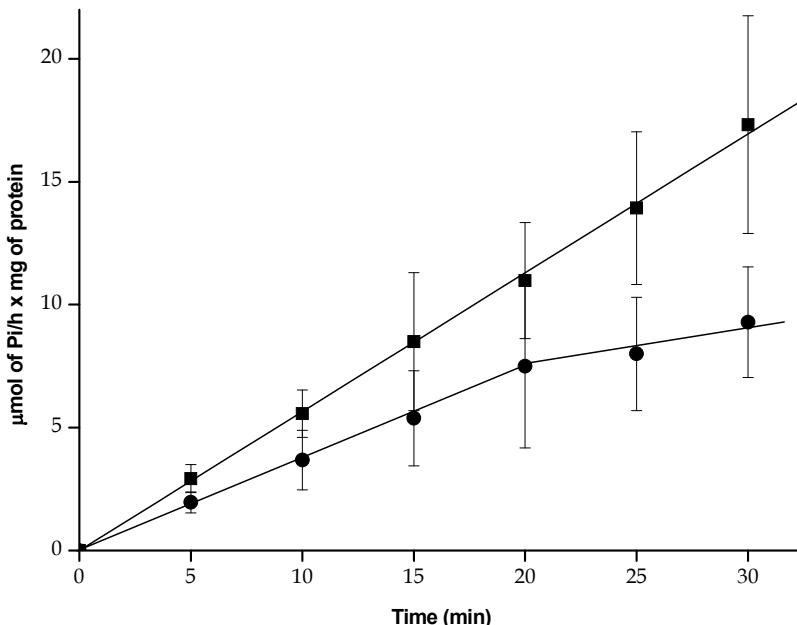


Fig. 5. Effects of *Bauhinia megalandra* leaf extract on the time course of hepatic microsomal glucose-6-phosphatase. The activity of the glucose-6-phosphatase was measured in the absence (■) or in the presence (●) of 20  $\mu\text{g}/\text{mL}$  of *Bauhinia megalandra* leaf extract at 15 mM glucose-6-phosphate; each point represents the mean of 5 experiments and the vertical bars correspond to standard deviation. The enzyme activity is expressed in  $\mu\text{mol}$  of phosphate (Pi) released /hour  $\times$  mg of protein. All the differences observed between control and extract treated microsomes were statistically significant at  $p < 0.05$  except that seen at 20 min.

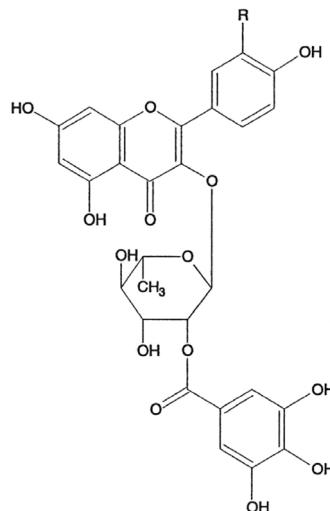
### **3. Isolation and identification of flavonoids from *Bauhinia megalandra* leaves. Characterization of their effects on hepatic microsomal glucose-6-phosphatase and gluconeogenesis**

We fractionated the methanol extract of fresh *Bauhinia megalandra* leaves using different solvents (methanol : water 1:1; ethyl acetate : acetone 8:2) and column chromatography on sephadex LH-20 and RP-18 (Estrada et al., 2005) to obtain the following flavonoids: astilbin (1), quercetin 3-O- $\alpha$ -rhamnoside (2), kaempferol 3-O- $\alpha$ -rhamnoside (3), quercetin 3-O- $\alpha$ -arabinoside (4), quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (5), kaempferol 3-O- $\alpha$ -(2"-galloyl)rhamnoside (6), quercetin (7) and kaempferol (8). The identification of the above mentioned flavonoids was established by comparing their  $^1\text{H}$  and  $^{13}\text{C}$

NMR chemical shifts and proton coupling constants in DMSO-d<sub>6</sub> with those reported (Agrawal, 1989; Markham and Geiger, 1994; De Brito et al., 1995; Méndez et al., 1995; Bilia et al., 1996) and the structure of compounds **5** and **6** is shown in Figure 6. To the best of our knowledge, this was the first report of the isolation of kaempferol 3-O- $\alpha$ -(2"-galloyl)rhamnoside from nature.

The effect of the isolated flavonoids on the activity of the hepatic glucose-6-phosphatase was carried out using intact and disrupted microsomes with glucose-6-phosphate as substrate as indicated above.

The inhibition of the enzyme by the flavonoids isolated from *Bauhinia megalandra* leaves in intact microsomes, but not in disrupted ones (Table 3), is a clear indication that they interact with the glucose-6-phosphate transporter (T1) of the glucose-6-phosphatase system with no effect on the catalytic subunit. It is interesting to point out that the flavonoids tested behave in a similar way to phlorizin (Table 3) only inhibiting the T1 transporter (Arion et al., 1980).



**5:** R = OH

**6:** R = H

Fig. 6. Structure of quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**5**) and kaempferol 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**6**)

As can be seen in Table 3, the aglycones, quercetin (**7**) and kaempferol (**8**), showed the lowest inhibitory effect on the enzyme and the percentage inhibition increased with the addition of the polar group rhamnose or arabinose as in the case of compounds **1**, **2**, **3** and **4**, which exhibited moderate effects. The flavanol glycoside evaluated (**1**) showed a similar behavior to compounds **2** and **3**, but to a smaller extent; this could be attributed to the saturation of the double bond in the C-ring of the flavonoid nucleus. The strongest inhibitory effect was observed when the galloyl moiety was present (**5** and **6**). This observation suggests the possibility of a particular interaction between this polyphenolic residue and the glucose-6-phosphate transporter (T1), because the complex formation

between proteins and tannins in which gallic acid is usually one of the main components, is well known (Haslam *et al.*, 1989).

Compound	Intact microsomes			Disrupted microsomes	
	Activity	%	IC <sub>50</sub>	Activity	%
Control	2.77 ± 0.28			5.66 ± 0.45	
Astilbin ( <b>1</b> )	2.23 ± 0.42 <sup>a</sup>	-19.5	9.59 ± 27	5.80 ± 0.43	+2.5
Quercetin 3-O- $\alpha$ -rhamnoside ( <b>2</b> )	1.95 ± 0.36 <sup>b</sup>	-29.6	246 ± 5	5.42 ± 0.53	-4.2
Kaempferol 3-O- $\alpha$ -rhamnoside ( <b>3</b> )	2.04 ± 0.25 <sup>b</sup>	-26.4	186 ± 4	5.84 ± 0.46	+3.2
Quercetin 3-O- $\alpha$ -arabinoside ( <b>4</b> )	2.04 ± 0.23 <sup>b</sup>	-26.4	1069 ± 31	5.77 ± 0.18	+3.0
Quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside ( <b>5</b> )	1.02 ± 0.22 <sup>c</sup>	-63.2	27 ± 1	6.43 ± 0.52 <sup>a</sup>	+13.6
Kaempferol 3-O- $\alpha$ -(2"-galloyl)rhamnoside ( <b>6</b> )	0.93 ± 0.27 <sup>c</sup>	-66.4	31 ± 2	6.61 ± 0.41 <sup>b</sup>	+16.8
Quercetin ( <b>7</b> )	2.43 ± 0.05 <sup>c</sup>	-12.3	1330 ± 39	5.81 ± 0.52	+2.7
Kaempferol ( <b>8</b> )	2.71 ± 0.49	-2.2	555 ± 6	5.98 ± 0.16	+5.7
Phorizin	2.00 ± 0.11 <sup>b</sup>	-27.8	4.66 ± 19	5.71 ± 0.40	+0.9

Table 3. Effects of flavonoids isolated from *Bauhinia megalandra* on glucose-6-phosphatase. The glucose-6-phosphatase was assayed using 5 mM glucose-6-phosphate as substrate in intact and disrupted microsomes in the absence (control) or presence of 50  $\mu$ M of each of the flavonoids isolated from *Bauhinia megalandra* leaves or 50  $\mu$ M phlorizin. The enzyme activity is expressed as  $\mu$ mol of phosphate released / h  $\times$  mg of microsomal protein and each value represents the means of 5-9 experiments  $\pm$  standard deviation. The percentage inhibition (-) or activation (+) was also calculated.

The IC<sub>50</sub> was carried out following Arion *et al.*, (1998) using intact microsomes with 1mM glucose-6-phosphate in the presence of increasing concentrations (including values below and above the IC<sub>50</sub>) of the isolated flavonoids; the result are the average of 3 experiments  $\pm$  standard deviation, and expressed in  $\mu$ M.

The increase in the glucose-6-phosphatase inhibition capacity with increase in the polarity of the flavonoids suggests a specific interaction with the glucose-6-phosphate transporter of the enzyme system because it cannot be related to a higher solubility in the endoplasmic reticulum membrane. Also, the interaction seems to be specific view of the fact that none of the flavonoids was able to inhibit the catalytic subunit of the glucose-6-phosphatase system (Table 3). The greater enzyme inhibition exerted by quercetin (**7**) in comparison to kaempferol (**8**)(Table 3) could be explained by the fact that the former has one additional vicinal hydroxyl group on the B-ring with respect to the latter. The difference in polarity between the aglycones is overcome by the presence of the carbohydrate and galloyl moieties, and in consequence, the inhibitory effect was also affected.

The compounds **5** and **6** showed the lowest IC<sub>50</sub> value and were very close to each other (Table 3). The lack of gallic acid in compounds **2** and **3** increased the IC<sub>50</sub> value 9 and 6 times respectively, with respect to compounds **5** and **6**. The aglycones, compounds **7** and **8**, exhibited high IC<sub>50</sub> values, which are 49 and 18 times higher than those obtained with compounds **5** and **6**, respectively (Table 3). Compounds **1** and **4**, although glycosylated, showed high IC<sub>50</sub> values, comparable to that observed with the aglycone quercetin (**7**). To

our knowledge, this has been the first report of the IC<sub>50</sub> for phlorizin and glucose-6-phosphatase. There is a good agreement between the percentages of glucose-6-phosphatase inhibition and the IC<sub>50</sub> produced by the flavonoids tested. The sugar bound to the flavonoid seems to be important due to the fact that substitution of rhamnose by arabinose increases by more than 4 times the IC<sub>50</sub> (Table 3). The reduction of the double bond of ring C, in astilbin, also increases the IC<sub>50</sub> by almost 4 times (Table 3).

All the flavonoid isolated, but one (2) produce activation of the glucose-6-phosphatase catalytic subunit, however, only compounds 5 and 6 (Table 3) do so to an appreciable magnitude and this could be due to modifications in the microsomal membranes that expose the active center of the enzyme, as a consequence of the presence of the tannic flavonoids.

Due to the fact, that quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (5) exerts high inhibition of the T1 transporter of the glucose-6-phosphatase system and showed the lowest IC<sub>50</sub> of all the flavonoids tested, we studied the effects of that compound on the kinetic parameters of the enzyme using glucose-6-phosphate or pyrophosphate as substrate. The enzyme assay was carried out as described above. The enzyme assays, with both substrates were carried out in the absence (control) or presence of 15  $\mu$ M quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (5).

#### Glucose-6-phosphate as substrate

	Intact microsomes		Disrupted microsomes	
	V <sub>MAX</sub>	K <sub>M</sub>	V <sub>MAX</sub>	K <sub>M</sub>
Control	7.59 ± 0.91	4.78 ± 1.10	11.57 ± 1.55	0.79 ± 0.27
Quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (5)	7.36 ± 1.79	7.44 ± 0.42*	10.47 ± 1.45	0.57 ± 0.17

#### Pyrophosphate as substrate

	Intact microsomes		Disrupted microsomes	
	V <sub>MAX</sub>	K <sub>M</sub>	V <sub>MAX</sub>	K <sub>M</sub>
Control	7.61 ± 2.45	2.28 ± 1.35	8.40 ± 1.73	0.40 ± 0.11
Quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (5)	6.72 ± 1.33	1.87 ± 0.41	7.58 ± 0.73	0.24 ± 0.07

Table 4. Effects of quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (5) on the kinetic parameters of the glucose-6-phosphatase. Microsomal glucose-6-phosphatase was assayed using glucose-6-phosphate (1-30 mM) or pyrophosphate (0.5-5 mM) as substrate in the absence (control) or presence of 15  $\mu$ M quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (5) as described in the text. The kinetic parameters were calculated from the Michaelis and Menten graph using the Enzfitter program. The V<sub>MAX</sub> is expressed as  $\mu$ mol of phosphate released /h/mg proteins when glucose-6-phosphatase was the substrate and in  $\mu$ mol of pyrophosphate hydrolysed /h/mg proteins and the K<sub>M</sub> in mM. Each value represents the means of 3-5 experiments ± standard deviation and in each experiment, the livers of 3-4 rats were used. \* Means differences statistically significant at p < 0.005.

Table 4 show the effects of quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (5) on the kinetic parameters of the microsomal glucose-6-phosphatase using glucose-6-phosphate or pyrophosphate as substrate. In controls using glucose-6-phosphate as substrate, when the microsomes were disrupted by the presence of histones, there was an increase in the V<sub>MAX</sub> with a latency of 34.4% and the K<sub>M</sub> was 1/6 of the value observed in the intact system; these results are in good agreement with those reported elsewhere (Blair and Burchell, 1988;

Gonzalez-Mujica *et al.*, 1993, 1998). In intact microsomes the presence of quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**5**) increased the  $K_M$  for glucose-6-phosphate by nearly 56%, a change that was statistically significant at  $p < 0.005$ . On the other hand, there were no changes in the  $V_{MAX}$  of intact microsomes neither in the  $V_{MAX}$  nor in the  $K_M$  of the disrupted system. When pyrophosphate was the substrate, the activity showed a lower latency (9.4%) and a similar decrease of the  $K_M$  (1/6) compared with that shown when glucose-6-phosphate was the substrate and the microsomes were disrupted, results that are similar to those reported by others (Blair and Burchell, 1988; Gonzalez-Mujica *et al.*, 1993, 1998). The kinetic parameters of the enzyme using pyrophosphate as substrate in intact and disrupted microsomes were not altered by the inclusion of the flavonol.

From the above results: increase of the  $K_M$  for glucose-6-phosphate in intact microsomes without affecting the other kinetic parameter in intact or disrupted microsomes using glucose-6-phosphate or pyrophosphate as substrates, is a clear indication that quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**5**) behaves as a competitive inhibitor of the T1 transporter with out altering the catalytic subunit nor the other transporters of the glucose-6-phosphatase system. It is interesting to point out that a synthetic derivative of chlorogenic acid, a compound without any structural relationship with glucose-6-phosphate has been reported as a competitive inhibitor of T1 (Arion *et al.*, 1998)

Hepatic gluconeogenesis was studied using 48 h fasted rat liver slices of 200  $\mu\text{m}$  thick incubated in Krebs-Ringer bicarbonate buffer, as described above, in the absence (control) or the presence of 30  $\mu\text{M}$  quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**5**). The incubations were carried out with 10 mM lactate as gluconeogenic substrate, at 37 °C, under an O<sub>2</sub>:CO<sub>2</sub> (95:5) atmosphere, with continuous shaking in an orbital bath (60 cycles/min), for a total time of 90 min. Samples for glucose determination were taken every 30 min. At the end, the slices were dehydrated with acetone and dried at 70 °C for 24 h and the dry weight of the slices was determined. Glucose determination was made by the glucose oxidase method (Trinder, 1969) and the gluconeogenic activity was expressed as nmol of glucose produced/mg liver dry weight.

The gluconeogenic capacity of the rat liver slices in the absence (control) and presence of quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**5**) is shown in Fig. 7. The glucose production by the control liver slices was almost linear with time and similar to that reported earlier (Gonzalez-Mujica *et al.*, 1998). Quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**5**) drastically reduced the gluconeogenic capacity of the liver slices at all the times studied: at 30 min the inhibition was approximately 85%; the inhibition was nearly 50% at 60 min. and 41% at 90 min.

