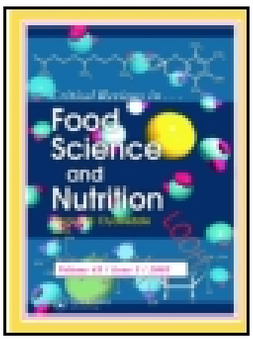

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REVIEW

Research progress of black tea thearubigins: a review

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ABSTRACT

As the most abundant component in black tea, thearubigins (TRs) contribute a lot to black tea's characteristic color, mouthfeel, and potential health benefits. But compared to lower molecular weight black tea polyphenols, there are fewer researches that focus on TRs because of their heterogeneity. This review summarized recent research progress on (1) isolation method of TRs; (2) structure analysis and formation mechanism of TRs; (3) biofunctions of TRs, including antioxidation, antimutagenic and anticancer effects, effects on mitochondrial activation, gastrointestinal motility and skeletal health, to show some future research aspects and prospects of TRs.

KEYWORDS

Formation; function; isolation; structure; thearubigin

Introduction

Black tea, as one of the most consumed beverages in the world, has been focused by researchers for quite a long time because of its potential health benefits (Naveed et al. 2018; Sanlier, Gokcen, and Altuğ 2018). Being a fermented tea, black tea is generally made through four major steps: withering, rolling, fermentation, and drying. Among these four steps, fermentation is the most critical one and matters to black tea's characteristic color, smell, mouthfeel, and above all, its biofunctions (Muthumani and Kumar 2007; Owuor et al. 2006).

Through fermentation, catechins in fresh tea leaves are oxidized and polymerized to higher molecular weight polyphenols under the catalysis of endogenous polyphenol oxidase (PPO) and peroxidase (POD) (Finger 1994). For low molecular weight black tea polyphenols, such as theaflavins (TFs) and theaflavin derivatives, plenty of studies have been carried out on them. As summarized in the review by Drynan et al. (2010), structures and formation mechanisms of many low molecular weight black tea polyphenols are fairly clear and detail. Moreover, the study of low molecular weight polyphenols has become ever more mature with the development of research methods and devices (Delius, Frank, and Hofmann 2017).

By contrast, for high molecular weight black tea polyphenols, their chemistry properties and formation mechanisms are still quite obscure. The name thearubigins (TRs) was firstly given to include all the high molecular weight polyphenols with reddish-brown color and good water solubility by Roberts (1958). Since then, studies have shown that, same as low molecular weight polyphenols, TRs also affect the sensorial quality of black tea (Owuor et al. 2006) and contribute to the potential health benefits. Chemistry of TRs was once reviewed by Haslam (2003), and that review motivated more researchers to study these tough but valuable mixtures. However, due to heterogeneity, the solubility and chromatographic behaviors of TRs are complicated, making both isolation and structural analysis difficult.

This review mainly summarizes recent research progress on TRs, including developments of isolation method, structure analysis, formation mechanism and potential biofunctions, with more focus on the past 15 years. And we hope this review could offer a comprehensive empirical study of TRs and draw some light on the future study and utilization of high molecular weight polyphenols.

Research progress on the chemistry of TRs

To date, there is still no consistent definition on TRs, but generally they are mixtures of heterogeneous phenolic polymers condensed from catechins and TFs (Haslam 2003). TRs are thought to be the most abundant components in black tea and account for more than 60% of the solids in a typical black tea infusion (Haslam 2003; Roberts, Cartwright, and Oldschool 1957). They are water soluble, acidic and reddish-brown colored polyphenols which perform similar ill-defined chromatography behaviors (Haslam 2003; Roberts 1958). In terms of molecular weight, studies resulted differently, varies from 700 Da to 40,000 Da, due to different analytical methods were applied (Fujihara et al. 2007; Haslam 2003). The classification of TRs is also not fixed. Generally, there are two approaches to classify TRs. According to the solubility in solvents, Roberts classified TRs into three groups: ethyl acetate insoluble SII part, ethyl acetate soluble SI part, and more neutralized SIa part (Roberts 1958). Another classification method depends on their chromatographic behavior in reverse phase high-performance liquid chromatography (HPLC). Group I covers TRs that excluded from HPLC column, group II is resolved TRs, and group III is TRs that eluted as unresolved

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"Guassian-shape hump" (Bailey, Nursten, and McDowell 1994; Kuhnert et al. 2010). The ambiguous classification of TRs also confirms the difficulty involved in their isolation and structural analysis.

Research progress on the isolation of TRs

Basically, all existing isolation methods of TRs are developed form two fundamental methods: solvent extraction and chromatography. The liquid-liquid extraction with ethyl acetate and *n*-butanol, as a traditional method which was firstly applied by Roberts, Cartwright, and Oldschool (1957) to separate and define TRs, has been improved in varies ways since then. For example, chloroform was added to effectively remove caffeine before the black tea infusion was extracted by ethyl acetate and *n*-butanol (Brown et al