The inhibition of the T1, the glucose-6-phosphate transporter, of the hepatic microsomal glucose-6-phosphatase system by quercetin 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**5**), might explain the inhibition of the gluconeogenic capacity of the rat liver slices in the presence of the flavonol and, in turn, could reduces the hepatic glucose production; this antihyperglycaemic effect serve to decrease the blood glucose level in diabetic patients.

#### 4. Effects of *Bauhinia megalandra* leaf aqueous extract on glycogenolysis

An aqueous extract of *Bauhinia megalandra* leaves was administered by a gastric tube to rats anesthetized with sodium pentobarbital (30 mg/Kg body weight), 1 h later a blood sample was taken from the tail and 0.15 mg/Kg body weight of epinephrine (Cori and Cori 1928) was

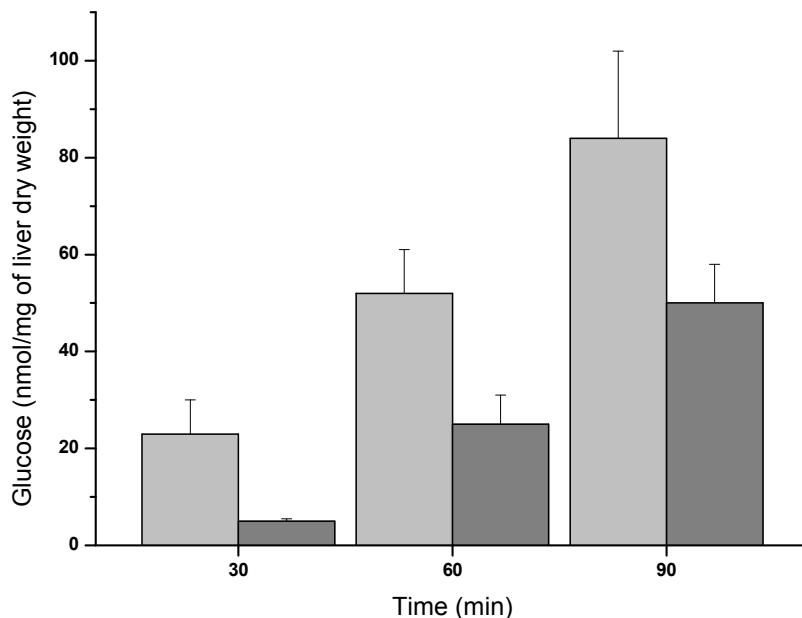


Fig. 7. Effects of quercetin 3-O- $\alpha$ -(2''-galloyl)rhamnoside (5) on hepatic gluconeogenesis. Liver slices from 48 h fasted rats were incubated in 4 mL Krebs-Ringer bicarbonate buffer supplemented with oleate saturated albumin using lactate as gluconeogenic substrate, in the absence (light grey) or presence of 30  $\mu$ M of Quercetin 3-O- $\alpha$ -(2''-galloyl)rhamnoside (5) (dark grey). At the indicated times, medium samples were withdrawn in order to measure glucose by the glucose oxidase method. The results are the average of 7–9 separate experiments  $\pm$  standard deviation. All the differences between the gluconeogenesis of controls and quercetin 3-O- $\alpha$ -(2''-galloyl)rhamnoside (5) treated liver slices were statistically significant at  $p < 0.005$ .

given by intraperitoneal injection, and afterwards blood samples were taken, as before, every 30 min for 2 h; glucose was estimated by the glucose oxidase-peroxidase method (Trinder 1969).

As shown in Figure 8, the administration of epinephrine substantially increases the glycaemia with a maximum at 60 min (over 80 % increases). When the plant extract was given before the catecholamine there was an important reduction of the hyperglycaemia induced by epinephrine (over 24 % reduction), been statistically significant at 60 min (Fernández-Peña, et al., 2008). These results suggest the intestinal absorption of compounds present in the *Bauhinia megalandra* leaves, which reach the liver via the portal vein and are capable of inhibiting the glycogenolysis direct or indirectly.

In order to establish the mechanism of action of such compounds, we incubated fed rats liver slices in the same way as described before (Figure 7) with just buffer alone (control) or in the presence of epinephrine (3.3  $\mu$ M) or dibutyryl cyclic AMP (10 nM) or the plant extract (3.44 mg/L) or the combinations of epinephrine and the vegetal extract or dibutyryl cyclic AMP and the plant extract.

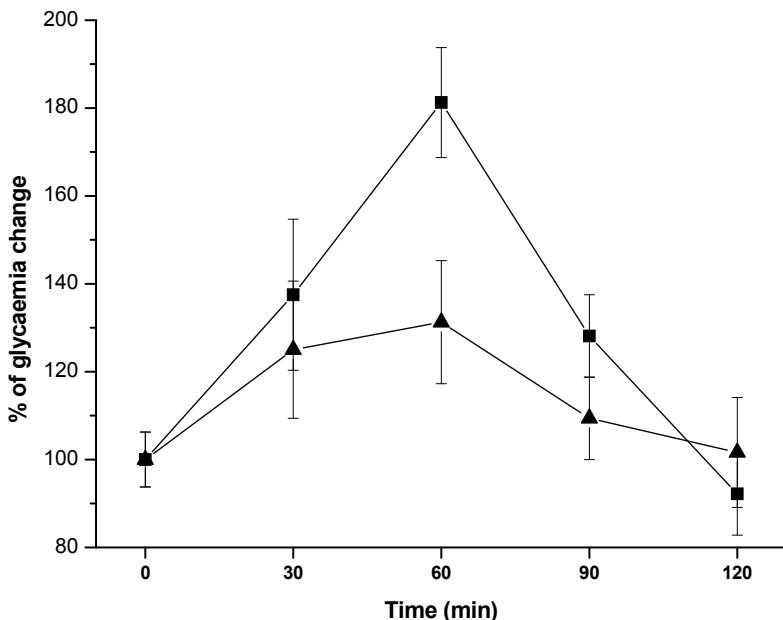


Fig. 8. Effects of *Bauhinia megalandra* aqueous extract on the hyperglycaemia induced by epinephrine. Male rats were anaesthetized (sodium pentobarbital 30 mg/Kg body weight) before the administration by a gastric tube of water (control -■-) or 3.44 g/Kg of body weight of the plant extract (-▲-). One h later the animals receive by intraperitoneal injection 0.15 mg/Kg body weight of epinephrine and the glycaemia was measured every 30 min for 2 h. The basal glycaemia of fed rats (100 %) was  $7.2 \pm 0.5$  mM. The values represent the means  $\pm$  standard deviation of 5 – 6 experiments and in each 3 rats were used. At 60 min the differences between control and experimental was statistically significant  $p < 0.001$ .

As shown in Figure 9 the amount of glucose released by the rat liver slices was increased by the presence of the catecholamine, being 60 and 54% of the control values at 60 and 90 min respectively. When epinephrine and the plant extract were present simultaneously, the glucose production by the liver slices was almost parallel with, but significantly lower than that observed with the hormone alone (Figure 9), being 35; 25 and 24% of the control values at 30; 60 and 90 min respectively. It is interesting to point out, that at 30 min the glucose production in the joint presence of epinephrine and the plant extract was 35% lower than that observed in the control.

As shown in Figure 10, the presence of dibutyryl cAMP produced an increase in the glucose release by the liver slices (Fernández-Peña, *et al.*, 2008) at all times studied, being more evident at 60 min, when it was 2.6 times the control value.

In the joint presence of the cyclic nucleotide and the plant extract there was a drastic reduction of the dibutyryl cAMP effect, at 60 min the glucose production was decreased by almost 50 %. The results presented (Figures 8; 9 and 10) strongly suggest that in *Bauhinia megalandra* leaf extract there are compounds that inhibit hepatic glycogenolysis. We also presented evidences that the plant extract inhibits the gluconeogenesis (Table 1 and Figure 7),

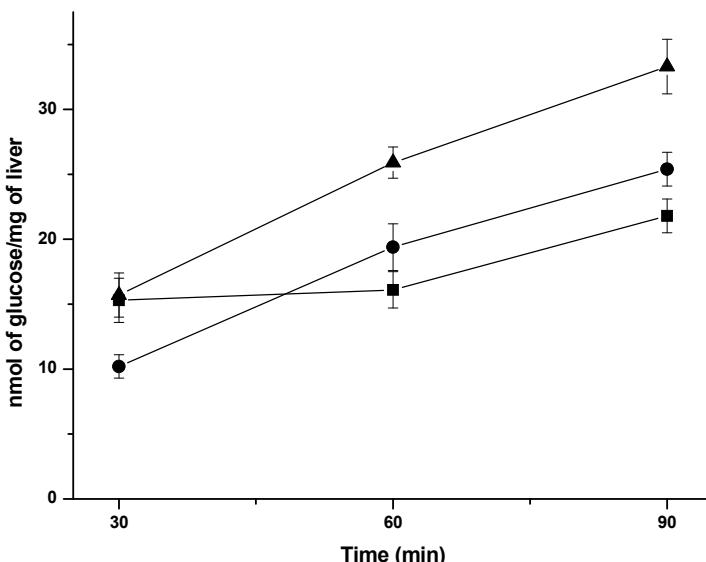


Fig. 9. Effects of epinephrine and of *Bauhinia megalandra* leaf extract on hepatic glycogenolysis. Fed rats liver slices were incubated in 4 mL of Krebs-Ringer bicarbonate buffer pH 7.4 in an atmosphere of O<sub>2</sub>/CO<sub>2</sub> 95/5 without additions for control (-■-), or in the presence of 3.3 µM epinephrine (-▲-), or the combination of 3.3 µM epinephrine and 13.8 µg of the plant extract (-●-). At the indicated times glucose was measured in samples of the incubation medium as before. The values are the means ± standard deviations of 5–6 experiments, in each 3 rats were used. At 60 and 90 min, the differences between epinephrine and control and the combination of epinephrine and the plant extract were statistically significant at p<0.001.

and due to the fact that the only enzyme common to both metabolic pathways, glycogenolysis and gluconeogenesis is the glucose-6-phosphatase in consequence this enzyme should be the target of the compounds present in *Bauhinia megalandra* leaf extract. Furthermore, the plant extract and the flavonoids isolated from it, inhibit the T1, the glucose-6-phosphate transporter of the hepatic microsomal glucose-6-phosphatase system. The above result, strongly suggest that the flavonoids present in *Bauhinia megalandra* leaf extract inhibit hepatic glycogenolysis and gluconeogenesis because of inhibition of the glucose-6-phosphatase and in consequence there is a reduction in the glucose production by the liver. These flavonoids might be useful in the treatment of non insulin dependent diabetes because of its antihyperglycaemic activity.

##### **5. Effects of *Bauhinia megalandra* aqueous extract and Kaempferol 3-O-α-rhamnoside on glucose intestinal absorption**

The glucose intestinal absorption was measured by the method described by Gonzalez-Mujica *et al.*, (2003). In rats anaesthetized with sodium pentobarbital (60 mg/Kg body weight), the intestine was exposed and divided *in situ* into 4 cm segments by ligatures; 1 mL of 10 mM glucose, 0.9 % NaCl alone or with the plant extract (1.14-9.10 mg) or extract

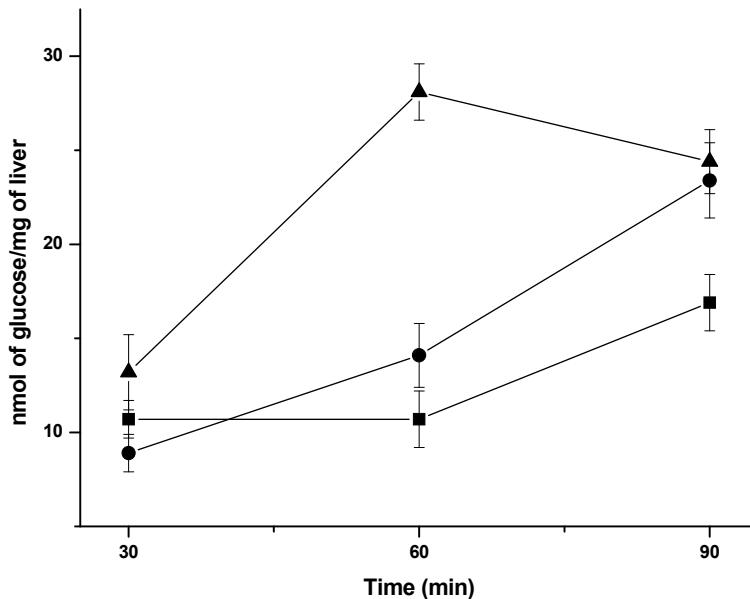


Fig. 10. Effects of dibutyryl cAMP and of *Bauhinia megalandra* leaf extract on hepatic glycogenolysis. All conditions were the same as those indicated in Figure 9, without addition for control (-■-), or in the presence of 10 nM of dibutyryl cAMP(-▲-), or the combination of 10 nM of dibutyryl cAMP and 13.8 µg of the plant extract (-●-). At 60 min the differences observed between the values obtained in the presence of the cyclic nucleotide alone and those in controls and in the presence of the mixture of dibutyryl cAMP and the plant extract were statistically significant at  $p < 0.001$ .

fractions (10 mg/mL) or pure compounds (5 mM), as indicated in the figures, was injected. Phlorizin, a well known inhibitor of sodium-glucose cotransporter 1 (SGLT1)(Panayatova-Heiermann, *et al.*, 1995), at a concentration of 0.1 mM was used as a positive control. After 30 min the intestinal segment contents were recovered without significant change in volume, and the glucose was measured (Trinder, 1969).

As shown in Table 5, the aqueous extract of *Bauhinia megalandra* leaves inhibits the glucose intestinal absorption in a concentration- dependant way. When the plant extract (4.55 mg) was injected together with 0.1 mM phlorizin, an additive inhibitor effect was observed. The concentration dependent inhibition exerted by the plant extract indicates a specific action and due to the fact that phlorizin is a known inhibitor of the sodium-glucose cotransporter 1 (Panayatova-Heiermann, *et al.*, 1995), the observed additive effect of *Bauhinia megalandra* leaf extract and phlorizin suggest that in the plant there are compounds that inhibit that cotransporter.

Vesicles from enterocyte apical membrane were prepared by the  $\text{Ca}^{++}$  precipitation method described by Kessler *et al.*, (1978). The intestinal membrane vesicles obtained contained maltase measured as described by SaMoita, *et al.*, (1989) in a ratio vesicles/homogenate 9.84; a small amount of  $\text{Na}^{+}/\text{K}^{+}$  ATPase sensitive to ouabain, estimated following Del Castillo & Robinson (1982) in a ratio vesicles/homogenate 0.42; succinate-cytochrome c reductase

<i>B megalandra</i> Extract	A		B	
	nmol of glucose absorbed	Addition	nmol of glucose absorbed	
0 mg	774.2 ± 34.4*	0	773.6 ± 70.3	
1.14 mg	602.2 ± 103.2**	4.55 mg <i>B megalandra</i> extract	348.9 ± 79.1**	
2.28 mg	464.5 ± 60.2**	0.1 mM phlorizin	392.1 ± 80.9**	
4.55 mg	395.7 ± 51.6**	4.55 mg <i>B megalandra</i>		
6.5 mg	223.7 ± 36.1**	extract + 0.1 mM phlorizin	87.9 ± 35.2**	
9.10 mg	141.1 ± 25.8**			

Table 5. Effects of *Bauhinia megalandra* extract on glucose intestinal absorption. In each of four consecutive intestinal segments were injected, *in situ*, 1mL of 10 mM glucose, 0.14% NaCl alone (0 mg) or in the presence of increasing amounts of *Bauhinia megalandra* leaf extract (A); or in the presence of 4.55 mg of the plant extract, or 0.1 mM phlorizin or the combination of 4.55 mg of plant extract and 0.1 mM phlorizin (B). After 30 min the glucose was measured in the intestinal segments content, expressed in nmol of glucose absorbed in 30 min and each value corresponds to the mean ± standard deviation of eight experiments. \* Means p< 0.005 and \*\* means p<0.0005.

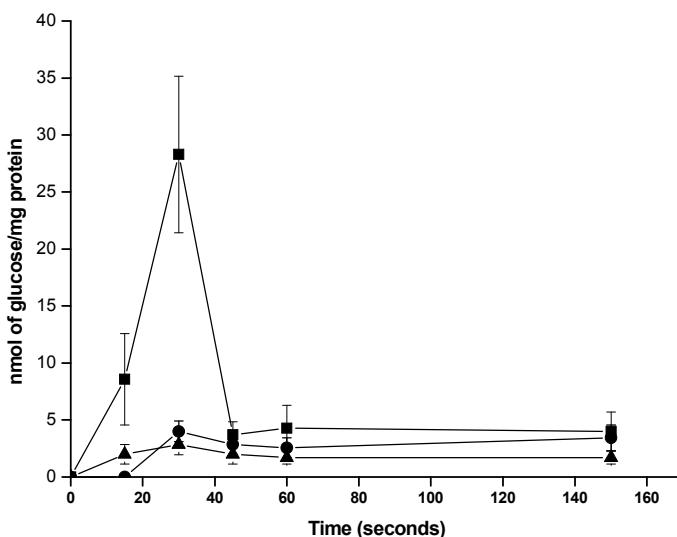


Fig. 11. Effects of *Bauhinia megalandra* on  $^{14}\text{C}$ -glucose uptake by enterocyte brush border membrane vesicles. Rat enterocyte brush border membrane vesicles internally filled with 100 mM KCl were incubated with 2 mM  $^{14}\text{C}$ -glucose (0.1  $\mu\text{Ci}$ ) and 100 mM NaCl for the control (-■-) or with the addition of 4.55  $\mu\text{g}$  of *Bauhinia megalandra* aqueous extract (-▲-) or with the addition of 1 mM phlorizin (-●-). At the indicated times the reaction was stopped by the fast filtration method (Hopfer, *et al.*, 1973) and the radioactivity was measured. The values are expressed in nmol of glucose uptake/ mg of vesicles protein and correspond to the mean ± standard deviation of 9 experiments with at least 2 rats in each one.

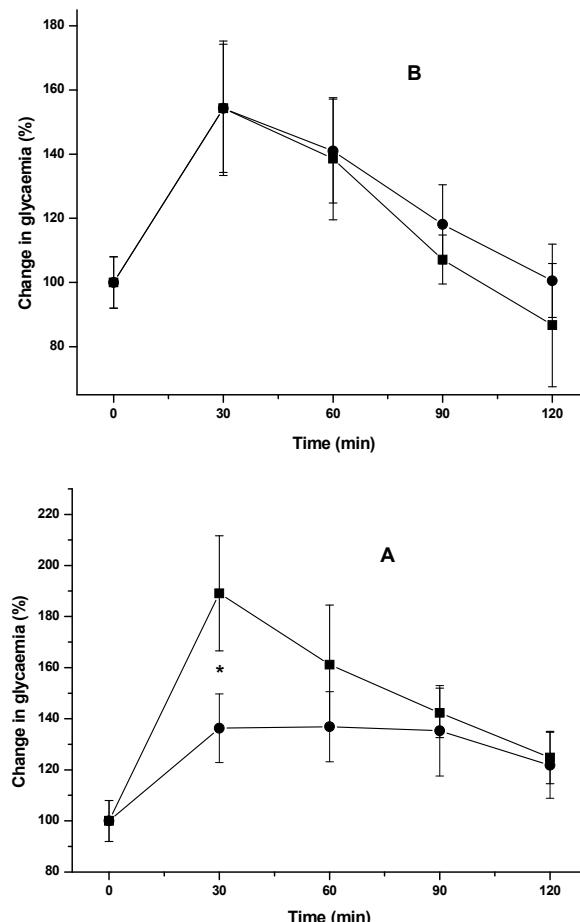
quantified by the method of Green, *et al.*, (1955) in a ratio vesicles/homogenate 0.36 and  $\beta$ -glucuronidase measured as described by Bergmeyer (1965) in a ratio vesicles/homogenate 0.09. Electronmicroscopy showed vesicles with a single membrane without electron dense material inside. These results are in good agreement with those reported by Kessler *et al.*, (1978) and indicate that the vesicles fraction obtained was enriched with brush border membrane, with low contamination with other cellular membrane. The  $^{14}\text{C}$ -glucose uptake by the intestinal brush border membrane vesicles, obtained as described above, was performed by the fast filtration method (Hopfer, *et al.*, 1973) in the absence or presence of the *Bauhinia megalandra* leaves extract or 1 mM phlorizin.

As shown in Figure 11 the  $^{14}\text{C}$ -glucose uptake by intestinal brush border membrane vesicles exhibits a peak at 30 s followed by a plateau between 45 and 150 s, a result very similar to that previously published (Kessler *et al.*, 1978). When the intestinal brush border membrane vesicles were incubated in the presence of 4.55  $\mu\text{g}$  of the plant extract or 1 mM phlorizin there was complete inhibition of the 30 s  $^{14}\text{C}$ -glucose peak uptake. These results clearly indicate that in the plant extract there are compounds that inhibit the sodium-glucose cotransporter 1 and in consequence are similar to those reported for green tea polyphenols (Kobayashi, *et al.*, 2000) and soyabean isoflavone (Vedavanam, *et al.*, 1999).

As shown in Figure 12-A, the simultaneous administration of *Bauhinia megalandra* leaf extract (260 mg) together with glucose (1 g/Kg body weight) by gastric catheter produced an almost flat glucose tolerance, with a decrease in nearly 28 % in the 30 min peak which was statistically significant in comparison with the control receiving water instead of the plant extract. On the other hand, when the *Bauhinia megalandra* leaf extract (260 mg) was administrated orally and the glucose (1 g/Kg body weight) by subcutaneous injection (Figure 12-B) there was no difference with the control that received water instead of the plant extract. The flat oral glucose tolerance (Figure 12-A) in comparison with the normal subcutaneous glucose tolerance (Figure 12-B) when the glucose was administered simultaneously with plant extract, strongly suggest that the main effect of *Bauhinia megalandra* extract is on the glucose intestinal absorption without any important effect on insulin release nor on the tissue glucose consumption.

We tested the effects of: quercetin 3-O- $\alpha$ -rhamnoside (**2**), kaempferol 3-O- $\alpha$ -rhamnoside (**3**) and kaempferol 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**6**), presented in Table 3, with the intention to establish the compound, present in *Bauhinia megalandra* leaves, responsible for the inhibition of the glucose intestinal absorption. The glucose intestinal absorption was measured using the method described above (Gonzalez-Mujica, *et al.*, 2003) in the absence (control) or the presence of 5 mM of the compounds **2**, **3** or **6**; phlorizin at a concentration of 0.1 mM was used as a positive control. The combined effect of kaempferol 3-O- $\alpha$ -rhamnoside (**3**) and 0.1 mM phlorizin was also studied (Rodríguez *et al.*, 2010). The kinetic parameters,  $K_M$  and  $V_{MAX}$  of glucose intestinal absorption were measured using increasing glucose concentration (12.5 – 50 mM), in the absence and in the presence of 5 mM kaempferol 3-O- $\alpha$ -rhamnoside (**3**), but reducing the time of absorption to 15 min in order to guarantee measurement at the initial velocity.  $K_M$  and  $V_{MAX}$  were calculated from the Michaelis and Menten graph (Rodríguez *et al.*, 2010) using the Enzfitter program (Leatherbarrow, 1989).

As shown in Table 6, kaempferol 3-O- $\alpha$ -rhamnoside (**3**) exerts a statistically significant inhibition of glucose intestinal absorption, which was more than double that exhibited by quercetin 3-O- $\alpha$ -rhamnoside (**2**), inhibition also statistically significant; on the other hand kaempferol 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**6**) lacked any effect.



\* Means difference statistically significant at  $p < 0.005$ .

Fig. 12. Effects of *Bauhinia megalandra* extract on oral and subcutaneous glucose tolerance. Rats under light anesthesia (30mg of sodium pentobarbital/Kg of body weight) received 1 g/Kg body weight of glucose by gastric catheter (A) or by subcutaneous injection (B) together with 260 mg of *Bauhinia megalandra* leaf extract (-●-) or an equivalent amount of water (-■-) by gastric catheter. At the indicated times, blood samples were taken for glucose determination. The glycaemia at time zero was  $75.0 \pm 9.4$  mg/dl. The results are expressed as the percentage of change in relation to zero-time value (100 %) and represent the means  $\pm$  standard deviation of 7 experiments with at least 2 rats in each one.

Regarding chemical structure and related biological activity, it is interesting to note that the presence of the galloyl group bound to the rhamnosyl moiety in kaempferol 3-O- $\alpha$ -(2''-galloyl)rhamnoside (**6**) annuls the inhibitory effect of kaempferol 3-O- $\alpha$ -rhamnoside (**3**). The rhamnosyl moiety is present in both kaempferol 3-O- $\alpha$ -rhamnoside (**3**) and quercetin 3-O- $\alpha$ -rhamnoside (**2**) but only the first compound efficiently inhibit glucose intestinal absorption,

indicating that the hexose is not a determinant for biological activity. The presence of an H (kaempferol 3-O- $\alpha$ -rhamnoside) in stead of an OH (quercetin 3-O- $\alpha$ -rhamnoside) in the 3' position of the flavonoid B ring is a determinant for the biological activity.

Compound	Glucose absorption nmol/30 min	% inhibition
Control	933, 4 ± 44.3	
Quercetin 3-O- $\alpha$ -rhamnoside ( <b>2</b> )	822.2 ± 22.1 *	12
kaempferol 3-O- $\alpha$ -rhamnoside ( <b>3</b> )	666.7 ± 4.5 **	29
Kaempferol 3-O- $\alpha$ -(2"-galloyl)rhamnoside ( <b>6</b> )	895.6 ± 17.8 NS	4
Phlorizin	700.0 ± 71.1 **	25
kaempferol 3-O- $\alpha$ -rhamnoside ( <b>3</b> ) + Phlorizin	300.0 ± 44.3 ***	68

Table 6. Effects of flavonoids purified from *Bauhinia megalandra* leaves and phlorizin on the glucose intestinal absorption. Isolated intestinal segments were injected *in situ* with 1 mL of 10 mM glucose and 0.9 % NaCl for control. In the test procedures the flavonoids: quercetin 3-O- $\alpha$ -rhamnoside (**2**), kaempferol 3-O- $\alpha$ -rhamnoside (**3**) or Kaempferol 3-O- $\alpha$ -(2"-galloyl)rhamnoside (**6**) were added at a final concentration of 5 mM. The final concentration of phlorizin used was 0.1 mM, and the combination of 5 mM kaempferol 3-O- $\alpha$ -rhamnoside (**3**) and 0.1 mM phlorizin was also used. After 30 min the glucose absorbed was estimated. The results correspond to the means ± standard deviation of 4 experiments. \* Indicate p< 0.05, \*\*Indicate p< 0.005 \*\*\*indicate p<0.001 and NS not significant, according to the Student t test.

As it can be seen in Table 6, the combined use of kaempferol 3-O- $\alpha$ -rhamnoside (**3**) and phlorizin inhibits glucose intestinal absorption by more than double the effect of either of these alone, indicating an additive effect of both compounds. Due to the fact that phlorizin is a known inhibitor of the sodium-glucose cotransporter 1 (Panayatova-Heiermann, *et al.*, 1995) in consequence kaempferol 3-O- $\alpha$ -rhamnoside (**3**) should be acting on the same transporter.

	K <sub>M</sub> in mM	V <sub>MAX</sub> in $\mu$ mol/min
Control	38.93 ± 4.92 *	42.33 ± 5.02 NS
Kaempferol 3-O- $\alpha$ -rhamnoside ( <b>3</b> )	67.24 ± 11.12 *	50.04 ± 4.59 NS

Table 7. Effect of kaempferol 3-O- $\alpha$ -rhamnoside (**3**) on the kinetic parameters of glucose intestinal absorption. Isolated intestinal segments were injected *in situ* with 0.9 % NaCl and increasing concentration of glucose (12.5-50 mM) for control, and in the test procedure 5 mM kaempferol 3-O- $\alpha$ -rhamnoside was added. After 15 min the glucose absorbed was estimated and the K<sub>M</sub> and V<sub>MAX</sub> were calculated from the Michaelis and Menten graph using the Enzfitter program (Leatherbarrow, 1987). The results correspond to the means ± standard deviation of 4 experiments. \* Means p< 0.005, NS means not significant according to the Student t test.

As shown in Table 7 the control values of the kinetic parameters of glucose intestinal absorption are different to those reported by Ader, *et al.*, (2001) and Li, *et al.*, (2006), however it is important to point out that in these two cases the  $K_M$  and  $V_{MAX}$  correspond to the sodium-glucose cotransporter function and in our case the kinetic parameters correspond to the total process of the glucose intestinal absorption. The presence of 5 mM kaempferol 3-O- $\alpha$ -rhamnoside (**3**) increases in 1.7 times the  $K_M$  without a significant change by the  $V_{MAX}$  of the glucose intestinal absorption, these results strongly suggest that kaempferol 3-O- $\alpha$ -rhamnoside (**3**) behaves as a competitive inhibitor of the intestinal sodium-glucose cotransporter 1.

## 6. Intestinal absorption of flavonoids from *Bauhinia megalandra*

The compounds present in the plant extract, prior to develop any biological function, have to be absorbed by the intestine and pass through the blood to reach the target tissue. With the intention of obtaining information on the intestinal absorption of the compounds present the *Bauhinia megalandra* leaves; we studied the urine of rats that drank, during a week, the plant extract instead of water. The urine was treated with acid acetone, the supernatant dried and fractionated using different solvents and column chromatography on silica gel to yield a compound that was present neither in the plant extract nor in the urine of the control rats. The structure of the compound was established using  $^1H$  and  $^{13}C$  NMR as being an ortho-substituted benzene; however, currently we do not know the types of radicals present (results not published). Due to the fact, that the ortho-substituted benzene was present neither in the plant extract nor in the *Bauhinia megalandra* leaf extract, this must correspond to a compound, probably a flavonoid, present in the plant extract that was absorbed at the intestine level, afterwards metabolized and excreted in the urine. This ortho-substituted benzene, is different to the phenolic acid that has been reported (Touriño, *et al.*, 2009) are eliminated by the urine of animals that ingest flavonoids. This is direct evidence of the intestinal absorption of compounds present in the *Bauhinia megalandra* leaf extract.

## 7. Conclusions

The aqueous extract of *Bauhinia megalandra* leaves inhibited the gluconeogenesis and glycogenolysis, stimulated by epinephrine or dibutyryl AMPc, activity of liver slices, reduced the hyperglycaemia promoted by epinephrine in the intact animal and inhibited the glucose intestinal absorption in a dose dependant way by affecting the SGLT 1. From the methanol extract of the *Bauhinia megalandra* leaves, we purified and characterized 8 flavonoids for one of which, kaempferol 3-O- $\alpha$ -(2"-galloyl)-rhamnoside (**6**), to the best of our knowledge, this was the first report of its being isolated from nature; all of the flavonoids isolated were able to inhibit the enzyme glucose-6-phosphatase, the most active ones being those with the galloyl group with IC<sub>50</sub> of approximately 30  $\mu$ M. Quercetin 3-O- $\alpha$ -(2"-galloyl)-rhamnoside (**5**) behaved as a competitive inhibitor of the glucose-6-phosphatase transporter of the glucose-6-phosphate system and drastically reduced the liver gluconeogenic activity. The reduction of the glycogenolysis and gluconeogenesis might be a consequence of the inhibition of the hepatic glucose-6-phosphatase by the flavonoids present in the plant extract. Kaempferol 3-O- $\alpha$ -rhamnoside (**3**) was a competitive inhibitor of

the glucose intestinal absorption. In the urine of the rats that drank the *Bauhinia megalandra* leaves aqueous extract we identified an ortho-substituted benzene, probably a product of the metabolism of one of the flavonoides.

We have presented direct and indirect evidences of the intestinal absorption of compounds present in the *Bauhinia megalandra* leaf extract which are responsible for the reduction in the hepatic production of glucose and of the glucose intestinal absorption, events that are important for the reduction of the supply of glucose to the blood. As a consequence the compounds present in this plant may well be useful in the treatment of diabetic patients due to their antihyperglycaemic activity.

## 8. Acknowledgement

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# First-Pass Metabolism Changes After Long-Term Garlic Supplementation

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## 1. Introduction

The suboptimal penetration of highly active antiretroviral therapy (HAART) drugs into the virus reservoirs and sanctuary sites ensures that the replication-competent HIV viruses or integrated HIV virions remain sheltered from HAART's pharmacological activity (Saksena et al, 2010). Persistent virus replication thus sustains continuous colonization of different tissues and cellular targets, which contributes to the disease relapse and to the emergence of virus resistance to HAART therapy (Saksena et al, 2010; Hoggs et al, 2006). Drug resistance is usually triggered by increased gene expression of the efflux transporters in the infected cells, resulting in even lower amounts of antiretrovirals residing in the HIV reservoirs (Löscher&Potschka, 2005; Kis et al, 2009). The persistent infection of the immune cells subjects HIV-infected to opportunistic infections (Orenstein et al, 1997), which alongside the metabolic syndrome, induced by long-term HAART therapy (Unger, 2003), contributes to the concomitant consumption of garlic supplements to ameliorate these symptoms (Amagase, 2006). However garlic phytochemicals have been shown to modulate the activities of intestinal (Berginc et al, 2009), and hepatic transporters (Berginc et al, 2010a) and CYP3A4 enzymes (Foster et al, 2001), the pivotal impediment to attaining therapeutic plasma concentrations after peroral administration and gastrointestinal absorption (Piscitelli et al, 2002; Vermier et al, 2009) of the protease inhibitors (i.e. saquinavir - Saq, darunavir - Dar) of human immunodeficiency virus (HIV-PI). Following that, concomitant application of antiretrovirals and garlic supplements could lead to pharmacokinetic interactions and possibly to therapy failure or to intensification of undesired effects. Piscitelli (Piscitelli et al, 2002) investigated the impact of long-term garlic supplements consumption (garlic caplets GarliPure® from Natrol) on the pharmacokinetics of Saq by conducting a clinical study on healthy male volunteers. A significant decline in the extent of saquinavir absorption was noted, which even after 10-day washout period did not return to the baseline values. The volunteers were administered higher Saq doses than needed for therapy (1200 mg 3-times daily); therefore the potential garlic modifying effects on the Saq pharmacokinetics could be even more pronounced among the infected patients, because they receive lower HIV-PI doses. Investigators suggested that the observed detrimental effect of garlic on the Saq pharmacokinetics could be caused by the formation of garlic metabolite(s) with enzyme-

transporter induction properties. Hypothetically, this would lead to the increased formation of Saq metabolites capable of inducing the metabolism of the parent drug. Plasma samples of the volunteers were not analyzed for the presence of potential garlic metabolites and the necessary *in vitro* studies to elucidate the hypothetical interaction mechanisms were never conducted. Therefore, these assumptions could not be verified. Furthermore, it has never been resolved, whether gut wall could represent an important hindrance to the absorption (Kis et al, 2009) and consequently to the therapeutic efficacy of HIV-PIs.

To address these questions, we performed *in vitro* studies with hepatic and intestinal tissues of rats, fed garlic supplements for two weeks. This approach allowed us to distinguish the influence, which garlic supplements could exert on first-pass Saq's metabolism in the gut and in the liver. Based on the knowledge of short-term influence of garlic phytochemicals and/or supplements on transporter-enzyme interplay (Berginc et al, 2009; Berginc et al, 2010a) and clinical data for Saq (Piscitelli et al, 2002), the mechanisms behind pharmacokinetic interactions between Saq and garlic were elucidated.

According to the clinical experience with Saq, a warning regarding the possibility of interactions with garlic or garlic supplements has been extended also to other HIV-PIs. Therefore, our research also aimed to identify, whether the "*in vitro*" transport and metabolism and perhaps therapeutic efficacy of Dar, a novel, second-generation HIV-PI, could really be affected by garlic consumption in a similar fashion. Furthermore, we hypothesized that differences may exist between the influences of different garlic supplements on HIV-PI pharmacokinetics. Rats were thus fed two garlic supplements with very distinct composition; aged garlic extract - AGE, a preferentially hydrophilic product rich in water-soluble  $\gamma$ -glutamyl derivatives of organosulfur compounds, flavonoids and other substances (Berginc et al, 2010b) and garlic oil macerate - GOM, containing preferentially oil-soluble allicin degradation products (i.e. various sulfides, ajoene, vinyl dithiins and other) (Yoschida et al, 1999). We also analyzed plasma samples of fed rats to identify potential garlic metabolites/phytochemicals, which could modify gene expression as suggested by Piscitelli (Piscitelli et al, 2002).

## 2. Methods and materials

### 2.1 Materials

Salts for incubation salines, sunflower seed oil from *Helianthus annuu*, Williams medium E, L-glutamine, insulin, gentamicin and ampicillin, analytical standards for constituents of garlic supplements (rutin, tangeretin, bergamottine, adenosine, S-allyl cisteine and quercetin) were from Sigma Aldrich Chemie (Deisenhofen, Germany). Saq and ritonavir (Rito) were purchased at Sequoia Research Products. All chemicals used in this study were of the highest grade available. Kyolic® liquid aged garlic extract (AGE) was produced by Wakanuga of America CO., LTD (Mission Viejo, CA, USA), lot number 5H04A. The AGE used in this study was standardized to 1.27 g/l S-allylcysteine content, the compound normally used for the standardization of AGE. Commercially available garlic's soft-gelatin capsules filled with garlic oil macerate (GOM) were purchased in a local pharmacy. Dar was extracted from Prezista® tablets (300mg) by ethanol extraction, evaporation and reconstitution in DMSO. The purity of extracted Dar was confirmed by HPLC analysis at 240 nm; the Dar peak surface represented 97% of the total chromatogram surface area.

## 2.2 Methods

### 2.2.1 Test system

The experiment was conducted with adult, at the start 12 week old, male Wistar rats. The study conformed to the Law for the Protection of Animals (Republic of Slovenia) and was registered at the Veterinary Administration of Republic of Slovenia No. 34401-41/2008/2.

Animals were treated according to the principles of Convention ETS 123 (The convention for the protection of animals used for experimental and other scientific purposes) and Directive 86/609/EEC (Council directive on the protection of animals used for experimental and other scientific purposes).

Group	Supplemented with	N	Dosage	M <sub>before</sub> (g)	M <sub>after</sub> (g)
Sham-A group	saline	3	3.6 mL/kg	357	363
AGE group	AGE	3	1 % AGE in 3.6 ml of saline per kg	369	376
Sham-B group	sunflower seed oils	3	2.6 mL/kg	387	391
GOM group	GOM	3	0.3 % garlic oil macerate in 2.6 mL per kg	371	386

N – The number of fed rats; M<sub>before</sub> - the mean rat weight before supplementation; M<sub>after</sub> – the mean rat weight on day 15.

Table 1. The experimental design of chronic/long-term garlic supplementation to rats.

Animals were fed with pelletized feed Global Diet 2018 ad libidum and had free access to the drinking water through the experimental period. Animals were weighed before first application, on day 7 and at the end of the study. The first weighing was used for randomization, group formation and dose adjustments. From the first and last weighing of animals the mean body weight at the start and at the end of the study were calculated. The experiment was designed with 4 groups of animals. Two different garlic supplements (aged garlic extract – AGE or soft gelatin capsules with garlic oil macerates - GOM) were given orally to two groups daily by gavage for 14 days. The recommended human single oral doses of garlic supplements (2.5 mL of liquid AGE and 2 soft gelatin capsules of GOM, respectively) were adjusted according to the rat's weight; AGE was diluted by saline and the content of soft gelatin capsules was diluted by sunflower seed oil (Table 1). Separately we also evaluated the impact of intragastric gavage of saline (sham-A group) and of sunflower seed oil (sham-B group), because they were used to dilute both garlic supplements. The oil was chosen in compliance with OECD repeated dose methods where oil is recommended as vehicle for worse soluble items (OECD, 2008). Apart from intragastric gavage of different supplements/saline/sunflower seed oil, all groups had access to standard rat chow and water ad libidum. On day 15, rat small intestine, liver and plasma were obtained from rats after short fastening period before the sacrificing. After euthanasia and laparotomy, the intestine was rinsed with ice-cold 10 mM glucose Ringer solution and jejunum was used. Rat liver was preserved in ice-cold transportation medium, consisting of Williams medium E supplemented with L-glutamine (0.29

mg/mL), insulin (0.13 I.U./mL), gentamicin (50 µg/mL) and ampicillin (100 µg/mL). Plasma samples of rats fed with AGE, GOM and of reference animals were withdrawn and stored at -70°C until LC/MS/MS analysis.

### 2.2.2 Saq and Dar permeability through rat jejunum

The intestinal tissue was cut into 3 cm long segments, excluding visible Peyer's patches. Intestinal segments were opened along the mesenteric border, stretched onto inserts with exposed tissue area of 1 cm<sup>2</sup> and placed between two compartments of EasyMount side-by-side diffusion chambers (Physiologic Instruments, San Diego, USA). 2.5 mL of bathing solution (Ringer buffer, pH 7.4) on each side of intestine was maintained at 37°C and continuously oxygenated and circulated by bubbling with carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>). 625 mM glucose and 625 mM mannitol were always added to the serosal (S) and mucosal (M) sides, respectively, to give 10 mM final concentrations. After 25 min of pre-incubation, incubation salines were replaced by donor solutions containing HIV-PIs at the highest concentrations, which could be prepared in Ringer buffer; Saq (25 µM) or Dar (100 µM). If monitoring mucosal-to-serosal (M-S) permeability, donor solution also contained 10 mM mannitol, whereas if monitoring serosal-to-mucosal (S-M) permeability, 10 mM glucose was present in the donor solution. 250 µL of samples were withdrawn from the acceptor (M or S) side every 20 min up to 80 min and replaced by the fresh Ringer buffer containing all necessary ingredients at appropriate concentrations. The donor solutions were also replaced before each sampling time to avoid the loss of investigated compound (Saq, Dar) due to its intestinal metabolism. Immediately after sampling, the withdrawn samples were precipitated by methanol (1:3), which contained haloperidol (100 µg/L) as internal standard, vortexed and kept on ice for at least 15 min. Afterwards the proteins were removed by centrifugation for 10 minutes at 15 000 g and 4°C. The supernatant was analyzed by LC/MS/MS for the parent drugs (Saq or Dar) and their metabolites.

During the permeability experiments, the diffusion chambers were equipped with two pairs of Ag/AgCl electrodes for measuring transepithelial potential difference and short circuit current with a multi channel voltage-current clamp (model VCC MC6, Physiologic Instruments) enabling the evaluation of tissue integrity and viability during the experiments. Only tissues that retained their integrity and viability (described in details in Berginc et al (Berginc et al, 2010b)) were used for the analysis.

During *in vitro* permeability experiments short-term and long-term effects were monitored. The expression short-term used throughout the text refers to the instant influence the addition of AGE supplement in 1 v/v % to the mucosal side of intestine from AGE or sham-A group exerted (i.e. allosteric modifications). On the other hand, the expression long-term effects refer to the transcriptional/translation changes resulting in different protein expression due to 2 week supplementation of rats.

### 2.2.3 Saq and Dar metabolism and transport in the rat liver slices

Rat liver slices (250 µm thick) for the experiments were prepared and pre-incubated for 30 min in the incubation medium (Williams medium E supplemented with L-glutamine (0.29 mg/mL), insulin (0.13 I.U./mL), gentamicin (50 µg/mL) and ampicillin (100 µg/mL)) at 37°C. Afterwards, each liver slice was incubated in 1.5 mL of the same medium, which also

contained 20 µM Saq or Dar. The samples of incubation medium were withdrawn after 30 and 60 min for Saq and after 45 and 90 min for Dar to avoid steady state. After the incubation, liver slices were weighted and homogenized. All the samples were immediately precipitated with methanol (1:3) containing haloperidol as internal standard (100 µg/L). The samples were afterwards prepared for the analysis in the same manner as described for permeability experiments. The LC/MS/MS analysis of the liver extract samples and of the withdrawn samples from the incubation medium at different time points was performed. The transport rate (the rate of metabolite concentration increase in the incubation medium) of two Saq and two Dar metabolites (Saq-M1, Saq-M3, Dar-M1, Dar-M4) was determined. In the liver extracts concentrations of Saq/Dar and their metabolites were measured. By comparing concentrations of the parent drugs and their metabolites in the intracellular cell compartment obtained by extraction and the metabolite transport rates with the corresponding reference values (i.e. concentrations of Saq/Dar and their metabolites or metabolite transport rates determined without garlic phytochemicals) we were able to determine the impact of garlic supplements on the activity of efflux/uptake transporters into bile or back into plasma and CYP3A4.

#### **2.2.4 Plasma preparation**

Prior to the LC/MS/MS analysis, each 200µL plasma sample was precipitated with 3 volumes of ice-cold methanol, briefly vortexed, left to stand on -20°C for two days and then centrifuged at 10 000 g for 10 min at 4°C. The supernatant was transferred to autosampler vials for subsequent LC/MS/MS analysis.

#### **2.2.5 LC/MS/MS sample analysis from studies with rat jejunum and liver slices**

LC/MS/MS analysis of the samples obtained in the *in vitro* transport/metabolism experiments was performed on a Varian 1200L triple-quadrupole LC-MS Varian (Palo Alto, CA, USA). The mass spectrometer was coupled to ProStar 210 binary pumps, a Varian 420 autosampler and Varian 510 column oven. For chromatographic separation, a Phenomenex Kinetex 50x2.0 mm C-18 column with 2.6 µm particles was used for Dar, and a Phenomenex Gemini 150x2.0 mm C-18 column with 3 µm particles for Saq. The injection volume was 10 µL and the column temperature was 50 °C. Mobile phase consisted of water and acetonitrile containing 0.1% of formic acid. The elution for Dar and its metabolites was performed using a linear gradient from 20% to 65% of organic phase in 4 minutes with a flow rate of 0.4 mL/min, whereas that of Saq was performed using a linear gradient from 20% to 36% organic phase over 12 minutes with a flow rate of 0.5 mL/min. For the purpose of this study, two Dar (Dar-M1 - Dar carbamate hydrolysis ( $t_r$  3.3 min), Dar-M4 - Dar hydroxylation ( $t_r$  5.2 min)) [12] and two Saq (Saq-M1 - Saq oxidation at (1,1-dimethylethyl)amino group ( $t_r$  8.1 min), Saq-M3 - Saq oxidation at benzyl group ( $t_r$  5.9 min)) (Fitzsimmons&Collins, 1996) metabolites were monitored.

#### **2.2.6 LC/MS/MS analysis of plasma samples**

Rat plasma samples were analyzed by a triple-quadrupole LC/MS/MS Agilent 6460 (Agilent Technologies, Waldbronn, Germany) equipped with a Jet-Stream™ ESI interface coupled to an Agilent 1290 Infinity UPLC. 1µL of acetonitrile-precipitated rat plasma sample was injected on

a Phenomenex Synergi Polar-RP 100x3mm 2.5 $\mu$ m column. The mobile phase A consisted of water with 0.1% formic acid and mobile phase B consisted of 98% acetonitrile with 2% water. The gradient elution was linear from 1% B to 90% B over 10 minutes and the flow rate was 0.4 mL/min. After the column, the eluent was mixed with 0.2mL/min 100% acetonitrile via a zero-dead volume mixing tee in order to facilitate the evaporation and ionization process in the ESI Jet-Stream interface. The multiple reaction monitoring mode was used for the identification and relative quantification of AGE and GOM constituents in rat plasma (Table 4).

### 2.2.7 Data analysis

The apparent permeability coefficient ( $P_{app}$ ) of HIV-PIs was calculated according to Eq. (1)

$$P_{app} = \frac{dc}{dt} \frac{V}{c_0 A} \quad (1)$$

where  $dc/dt$  represents changes in HIV-PI concentration in the acceptor compartment per unit time under steady state conditions,  $V$  is the volume of the acceptor compartment,  $A$  is the exposed surface area (1 cm<sup>2</sup>) and  $c_0$  is the initial, donor concentration of HIV-PIs. Afterwards the ratios ( $R_{ex}$ ) were determined according to the Eq. (2) by dividing S-M  $P_{app}$  ( $P_{app}^{S-M}$ ) values with the corresponding M-S  $P_{app}$  ( $P_{app}^{M-S}$ ) values.

$$R_{ex} = \frac{P_{app}^{S-M}}{P_{app}^{M-S}} \quad (2)$$

Results in Tables and Figures are presented as means  $\pm$  SD of at least 3 measurements. Data were evaluated statistically using SPSS 16.0 for Windows. Where appropriate, F-test for testing the equality of variances and, afterwards, 2-tailed Student t-test ( $\alpha = 0.05$ ), were used. Otherwise, one way ANOVA, followed by Bonferroni post-hoc test were applied.

## 3. Results and discussion

### 3.1 The impact of long-term garlic supplementation on HIV-PIs intestinal permeability

The intestinal transporter-enzyme interplay (i.e. PGP and CYP3A4 or MRP-2 and UGT) can be responsible for lowering the fraction of the absorbed drugs that are subjected to the metabolism or transport with these proteins (Zhang et al, 2008). The PGP/MRP-2 mediated drug efflux from absorptive cells into the GIT lumen prolongs the overall residence time of drugs in the enterocytes through the more intense entero-enteric circulation, subjecting drugs to a more extensive intracellular phase I or phase II metabolism (Wu&Benet, 1999). The impact of long-term garlic supplementation on the activity of intestinal transporters and thus on the fraction of HIV-PIs (Saq, Dar) absorbed were assessed by evaluation of *in vitro* parent drug intestinal permeability through the rat jejunum (Table 2A, 2B). 100  $\mu$ M Dar donor concentrations were chosen to simulate intraluminal Dar concentrations after peroral administration of clinical dose [12]. Since low water-solubility limits achieving high Saq concentrations (Aungst, 1999), the highest possible Saq donor concentrations (25  $\mu$ M), which could be prepared in Ringer buffer pH 7.4, were applied.

Both HIV-PIs exerted low intestinal permeability regardless of whether rats were fed garlic supplements or not. Their M-S permeabilities were below 10 $\times$ 10<sup>-6</sup> cm/s (Table 2A, 2B), the

limit above which drugs could be classified as highly permeable compounds according to our intra-laboratory experiments and FDA suggested highly permeable standards (Yu et al, 2002). Tested HIV-PIs were also profoundly effluxed from enterocytes into the lumen (high  $R_{ex}$  values, Table 2A and 2B - 1<sup>st</sup> phase designated "No Garlic"), indicating that at clinical Dar doses or the highest Saq doses, efflux transporters (Pgp, Mrp-2) were not saturated. This is in accordance with the results obtained by Holmstock (Holmstock et al, 2010). Namely, their Caco-2 permeability experiments and *in situ* intestinal perfusions of Pgp knock-down mice indicated a profound participation of Pgp in Dar's secretion from absorptive cells even at the highest tested - clinical 100  $\mu\text{M}$  dose. Transporter saturation was also not achieved with 25  $\mu\text{M}$  Saq, as evident from significantly higher  $R_{ex}$  values (higher than 3, Table 2B) compared to the value 1, which usually indicates complete transporter inhibition. Since Pgp/Mrp-2 were not inhibited *in vitro*, the *in vivo* impact of efflux transporters on limiting drug absorption could also be significant, owing to the higher expression of transporters in human small intestine than in the rat's (Cao et al, 2006). Therefore, gut represents an important biological barrier in achieving therapeutic plasma HIV-PI levels.

**Table 2A**

Darunavir - $P_{app}$ GIT (100 $\mu\text{M}$ ) [cm/s]	No garlic			AGE addition		
	M-S (*10 <sup>-7</sup> )	S-M (*10 <sup>-6</sup> )	$R_{ex}$	M-S (*10 <sup>-7</sup> )	S-M (*10 <sup>-6</sup> )	$R_{ex}$
<b>Sham-A group</b>	7.7 ± 0.7	20.4 ± 1.0*	26	10.4 ± 4.4	20.6 ± 1.6*	20
<b>AGE group</b>	8.6 ± 0.5	13.4 ± 1.0 <sup>a</sup>	16	9.2 ± 1.1	11.8 ± 3.1*	13
<b>Sham-B group</b>	9.5 ± 2.1	17.9 ± 1.8*	19			
<b>GOM group</b>	8.1 ± 2.1	5.8 ± 1.7 <sup>a</sup>	7.2			

**Table 2B**

Saquinavir - $P_{app}$ GIT (25 $\mu\text{M}$ ) [cm/s]	No garlic			AGE addition		
	M-S (*10 <sup>-7</sup> )	S-M (*10 <sup>-6</sup> )	$R_{ex}$	M-S (*10 <sup>-7</sup> )	S-M (*10 <sup>-6</sup> )	$R_{ex}$
<b>Sham-A group</b>	6.7 ± 0.3	2.6 ± 0.7*	3.9	5.9 ± 0.4	3.7 ± 0.3 <sup>b</sup>	6.3
<b>AGE group</b>	7.0 ± 2.0	5.6 ± 1.0 <sup>a</sup>	4	5.9 ± 0.5	7.4 ± 0.4 <sup>b</sup>	13
<b>Sham-B group</b>	6.9 ± 0.8	2.3 ± 0.2*	2.9			
<b>GOM group</b>	6.5 ± 2.7	4.9 ± 0.6 <sup>a</sup>	7.5			

HIV-PIs permeability was determined in two phases; first the permeability was measured in Ringer buffer pH 7.4 (20 min sampling intervals for 80 min - No garlic) and afterwards AGE was added to the mucosal side of rat ileum to give 1 % final AGE concentration and the experiment continued for additional 80 min (AGE addition).

\* - S-M  $P_{app}$  HIV-PI values are significantly higher than the corresponding M-S  $P_{app}$  values

<sup>a</sup> - S-M  $P_{app}$  values of treated rats (AGE or GOM group) significantly higher/lower than reference S-M  $P_{app}$  values (sham-A and sham-B group)

<sup>b</sup> - S-M  $P_{app}$  values determined after the addition of AGE significantly changed compared to S-M  $P_{app}$  values in the first phase

Table 2. Saq and Dar intestinal permeability through rat jejunum determined in rats fed different garlic supplements (AGE or GOM - AGE and GOM group), saline (sham-A group) or sunflower seed oil (sham-B group). The permeability values of Saq and Dar after the addition of AGE are also given (AGE addition) in the table.

The short-term impact (i.e. instant/allosteric changes of transporter/enzyme activities) induced by AGE phytochemicals on intestinal rat's ABC transporters (Pgp, Mrp-2), involved in HIV-PI (Saq, Dar) absorption, have been investigated in our previous studies, where no feeding protocols were applied (Berginc et al, 2009; Berginc et al, 2010c). In the presented study these effects were re-investigated with the intestine of AGE fed rats to elucidate the (ir)reversibility of garlic phytochemicals or phytochemical metabolites binding to the efflux transporters. We reported previously that activities of Pgp and Mrp-2 significantly changed owing to allosteric modulations exerted in the presence of AGE components (Berginc et al, 2009; Berginc et al, 2010c). The impact of Pgp on the absorption of HIV-PIs was found to exceed that of Mrp-2, which has also been corroborated by Usansky (Usansky et al, 2008) in their *in vivo* study with Sprague-Dawley rats and Saq. In the case of Dar, mixed reports can be found regarding MRP-2/Mrp-2 importance in Dar absorption (Berginc et al, 2010c; Kakuda&Kiser, 2006). Based on this and the Mrp-2 expression pattern along GIT tract in rats (Mrp-2 expression decreases in more distal parts of rat's small intestine) (Berginc et al, 2010c; Usansky et al, 2008), the participation of Mrp-2 in HIV-PIs intestinal absorption is minor and the observed short-term effects in our studies were predominately caused by modified Pgp's activity. The noted short-term activity changes of the studied ABC transporters in our previous studies were explained by multiple binding sites in these transporters (four in Pgp and two in MRP-2) (Martin et al, 2000), which allow simultaneous binding of more substrates leading to allosteric modifications. Allosteric modifications induced by AGE phytochemicals in these studies were observed either as competitive inhibition between compounds (phytochemicals and drug) for an identical binding site (i.e. Dar – unchanged efflux) or as positive-cooperative effect and increased efflux (i.e. Saq), when the preferences of all involved substances for binding sites are distinct (Berginc et al, 2009; Berginc et al, 2010c). Similar allosteric modification of intestinal transporters involved in Saq and Dar efflux were also observed using small intestine of AGE fed rats in this study; Saq efflux significantly increased, whereas Dar efflux remained unchanged (slight but statistically insignificant decrease - Table 2A and 2B – phase "AGE addition"). The content of soft-gelatin capsules containing GOM could not be tested for their short-term influence on efflux transporters due to its poor miscibility with the incubation buffer, which would prevent adequate diffusion of oily phytochemicals from emulsion to the mucosal surface. The short-term modifications of efflux transporter activities were equally pronounced in rats, fed AGE, and in the control rats (sham-A group). Namely the average ratios between S-M  $P_{app}$  values after and before AGE addition were ca 1.0 for Dar and 1.5 for Saq, regardless which rats were used (treated or untreated - Table 2; sham-A and AGE group). This means that in the time-frame from the last intragastric gavage of garlic supplement and animal euthanization (18 h), garlic phytochemicals must have completely dissociated from intestinal efflux transporters to determine identical magnitude of permeability changes. Intestinal transporters in fed rats therefore could remain occupied with garlic phytochemicals after intragastric gavage but for at most 18 h per day. This could be sufficient to change transporter expression in the long run. However, transcriptional/translational effects and consequently elevated/decreased transporter expression could also be induced by binding of the absorbed xenobiotics on the transporter's gene promoters or to the corresponding transcriptional factors. Both scenarios could thus contribute to potential long-term expression changes. In the presented study mRNA or protein levels to quantify Pgp/Mrp-2

expression levels in (un)treated rats were not performed, because increased mRNA levels for Pgp have been found to correlate well with increased protein levels and its functionality (MacLean et al, 2008; Englund et al, 2006). Although such correlation between mRNA and protein levels for Mrp-2 has not yet been established (MacLean et al, 2008), diallyl disulfide, garlic sulfide present in oily (i.e. GOM) preparations and in smaller amounts also in aqueous/hydrophilic garlic supplements (i.e. AGE) (Berginc et al, 2009), induced transcription of Mrp-2, leading to increased Mrp-2 protein levels in rat renal brush-border membrane vesicles. Owing to the established positive correlation between increased Pgp/Mrp-2 levels (elevated protein and mRNA levels) and their functionality, the long-term impact of garlic constituents on the Saq and Dar intestinal permeability was assessed directly by measuring their permeabilities.

A 14-day AGE or GOM supplementation to rats significantly affected intestinal permeability of both antiretrovirals (Table 2A and 2B – AGE, GOM group); the efflux of Saq significantly increased and that of Dar significantly decreased compared to reference values (S-M  $P_{app}$  values of sham-A group – Table 2; see column "No garlic"). M-S permeabilities in both cases did not change (M-S  $P_{app}$  values sham-A or sham-B group). Sunflower seed oil, used to dilute GOM doses, had no impact on the permeability of tested HIV-PIs.

The increase of Saq's S-M  $P_{app}$  values observed in rats fed with both garlic supplements as mentioned, implies that the expression of efflux transporters in the mucosal enterocytic membrane must have increased during long-term garlic supplementation due to the repeated exposure of enterocytes to garlic phytochemicals or perhaps their metabolites. In fact, our results partially confirm a hypothesis which has been proposed by Piscitelli (Piscitelli et al, 2002) to explain lower amounts of absorbed Saq in a clinical study. Our results are in accordance with the part of hypothesis, which claims that the transporter expression changes could be induced by garlic phytochemicals or their metabolites. Since rats were not fed HIV-PIs and garlic supplements simultaneously as performed in clinical study by Piscitelli, the second part of his hypothesis that the parent drug (Saq) or its metabolites could also be responsible for the observed effects, could not be corroborated. However, in the case of Dar, in spite of the suggested increased transporter expression, the efflux of this HIV-PI was significantly lower than that determined for the untreated rats (sham-A group). As we mentioned previously, Dar and Saq Pgp binding sites are not identical, which was confirmed in previous *in vitro* study on rat intestine using ritonavir (Rito) as potent Pgp and Mrp-2 inhibitor (Berginc et al, 2010c). While Rito significantly inhibited Dar efflux from enterocytes regardless of the applied Rito concentration, that was not true for Saq. Instead of inhibiting Pgp-mediated Saq efflux, low Rito concentrations stimulated active Saq excretion and the inhibition was achieved only at saturable Rito concentrations. This undoubtedly indicated that Dar and Rito share identical binding site on Pgp, whereas Saq and Rito do not; only after Rito-Pgp binding site(s) are completely occupied by Rito, Rito can displace Saq from its binding site on Pgp. Therefore, significant decrease of Dar efflux after 2-week rat supplementation with garlic products observed in this study was most probably due to the occupation of Dar-binding site on Pgp by garlic phytochemicals or their metabolites with longer half-life(s). This indicates that the compounds from garlic supplements remained bound to intestinal transporters for at least 18h, additionally confirming Piscitelli's assumptions (Piscitelli et al, 2002).

### 3.2 The impact of long-term garlic supplementation on HIV-PIs hepatic pharmacokinetics

Various high-throughput screening studies using human hepatic microsomes (Foster et al, 2001) and recombinant CYP3A4 enzymes (Pal&Mitra, 2006) indicated that lipophilic garlic supplements, AGE and individual garlic phytochemicals could significantly inhibit CYP3A4 activity (short-term effect) and consequently impair HIV-PI (Saq, Dar) metabolism. The short-term effects of AGE on the hepatic HIV-PI distribution and metabolism in rat liver slices have also been investigated in our previous studies, where we have shown that AGE allosterically activated Pgp transport (i.e. it decreased intracellular concentrations of Pgp substrate - Dar) in the same manner as in the intestine but inhibited hepatic Mrp-2 transport. We determined that Mrp-2 inhibition observed in those studies was caused by metabolism of garlic flavonoids to the corresponding glucuronide metabolites, which are good MRP-2/Mrp-2 substrates. These glucuronide metabolites bind to Mrp-2 and competitively displace other Mrp-2 substrates (i.e. Saq) from these transporters, causing an increase of intrahepatic Saq concentrations. The inhibition of CYP3A4 HIV-PI metabolism by AGE was also confirmed in that study (Berginc et al, 2010d).

However after 14 days of AGE supplementation, the concentrations of both tested HIV-PIs in rat liver slices significantly increased (Table 3). Similar results were obtained also with rats fed with GOM, but the impact of GOM supplementation on the hepatic pharmacokinetics was obscured by the high influence of reference treatment - sunflower oil alone (sham-B group). Namely, if one compares intracellular concentrations of parent drugs and of the corresponding metabolites in the extracts from the liver slices of rats fed only sunflower oil (Table 3, sham-B group) with the corresponding values obtained from liver slices of rats fed saline (Table 3: sham-A group), it is obvious that sunflower oil itself exhibited pronounced effects on HIV-PI hepatic distribution and metabolism. The effect of GOM was thus more difficult to evaluate than that of AGE (Table 3; sham-A and AGE group). The daily quantities of the sunflower seed oil used to dilute GOM for sufficiently exact dosing and in the appropriate reference group was ca. 1 mL. According to the guidelines, this amount added to the rat's dietary fat intake does not influence the animal's condition regarding the clinical examination, hematological, biochemical and histological parameters (OECD, 2008). However, the increased daily fat intake was obviously sufficient to induce transcriptional/translational changes, which finally led to changes in HIV-PIs hepatic distribution and metabolism (significantly higher intracellular HIV-PI concentrations in rat liver slices and significantly higher formation and excretion rate of metabolites) compared to the reference values (Sham A group). Sunflower seed oil represents a good source of monounsaturated fatty acids (i.e. oleic acid), which have been known to reversibly increase the transcription of enzymes (among them also CYP) in liver and gut (Niot et al, 1997; Chen et al, 2001). The intake of higher fat quantities also interferes with endogenous lipid synthesis in metabolism, leading to changes in the plasma lipids and bile acid composition. To avoid cytotoxicity of the increased amounts of bile acids formed; these acids need to be excreted into the bile. Therefore, the increased production of bile acids had to be accompanied with the increased transporter expression to ensure cell survival. The excretion of bile acids into gallbladder involves the uptake of these endogenous compounds from plasma into hepatocytes with the aid of uptake transporters (i.e. NTCP, OATPs) and their excretion into the gallbladder by sinusoidal efflux proteins (Pgp, Mrp-2, S-Pgp, MDR3) (Meier et al, 1997a). Therefore the expression of these transporters in organisms

fed high fat diet would also increase, explaining the effects observed in this study on the hepatic HIV-PI pharmacokinetics in sunflower seed oil fed animals (sham-B group).

<b>Table 3A</b>		<b>EXTRACT (mol/mg)</b>		<b>TRANSPORT RATE (<math>\mu\text{mol}/\text{min}^*\text{mg}</math>)</b>		
		Saq (*10 <sup>-11</sup> )	Saq-M1 (*10 <sup>-13</sup> )	Saq-M3 (*10 <sup>-12</sup> )	Saq-M1 (*10 <sup>-9</sup> )	Saq-M3 (*10 <sup>-8</sup> )
<b>Sham-A group</b>		2.9 ± 0.3	4.6 ± 1.0	2.3 ± 0.1	3.8 ± 0.4	3.7 ± 0.3
<b>AGE group</b>		8.2 ± 0.2*	3.9 ± 0.7	2.4 ± 0.4	3.4 ± 0.3	3.2 ± 0.2
<b>Sham-B group</b>		8.1 ± 0.4*	4.9 ± 0.2	4.7 ± 0.7*	5.6 ± 0.6*	6.4 ± 0.6*
<b>GOM group</b>		10.3 ± 0.4* <sup>a</sup>	7.8 ± 0.7*	4.3 ± 0.9*	4.8 ± 0.1*	6.0 ± 0.3*

<b>Table 3B</b>		<b>EXTRACT (mol/mg)</b>		<b>TRANSPORT RATE (<math>\mu\text{mol}/\text{min}^*\text{mg}</math>)</b>		
		Dar (*10 <sup>-11</sup> )	Dar-M1 (*10 <sup>-11</sup> )	Dar-M4 (*10 <sup>-12</sup> )	Dar-M1 (*10 <sup>-8</sup> )	Dar-M4 (*10 <sup>-8</sup> )
<b>Sham-A group</b>		7.8 ± 0.6	2.5 ± 0.2	3.4 ± 0.2	3.6 ± 0.8	2.1 ± 0.2
<b>AGE group</b>		10.3 ± 1.0*	1.7 ± 0.0*	1.7 ± 0.6*	2.7 ± 0.5	1.8 ± 0.3
<b>Sham-B group</b>		8.5 ± 0.5*	2.8 ± 0.3	6.0 ± 0.4*	6.1 ± 1.4*	5.9 ± 0.1*
<b>GOM group</b>		10.2 ± 1.1* <sup>a</sup>	3.2 ± 0.2*	5.0 ± 0.7*	4.7 ± 0.5	4.5 ± 0.3*

\* - the amounts of HIV-PIs and of the corresponding metabolites in the rat liver extracts or metabolite transport rates are significantly higher/lower than the corresponding reference values (sham-A and B group).

<sup>a</sup> - significantly higher HIV-PI amounts in the extract compared to the amounts of HIV-PIs, determined in the extracts of rat liver extracts from control sham-A group.

Table 3. Saq (Table 3A) and Dar (Table 3B) hepatic *in vitro* pharmacokinetics evaluated in rat liver slices obtained from rats fed with different garlic supplements (AGE or GOM groups), saline (sham-A group) or sunflower seed oil (sham-B group). The amounts of HIV-PIs and of the corresponding metabolites in the rat liver extracts and the metabolite transport rates from the rat liver slices into the incubation medium were determined.

Higher intrahepatic retention of HIV-PIs after prolonged garlic consumption could thus be explained by two factors. First, hepatocytes were obviously exposed to lower concentration of xenobiotics or their metabolites, responsible for the induction of Pgp compared to enterocytes. Namely after peroral absorption of garlic phytochemicals, lower amounts of garlic phytochemicals and their corresponding metabolites could reach hepatocytes due to incomplete/low xenobiotic absorption through the intestinal mucosa, binding to enterocyte's mucosal membranes, instability in GIT lumen and intraluminal metabolism, which altogether contributed to less profound effect on the expression of efflux transporters in hepatocytes than in the jejunum (Ioannides, 2003; Lawson&Wang, 2005b). Besides efflux transporters, Saq and Dar also utilize OATP1A2 (OATP-A), 1B1 (OATP-C) and 1B3 (OATP-8) solute uptake transporters to distribute into the human liver (Hartkoorn et al, 2010). The sinusoidal rat hepatocyte membrane embodies numerous Oatp isoforms with Oatp1, Oatp2 and Oatp4 being the most important ones (Treiber et al, 2004; Meier et al, 1997b). Because AGE has been shown to affect Oatp activity (Berginc et al, 2010b), increased intrahepatic HIV-PI concentrations observed in this study could also correspond to the increased Oatp expression in the liver, which by its influence on the HIV-PIs pharmacokinetics could have exceeded the effects of efflux transporters.

These results suggest that the observed hepatic physiological adaptations to supplementation of garlic products or sunflower seed oil were exerted by garlic phytochemicals, components of sunflower seed oil and/or the corresponding metabolites. Because AGE lacks oily compounds that constitute GOM and/or sunflower seed oil, the substances responsible for the changes in membrane transporter expression, were most probably different. The differences in garlic supplement composition and/or plasma levels of garlic phytochemicals notwithstanding, the same final effect regarding HIV-PIs and HIV-PI-metabolite hepatic distribution were observed. The composition dissimilarities of garlic supplements and consequently of the produced metabolites also affected HIV-PIs Cyp3A metabolism in the liver slices differently. According to our results (Table 3), less Saq and especially Dar metabolites were formed in AGE group compared to the control sham-A group. However, in the presence of GOM and sunflower seed oil, Cyp metabolism of both investigated drugs significantly increased (note the significant increase of Saq-M1 and Dar-M4 in Table 3). Our results are in accordance with experimental data obtained in studies with various oily garlic supplements and AGE (Fischer et al, 2007). Garlic oil constituents, diallyl sulfide, diallyl disulfide and diallyl trisulfide have been found to increase the transcription of several hepatic enzymes (Cyp1A1, 2B1 and 3A1, NAD(P)H quinone oxidoreductase 1, glutathione-S-transferase) in wild type Wistar-Kyoto rats (Fischer et al, 2007), similarly to our findings in GOM group (increased production of HIV-PI metabolites and consequently their faster transport from hepatocytes into the incubation media). However, another clinical study on volunteers taking garlic extract (GarliPure®) twice daily for 3 weeks indicated no impact of garlic phytochemicals on CYP3A4 expression in intestine or liver, while at the same time PGP expression significantly increased (Hajdaa et al, 2010). GarliPure® extract is labeled to contain  $\gamma$ -glutamylcysteines (12 mg), alliin (4.8 mg), sulfur (3.9 mg), thiosulfinate (3.8 mg) and to have an allicin releasing potential, meaning that after consumption, this product enables *in vivo* allicin synthesis from alliin in ca 3.6 mg quantities. AGE, which we used to feed rats in this study, also predominately contains  $\gamma$ -glutamylcysteines with S-allyl-L-cysteine being the main representative. However, AGE does not contain allicin because during the aging process, the content of lipophilic allicin degradation products declines and the compounds responsible for distinct garlic odor and unwanted side effects in AGE are thus minimized. However, based on our analysis, small amounts of these lipophilic phytochemicals are still present in AGE (Berginc et al, 2009). A recent analysis by Lawson and Wang (Lawson&Wang, 2001a) indicated that only one of 24 marketed allicin-releasing brands enabled the release of the designated amount of allicin, whereas the release from other supplements reached only 10 to 15% of the declared values. Therefore, one can assume that the compositions of GarliPure® and of AGE used in this study are very similar. Similar to observations in the study with GarliPure® impact on Saq pharmacokinetics in humans (Hajdaa et al, 2010) we also determined no impact of long term AGE supplementation on Cyp3A Saq metabolism in rats, indicating that the expression of this enzyme remained constant in spite of AGE administration. However in the case of Dar, AGE significantly decreased its metabolism. Similarly to multiple binding sites in efflux transporters, the CYP3A4 kinetic studies by Shou (Shou et al, 1994) performed on CYP3A4 transfected HepG2 cells indicated the existence of two binding sites in this enzyme. It is thus possible, that Saq and Dar, although metabolized by the same enzyme (i.e. CYP3A4), do not share identical binding sites on CYP3A4, which would be similar to their interaction with Pgp transporters. Phytochemicals or metabolites present in or derived from AGE (but not GOM) could have occupied the Dar but not Saq binding site on CYP3A enzyme, similarly as

in the case of Pgp transporter. This resulted in altered Dar but not Saq metabolism. However this hypothesis will be difficult to confirm until more structural and functional information is available about the binding sites of CYP3A enzymes.

### 3.3 Plasma levels of AGE and GOM constituents and their metabolites

The plasma levels of AGE and GOM constituents in plasma samples taken 24 hours after the last dosing were very low, resulting in signals near the limit of detection on a top-of-the line LC/MS/MS system. This fact primarily confirms that we were really observing the long-term influences of AGE and GOM exerted through altered enzyme and transporter expression in our experiments with the tissue samples rather than short-term allosteric or competitive effects of garlic constituents remaining in the isolated animal tissues. Nevertheless, significantly higher levels of garlic constituents were obtained in the plasma from AGE group compared to the plasma of GOM group (Table 4). The levels of S-allyl-L-cysteine, tangeretin and quercetin were 2, 5.4 and 8 times higher, respectively, in the plasma of AGE group compared to the GOM group. The relatively long period between the last dose and the plasma sampling allowed the majority of garlic-related metabolites to be excreted, so the only metabolite that was confidently quantified, was bergamottine glucuronide and it was present in the AGE group. The much higher concentration of garlic constituents and metabolites in the plasma of AGE group is in accordance with the results from the rat liver slices experiment, where it was shown that the influence of AGE supplementation on the Saq and Dar liver disposition was beyond that of GOM's (Table 3). It could be argued that the majority of GOM constituents and their metabolites were already eliminated especially through the lungs (they are volatile) before we performed experiments with the liver slice and before the plasma samples were taken, since the rats were fasting for 18 hours before the sacrifice. On the other hand, the long term changes of hepatic protein expression caused by GOM supplementation should have remained relatively unchanged after 2 weeks in spite of short period of food and GOM deprivation. Therefore, the conclusion of AGE's greater influence on HIV-PI's hepatic disposition remains unchanged.

Analyte	MRM [m/z] (polarity)	ret. time [min]	Plasma- AGE [Area cps]	Plasma- GOM [Area cps]	Plasma- REF [Area cps]
Rutin	611->303 (+)	4,81	34	N/D	N/D
Bergamottine	339->203 (+)	10,35	N/D	N/D	N/D
S-allyl-L-cysteine	162->145 (+)	2,32	1174	593	152
Adenosine	268->136 (+)	2,62	3041	2079	515
Tangeretine	373->343 (+)	8,55	1227	229	306
Quercetine	301->151 (-)	6,37	412	51	116
Bergamottin-glucuronide	515->339 (+)	6,96	1088	N/D	N/D

N/D - the response was below the limit of detection

Table 4. The multiple reaction monitoring mode (MRM) transitions used for the LC/MS/MS identification and quantification of garlic constituents in rat plasma (first two columns) and the peak areas obtained from the plasma samples of rats fed with AGE, GOM and the reference group (the last three columns: Plasma-AGE, Plasma-GOM and Plasma-REF, respectively).

Furthermore, based on the plasma profiles from rats exposed to different garlic supplements, it can be concluded that constituents and their metabolites, although structurally and chemically different, induced similar hepatic effects regarding the transporter expression but exerted significantly different effect on CYP3A metabolism of both compounds. The affinities of these metabolites or their parent phytochemicals from both supplements for CYP3A are evidently different, because AGE constituents/metabolites inhibited/did not affect Cyp3A HIV-PI metabolism, whereas HIV-PI Cyp3A metabolism increased after long-term GOM supplementation.

#### 4. Conclusion

Although applied at the highest possible donor concentrations, both drugs (Saq, Dar) displayed very low permeability in the absorptive direction and were subjected to a profound efflux from enterocytes back into intestinal lumen, indicating that the intestinal wall represents an important biological barrier for attaining therapeutic HIV-PIs plasma levels.

A concomitant consumption of garlic supplements together with HIV-PIs places the infected patients at higher risk for therapy failure, because the components of garlic supplements and/or their metabolites readily bind to efflux transporters. This binding changes the activities of efflux transporters by allosteric modifications in short-term and leads to an increased expression in the long run. Although two garlic supplements with different compositions were used, ABC transporter activity in the intestine increased similarly in both cases. Long-term garlic supplementation also affected hepatic metabolism and distribution of both HIV-PIs. Contrary to the effects in the intestine, the up-regulation of efflux transporters in the liver was less important than the increased expression of uptake Oatps transporters, which resulted in the overall increased intrahepatic HIV-PI amounts that could be further subjected to Cyp3A metabolism. Although the effects of AGE and GOM were similar regarding the transporter expression, their effects on Cyp3A metabolism were significantly different. AGE inhibited Dar but not Saq Cyp3A metabolism, while in GOM treated rats the Cyp3A metabolism of both drugs significantly increased, highlighting important dissimilarities in plasma profiles of garlic phytochemicals and the corresponding metabolites, as shown in this study. Given the fact that garlic supplements are so widely used in the HIV infected population because of their protective cardiovascular and anti-infective effects, further research should be directed at the identification of high- and low-risk supplement - drug combinations.

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# Anti-Trypanosomal Activity and Cytotoxicity of Some Compounds and Extracts from Nigerian Medicinal Plants

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## 1. Introduction

Human African trypanosomiasis, also known as sleeping sickness, is a vector-borne parasitic disease. The parasites concerned are protozoa belonging to the *Trypanosoma* genus. They are transmitted to humans by tsetse fly (*Glossina* genus) which have acquired their infection from human beings or from animals harbouring the human pathogenic parasites. Rural populations living in regions where transmission occurs and which depend on agriculture, fishing, animal husbandry or hunting are the most exposed to the disease. Sleeping sickness threatens millions of people in 36 countries in sub-Saharan Africa. Many of the affected populations live in remote areas with limited access to adequate health services. Treatment depends on drugs that can cross the blood-brain barrier to reach the parasite. Such drugs are toxic and complicated to administer. Suramin, discovered in 1921, is one of the drugs used for the treatment. However, it provokes certain undesirable effects, in the urinary tract and allergic reactions. Other drugs in use are equally toxic and difficult to administer. Thus the need to develop new drugs is imperative. Natural products and medicinal plants (Antia et al, 2009; Sara et al, 2004; Bizimana et al., 2006; Okpekon et al., 2004) have continued to contribute to the search for new and potent anti-trypanosomal drugs with minimal side effects. Their continued relevance in drug discovery and development is due to their bioactivity, biodiversity and reported safety in their use. An analysis of new and approved drugs for the treatment of human diseases indicated that natural products have continued to play a highly significant role in the drug discovery and development process. Thus biologically active natural products from plants, their derivatives or analogues contributed up to 57% of top selling prescription drugs in the United States in 1997 (Newman et al., 1997). This dominant role is due to the leads for the development of drugs that natural products give. Thus expanding, not decreasing, the exploration of nature as a source of novel active agents that may serve as leads and scaffolds for elaboration into desperately needed efficacious drugs for a multitude of disease indications is advocated (Newman & Cragg, 2007). Ethnobotanical surveys have revealed the use of *Alcornea cordifolia*, *Euphorbia poisonii*, *Monodora myristica*, *Prosopis africana*, *Spondias mombim*, *Nauclea pobeguini*, *Terminalia avicennioides*, *Cochlospermum planchonii*, *Nauclea latifolia* and *Withania somnifera* in the traditional medicine in parts of Nigeria (Igoli et. al., 2005). These plants are used as spices and medicinally for the treatment of diarrhoea,

dysentery, diabetes, bacterial infections, fevers, pain and snake bites amongst other ailments by the Igede people of Benue State in Nigeria. *Maytenus laevis* is used in traditional medicine in Colombia (Nagakawa et.al., 2004; Gonzalez et al., 1982) against pain and arthritis. Before the introduction of synthetically prepared medicines, herbal remedies were commonly prescribed and were often effective. Thus herbs and spices played very important, sometimes magical, roles in medicine. Hence these plants were collected and their extracts/ compounds isolated were evaluated for anti-trypanosomal and cytotoxic activities. A high throughput (HTS) method was used to screen the extracts and compounds. This has become very useful in modern drug discovery from natural products. It is a form of standardised and replicate screening method which employs 96 well plates which can be used to screen 80 extracts, compounds or fractions at the same time. It can also be adapted to obtain data to plot dose response curves and determine IC<sub>50</sub> and MIC values. Using a multilabel counter, suitable indicators, wells with extracts, cells, organisms and controls can be read quickly by examining fluorescence, luminescence and optical density or by radiometry giving exact and replicable values free from errors of visual determinations or measurements. The volumes used in these wells are between 200-250µL and for half well plates 20-40µL such that solutions of extracts or compounds as low as 1mg dissolved in 100µL gives a stock solution of 10mg/ml which can be diluted for all range of concentrations typically from 20-30µg/ml to ng or µM to nM values using serial dilution methods. This greatly enhances speed and efficacy of screening for hit molecules (Gray et al., 2011). Efforts were made to identify the active compounds contained in the extracts as much as possible. This is because it is the practice today to screen more and more pure compounds rather than crude extracts or fractions. Screening of crude extracts or mixtures/fractions does not allow understanding of the mode of activity of the test compounds against the target cells or tissues. Isolation of the active compounds is required before active concentrations can be determined. We hereby report on these plant extracts, the compounds obtained from them, their *in vitro* activity against the bloodstream form of *Trypanosoma brucei brucei* (*T.b.brucel*) and the cytotoxic activity of the active compounds/extracts against PNT2A cells.

## 2. Materials and methods

Standard methods of extraction and isolation or purification of compounds/extracts from plant materials were used while a standardised 96 well based assay was used to screen the extracts for anti-trypanosomal activity and cytotoxicity. The crude extracts as well as pure compounds obtained were screened *in vitro* for trypanosomal activity against *Trypanosoma brucei brucei* (S427) blood stream forms. Active compounds from the preliminary screens were further tested to confirm activity and determine their minimum inhibitory concentration (MIC) values. Cytotoxicity of the active compounds and extracts was then tested against PNT2A cells ("normal" prostatic cells).

### 2.1 General bioassay

The anti-trypanosomal and cytotoxicity assays were performed using the REDOX indicator Alamar blue™. The active component of which is resazurin (blue in colour) which in the presence of live parasites or cells is reduced to the bright pink fluorescent resorufin. The fluorescence values for the test plates are measured using a microplate reader in fluorescence mode with excitation and emission wavelengths of 560 and 590nm respectively.

Wells containing active compounds are easily identified as they remain blue in colour and have background levels of fluorescence. The test samples are initially screened at a single concentration and then MIC or IC<sub>50</sub> values are determined for the active compounds at n=2 or n=3. Positive and negative controls and a sterility checks are included in all assays. The incubation and treatment times, incubation conditions and seeding densities are optimised for each test species and cell line.

### 2.1.1 Anti-trypanosomal activity

Samples were tested against the bloodstream form of *Trypanosoma brucei brucei* (*T.b.brucel*) S427. The activity of the plant extracts and isolated compounds was determined using the well-established Alamar blue™ 96 well microplate assay. Samples were initially screened at a single concentration usually 20µg/ml for extracts or 20µM for pure compounds. The concentration of dimethylsulphoxide (DMSO) should not exceed 0.5% in the initial screen.

Samples for testing were prepared as 10mg/ml or 10mM stock solutions in 100% DMSO. These were diluted with HMI-9 medium to a concentration of 1mg/ml /1mM. 4µl of the test sample was added to the assay well then 96µl HMI-9 medium to give a final assay concentration after a 1:1 dilution of 20µg/ml / 20µM.

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	Sterility control	1	2	3	4	5	6	7	8	9	10	1.0µM suramin
<b>B</b>	control	11	12	13	14	15	16	17	18	19	20	0.5µM suramin
<b>C</b>	control	21	22	23	24	25	26	27	28	29	30	0.25µM suramin
<b>D</b>	control	31	32	33	34	35	36	37	38	39	40	0.125µM suramin
<b>E</b>	DMSO control	41	42	43	44	45	46	47	48	49	50	0.062µM suramin
<b>F</b>	DMSO control	51	52	53	54	55	56	57	58	59	60	0.031µM suramin
<b>G</b>	DMSO control	61	62	63	64	65	66	67	68	69	70	0.015µM suramin
<b>H</b>	DMSO control	71	72	73	74	75	76	77	78	79	80	0.008µM suramin

Table 1. Anti-trypanosomiasis assay plate layout.

The screening plate was arranged as illustrated in Table 1. Controls including a sterility control and DMSO controls in column 1; test samples in columns 2 to 11 and a concentration range of suramin as a positive control in column12. Trypanosomes were counted using a haemocytometer and diluted to a concentration of 3x10<sup>4</sup> trypanosomes / ml, 100µl of this suspension was added to each well of the assay plate with the exception of well A1 the

sterility check. The assay plate was incubated at 37°C, 5% CO<sub>2</sub> with a humidified atmosphere for 48 hours. After which 20µl of Alamar blue was added and the incubation continued for a further 24 hours. Fluorescence was then determined using the Wallac Victor microplate reader (Excitation 530nm Emission 590nm). The results were calculated as % of the DMSO control values. Minimum inhibitory concentration values (MICs) were determined for samples with less than 10% of control values. MIC determinations were carried out in duplicate. 200µg/ml test solutions were prepared in column 2 by pipetting 4µl of (10mg/ml) test stock solution and 196µl HMI-9 medium into each well. 100µl HMI-9 medium was pipetted into columns 1 and 3-12 and 1:1 serial dilutions were carried out from columns 2 to 11. 80µl of HMI-9 medium was added to column 12 and 20µl of x10 concentrations of suramin to give a final concentration range of 0.008 to 1.0µM. An inoculum of 100µl of trypanosomes at a concentration of 3x10<sup>4</sup>/ml was added to each well except A1, and the procedure continued as previously described.

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	control	100	50	25	12.5	6.25	3.1	1.55	0.78	0.34	0.17	1.0µM suramin
<b>B</b>	control	100	50	25	12.5	6.25	3.1	1.55	0.78	0.34	0.17	0.5µM suramin
<b>C</b>	control	100	50	25	12.5	6.25	3.1	1.55	0.78	0.34	0.17	0.25µM suramin
<b>D</b>	control	100	50	25	12.5	6.25	3.1	1.55	0.78	0.34	0.17	0.125µM suramin
<b>E</b>	control	100	50	25	12.5	6.25	3.1	1.55	0.78	0.34	0.17	0.062µM suramin
<b>F</b>	control	100	50	25	12.5	6.25	3.1	1.55	0.78	0.34	0.17	0.031µM suramin
<b>G</b>	control	1% DMSO	0.5% DMSO	0.25% DMSO	0.12% DMSO	0.06% DMSO	0.03% DMSO	0.01% DMSO	0.00% DMSO	0.00% DMSO	0.00% DMSO	0.015µM suramin
<b>H</b>	control	1% DMSO	0.5% DMSO	0.25% DMSO	0.12% DMSO	0.06% DMSO	0.03% DMSO	0.01% DMSO	0.00% DMSO	0.00% DMSO	0.00% DMSO	0.008µM suramin

Table 2. Final assay concentrations µg/ml

MIC values were determined as the concentration calculated to have < 5% of control values.

### 2.1.2 Cytotoxicity determinations

Cytotoxicity determinations were carried out using a modification the Alamar blue™ redox-based microplate assay (O'Brien *et al.*, 2000). The cytotoxic activity of the fractions and compounds was initially determined against PNT2A cells a "human" normal prostatic epithelial cell line. Initial screening was carried out at a concentration of 100µg/ml. Concentration response studies (300 to 0.3µg/ml) were then carried out in duplicate for samples with less than 60% of control values in the initial screen.

Cells were seeded into 96 well microplates at a density of  $0.5 \times 10^4$  cells/well in  $100\mu\text{l}$  Dulbecco's modified Eagle's medium (DMEM; Invitrogen) and incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  with a humidified atmosphere for 24 hours. After which DMEM solutions of test compounds at the desired concentrations and DMSO as a negative control and 0.1% Triton X as a positive control were added to give a total well volume of  $200\mu\text{l}$ . The microplates were incubated for 24 hours before the addition of  $10\mu\text{l}$  of alamar blue. After a further 20 hours incubation fluorescence was determined using a Wallac Victor microplate reader in fluorescence mode (Excitation 530nm; Emission 590nm). The results were calculated as % of the DMSO control values.  $\text{IC}_{50}$  values were calculated for the concentration response data using Graphpad prism software.

## 2.2 Isolation and structure elucidation of compounds

Melting points (uncorr.) were determined on a Buchi B-540 melting point apparatus. The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were run on a Bruker AV 300 (400MHz) and DRX 500 spectrometers using  $\text{CDCl}_3$  or DMSO-d6 as solvents and TMS as internal standard. HRESI-MS were run using Thermo LTQ Orbitrap or Thermo Exactive orbitrap mass spectrometer. A capillary voltage of 46.00V for the positive mode and -48.00V for the negative mode was used for HRESI-MS. Column chromatography were performed using silica gel MN-60 (Macherey-Nagel GmbH & Co. KG) and Gel filtration chromatography using Sephadex LH-20 (GE Health Lifescience UK) were performed in glass columns. Spots on TLC were visualised using Anisaldehyde- $\text{H}_2\text{SO}_4$  reagent or Dragendorff's reagent for the alkaloids. Developed TLC plates were also observed under UV lamp using short ( $\lambda = 254$  nm) and long ( $\lambda = 366$  nm) wavelengths. The structures of the pure compounds were determined using spectroscopic methods (1D/2D NMR and mass spectrometry). Column chromatography (CC) were carried out on wet packed silica gel in glass columns eluting gradient wise with hexane, hexane-ethyl acetate, ethyl acetate and ethyl acetate-methanol mixtures or with Sephadex LH-20 pre-soaked and wet packed in methanol. After the introduction of the extracts or fractions in methanol, the Sephadex columns were also eluted with methanol. TLC analysis was performed on pre-coated silica gel aluminum plates cut to desired size. NMR experiments were run in  $\text{CDCl}_3$  or DMSO-d6 with TMS as internal standard on Bruker AV 300 (400MHz) or DRX-500 spectrophotometer.

### 2.2.1 Plant materials

All plant materials were collected from Oju LGA Benue State, Nigeria between July and September 2008. The plants were authenticated by the Department of Forestry and Wildlife of the University where voucher specimens were deposited in the University of Agriculture Forest Herbarium. *Alcornea cordifolia* (bark), *Euphorbia poisonii* (stem), *Monodora myristica* (seeds), *Prosopis Africana* (bark), *Spondias mombim* (root), *Nauclea pobeguinii* (bark), *Nauclea latifolia* (bark), *Cochlospermum planchonii*(root) and *Withania somnifera* (whole plant), *Terminalia avicennioides* (bark). *Maytenus laevis* (root) was from samples already housed at the Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS). They were all sun dried in open air and ground using a mill.

### 2.2.2 Extraction and identification of the components

The plant materials (500g – 1.0kg) were air dried and extracted using hexane, ethyl acetate and methanol in a Soxhlet apparatus. The crude extracts were allowed to stand and sometimes cooled to allow for the precipitation of compounds. Where all precipitates have been produced or no (further) precipitation, the crude extracts or precipitates (where not a pure compound) were subjected to column chromatography (CC) eluting gradient-wise using hexane-ethyl acetate and subsequently ethyl acetate-methanol solvent mixtures. Using TLC as a guide, similar fractions were combined and allowed to evaporate under the fume hood to allow for the crystallisation of pure compounds. Where no pure compounds were separated and the fractions were active, such fractions were further purified via preparative thin layer chromatography or Gel filtration (GF) using Sephadex LH-20 to obtain the pure compounds. Methanol extracts which were usually viscous and complex were subjected to an initial vacuum liquid chromatography (VLC) to fractionate the extracts. Similar fractions (TLC) were also combined and further purified via column chromatography or Gel filtration. Flash chromatography using an iES Reveleris Automated Flash Chromatography system was used for the isolation of certain compounds. The column was dry packed after taking up the extracts with silica gel and then loaded onto the pre-packed flash column. The compounds were eluted starting with hexane with incremental additions of ethyl acetate, then with ethyl acetate with additions of methanol and finally with methanol. Using two variable length UV detectors and one ELSD detector, similar eluates were combined and confirmed by TLC and <sup>1</sup>H NMR. Pure compounds were then allowed to settle out from the combined fractions. All columns were exhaustively eluted and washed with methanol. Washings were allowed to stand to allow for the precipitation of compounds. The identification of pure compounds was carried out using one dimensional <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy (including J-mod, DEPT-q and DEPT 135 or 90). Spectral data of known compounds were compared with published spectral data and were thereby identified. Further 2D experiments (including correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC) and nuclear overhauser enhancement spectroscopy (NOESY)) were carried out for more complex molecules for accurate assignments of proton and carbon chemical shifts. Assigned structures were confirmed using ESI-MS and HRESI-MS to confirm their mass and molecular formula. The various extracts produced are listed in table 3 below. Extract code descriptions or extensions are: precipitate (P), latex (L), Sephadex (S), methanol wash (w), VLC fraction (V), flash chromatography fraction (F), root (R), bark (B).

## 3. Results

### 3.1 Anti-trypanosomal screening

The photographs below show the results from the concentration response studies to determine the MIC of the triterpenoids (compounds 11 and 12) from *M.laevis*. Well A1 is the sterility check, wells B1 to H1 negative controls and wells G2 to G11 and H2 to H11 are the DMSO 1:1 dilution controls. Column 12 contains a concentration range of 1 to 0.008 $\mu$ M of the positive control suramin. In plate 1 compounds 11 (rows C and D) and 12 (rows E and F) are tested n=2 over a concentration range of 100 to 0.17 $\mu$ M. In plate 2 compounds 11 (rows A, B and C) and 12 (rows D,E and F) are tested n=3 over a concentration range of 10 to 0.02 $\mu$ M.

S/No	Plant	Part	Extract codes			
			Hexane (H)	Ethyl acetate (E)	Combined hexane + ethyl acetate (HE)	Methanol (M)
1	<i>Alcornea cordifolia</i>	Bark (B)	ACBH	ACBE	ACBHE	ACBM
2	<i>Cochlospermum planchonii</i>	Root (R)	CPH	CPE	CPHE	CPM
3	<i>Euphorbia poisonii</i>	Stem	EPH	EPE	EPHE	EPM
4	<i>Maytenus laevis</i>	Root (R)	MLH	MLE	MLHE	MLM
5	<i>Monodora myristica</i>	Seed	MMH	MME	MMHE	MMM
6	<i>Nauclea latifolia</i>	Bark (B)	NLH	NLE	NLHE	NLM
7	<i>Nauclea pobeguinii</i>	Bark (B)	NPH	NPE	NPHE	NPM
8	<i>Prosopis Africana</i>	Bark (B)	PAH	PAE	PAHE	PAM
9	<i>Spondias mombim</i>	Root (R)	SMH	SME	SMHE	SMM
10	<i>Terminalia avicennioides</i>	Bark (B)	TAH	TAE	TAHE	TAM
11	<i>Withania somnifera</i>	Whole plant	WSH	WSE	WSHE	WSM

Table 3. Extracts produced from the plant materials

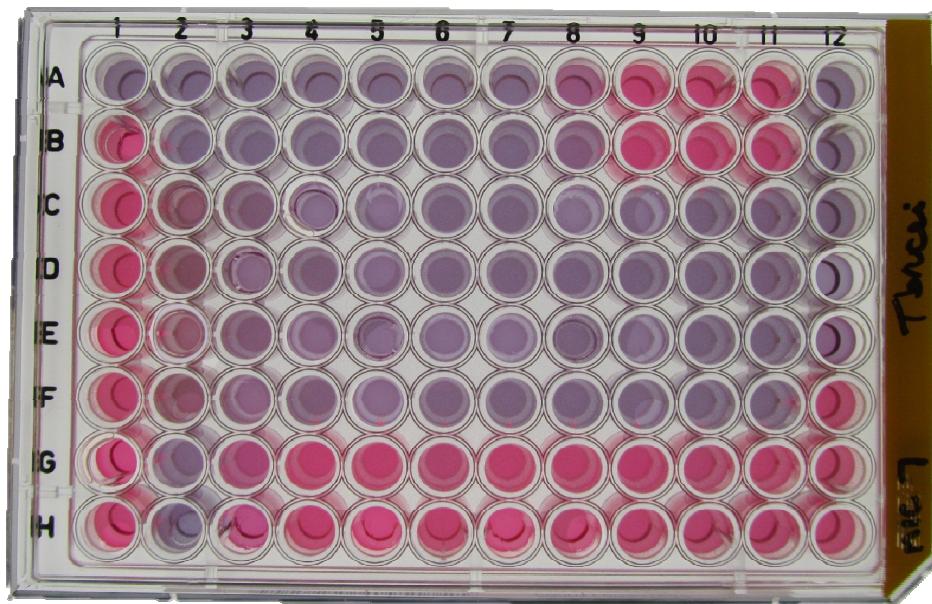


Plate 1. Photo showing MIC of compounds 11 and 12.

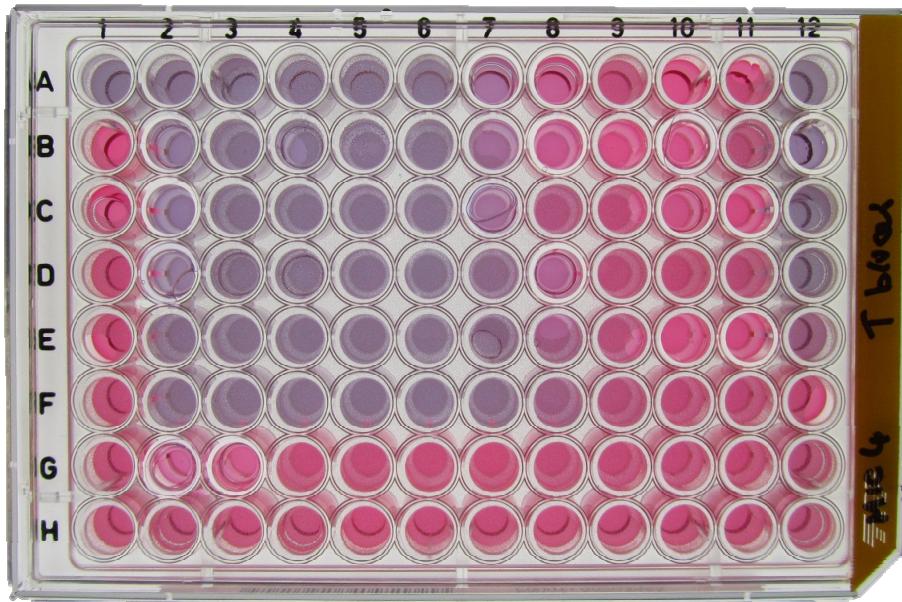


Plate 2. Photo showing MIC of compounds **11** and **12**.

The anti-trypanosomal activity and cytotoxicity against PNT2A cell lines observed for the extracts, fractions and compounds are given in the table below.

	Extract/ Fraction	Compound/ Remarks	Anti-trypanosomal activity		Cytotoxicity	
			% of Control (20 µg/ml)	MIC (µg/ml)	% of Control (100 µg/ml)	IC <sub>50</sub> (µg/ml)
1	ACBE-1	<b>8</b>	13.3		42.8	
2	ACBM-12	<b>3</b>	3.8	<0.2	29.9	1.5
3	ACBM-14	<b>7</b>	18.0		28.5	24.2
4	PAM-5	Column fraction	12.5			
5	PAM-6	Column fraction	19.4			
6	WSE-1a	Column fraction	12.5	12.5		
7	WSE-1b	Column fraction	12.5	12.5		
8	WSE-1c	Column fraction	3.12	3.12		
9	MMH-1	Crude extract	8.0	12.5		
10	MME-5	Column fraction	-1.2	3.125		
11	MME-10	Column fraction	-1.4	3.125		
12	MMS-28	<b>9 &amp; 10</b>	-1.4	6.25		
13	MME-29	Column fraction	-1.3	3.125		
14	MME-41	<b>10</b>	6.5	12.5		
15	MME-42	<b>9</b>	6.5	25		
16	EPL-1	Column fraction	0.8		64.5	

	Extract/ Fraction	Compound/ Remarks	Anti-trypanosomal activity		Cytotoxicity	
			% of Control (20 µg/ml)	MIC (µg/ml)	% of Control (100 µg/ml)	IC <sub>50</sub> (µg/ml)
17	EPLE-5	Column fraction	-0.1	6.25	29.6	
18	EPP-1	<b>6</b>	1.3	1.56		
19	EPH-1	Crude extract	-0.2			
20	EPHE-w	<b>5</b>	-0.7			
21	TASW-2	<b>14</b>	33.6			
22	TAHE-1	Crude extract	8.7			
23	TAHE-4	Column fraction	5.2	2.5		
24	NPE-5	Column fraction	0.8			
25	NPE-7	Column fraction	4.9	6.25	9.7	>100
26	NPE-13	Column fraction	4.4	12.5	44.2	>100
27	NPE-48	<b>1</b>	6.4	12.5	42.8	>100
28	NPM-10	Column fraction	28.1		43.9	7.7
29	SMHE-44	<b>2</b>	-0.2	25	87.5	
30	SMM-9	<b>4</b>	31.6		50	
31	MLHEF-14	<b>11</b>	3.8	0.625		
32	MLHEF-18	<b>12</b>	6.1	0.625		
33	WSM-w	<b>13</b>	78.7			
34	WSH-1	Crude extract	50.0	50.0		
35	WSH-2	Column fraction	25.0	25.0		
36	WSH-3	Column fraction	50.0	50.0		
37	MMH-3	Column fraction	6.25	6.25		
38	MMH-0	Crude extract	25.0	25.0		
39	MMH-4	Column fraction	50.0	50.0		
40	ACRHE-20	Column fraction	20		85.0	
41	NPE-57	<b>15</b>	100		87.8	
42	NPE-81	<b>16</b>	100			
43	EPH-1.4	Column fraction	100		81.2	
44	EPH-1.1	Column fraction	-0.2	12.5	76.4	
45	EPH-1.6	Column fraction	107.8		79	
46	ACBME-2	Column fraction	91.0		83.2	
47	ACBHE-6	Column fraction	80.3		89.3	
48	SMHE-7	Column fraction	92.3		92.7	
49	SMHE-13	Column fraction	96.3		87.5	
50	ACRM-1	Crude extract	95.8		87.5	
51	MMM-1	Crude extract	108.2			
52	MMEV-12	VLC fraction	-0.4			
53	MMEV-17	VLC fraction	0.9			

Table 4. Activity of crude extracts, fractions and compounds

### 3.2 Isolated compounds

The following compounds were isolated from the plants:

Compound	Reference	Plant
<b>1</b>	Hotellier et al. (1980)	<i>Nauclea pobeguinii</i> , <i>Nauclea latifolia</i>
<b>2</b>	Nonaka et al. (1982)	<i>Spondia mombin</i>
<b>3</b>	Lee et al. (1992)	<i>Alcornea cordifolia</i>
<b>4</b>	Nagle et al. (2006)	<i>Cochlospermum planchonii</i> , <i>Spondias mombin</i>
<b>5</b>	Gewali et al. (1990)	<i>Euphorbia poisonii</i>
<b>6</b>	Dubery et al. (1999)	<i>Euphorbia poisonii</i>
<b>7</b>	Bankova (1990); Saha et al. (1991)	<i>Alcornea cordifolia</i>
<b>8</b>	Koetter et al. (1989)	<i>Alcornea cordifolia</i>
<b>9</b>	Nkunya et al. (2004)	<i>Monodora myristica</i>
<b>10</b>	Ishii et al (1975)	<i>Monodora myristica</i>
<b>11</b>	Gonzalez et al. (1982)	<i>Maytenus laevis</i>
<b>12</b>	Jeller et al. (2004)	<i>Maytenus laevis</i>
<b>13</b>	Nandy et al. (1989)	<i>Withania somnifera</i>
<b>14</b>	Kim et al. (2001)	<i>Terminalia avecinoides</i>
<b>15</b>	Cerri et al.(1988)	<i>Nauclea pobeguinii</i> , <i>Nauclea latifolia</i>
<b>16</b>	Cerri et al. (1988)	<i>Nauclea pobeguinii</i> , <i>Nauclea latifolia</i>

Table 5. Isolated compounds and plant sources

Some of the active compounds were identified (Mass spec and NMR: 1D, 2D including COSY, HSQC, HMBC, NOESY) their spectral data were compared with literature reports. The structures of the compounds are given in the figure below.

### 3.3 NMR data for some isolated compounds

Position	Compound 9		Compound 10	
	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C
1	8.57 br.s (NH)	-	8.36 br.s (NH)	-
2	7.34 dd ( <i>J</i> =2.8, 3.2 Hz)	126.0 (CH)	7.27 <i>m</i>	125.4 (CH)
3	6.74 <i>ddd</i> ( <i>J</i> = 0.8, 2.0, 3.2 Hz)	104.5 (CH)	6.62 <i>m</i>	103.5 (CH)
4	8.20 <i>d</i> ( <i>J</i> = 1.2 Hz)	126.2 (CH)	7.85 <i>d</i> ( <i>J</i> = 0.8 Hz)	122.7 (CH)
5	-	129.8 (C)	-	126.5 (C)
6	7.80 <i>dd</i> ( <i>J</i> = 1.2, 8.4 Hz)	122.4 (CH)	7.48 <i>dd</i> ( <i>J</i> = 8.4, 1.6 Hz)	121.8 (CH)
7	7.50 <i>d</i> ( <i>J</i> = 8.4 Hz)	111.7 (CH)	7.43 <i>d</i> ( <i>J</i> = 8.4 Hz)	111.6 (CH)
8	-	138.8 (C)	-	137.9 (C)
9	-	127.8 (C)	-	128.2 (C)
1 <sup>c</sup>	10.06 <i>s</i>	192.5 (CHO)	2.41 <i>s</i>	27.4 (CH <sub>3</sub> )
2 <sup>c</sup>	-	-	-	198.6 (CO)
3 <sup>c</sup>	-	-	6.73 <i>d</i> ( <i>J</i> = 16.4 Hz)	124.7 (CH)
4 <sup>c</sup>	-	-	7.69 <i>d</i> ( <i>J</i> = 16 Hz)	145.6 (CH)

Table 6. The NMR spectra assignments for compounds **9** and **10**.

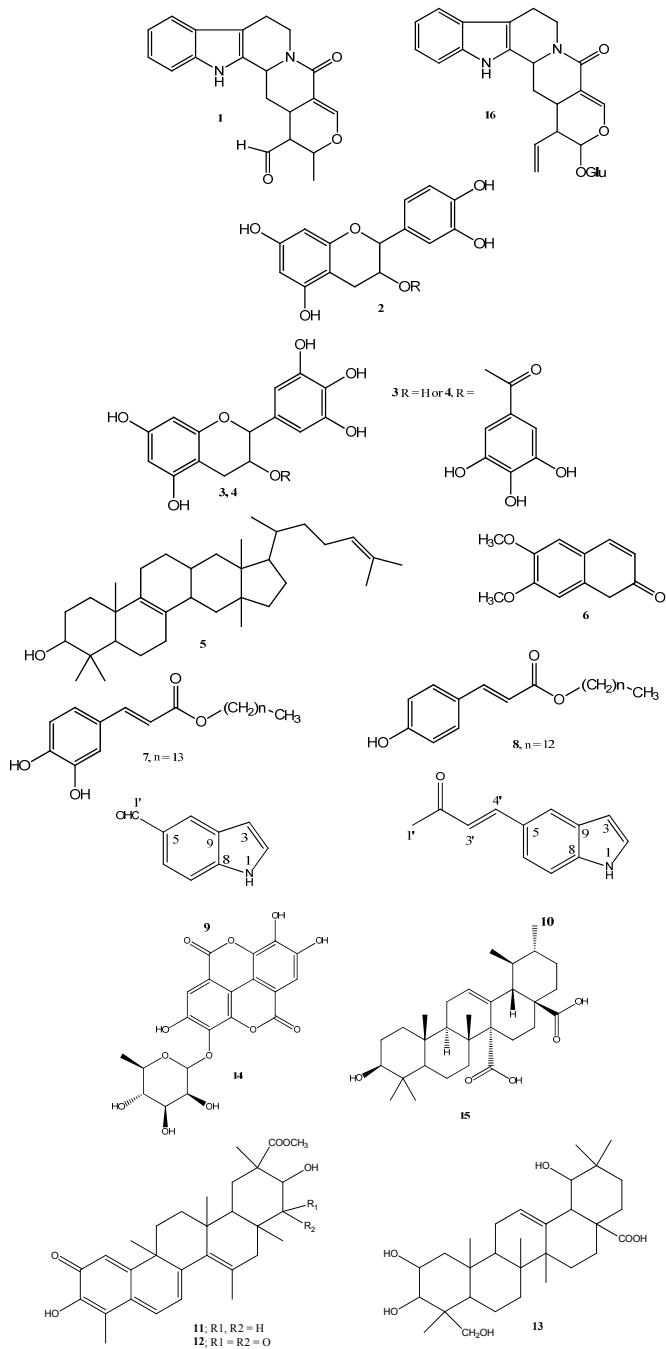


Fig. 1. Structures of isolated compounds

#### 4. Discussion

The beta carboline alkaloids were isolated from the two *Nauclea spp.* The catechins and their gallates were obtained from *A. cordifolia*, *S. mombim* and *C. planchonii*. Catechins including catechin, epicatechin, gallicatechin and five others have been reported to be active against nonproliferative bloodstream trypomastigote and intracellular replicative amastigote parasite forms of *Trypanosoma cruzi* (Paveto et al., 2004). The stem of *E. poisonii* afforded the steroid triterpene and scoroprone. The caffeic acid esters were isolated from *A. cordifolia* and there could be a mixture of more esters. The quinonemethide friedelene triterpenes were obtained from hexane and ethyl acetate extracts of *M. laevis*. They showed very good activity with MICs of 0.625 $\mu$ g/ml. Other pure compounds obtained are as listed on table 2. Some of the pure compounds were however not active when tested. Compound **13** is one of such as well as compound **14** which did not show very good activity. The results of these screening supports a further investigation of the active compounds identified. These could be confirmed as hit molecules especially when the compound's cytotoxicity is low showing selectivity which is vital for drug molecules. Compounds that are inimical to the growth and survival of microorganisms but do not affect the cells adversely are potential candidates for drug development. Some of the extracts and fractions show significant activity but the active compounds have not been identified in this study. Such extracts could be subject of further investigation to isolate or identify the active components. These results also lend credence to some of the ethnobotanical or traditional use of the plants as they are mainly used against fevers caused by malarial parasites. Interestingly, the same or similar compounds were isolated from the different plant samples. Some belong to the same families but others do not. This thus confirms their activity not being due to the crude extracts or fractions but the fact that these extracts or fractions contain the same or similar compounds. These results may easily pave way for a structure activity study as variations in structure and side chains or substituents on similar moieties vis-à-vis activity can easily be observed. For instance it can be observed that glycosidation knocks off activity from the test compounds.

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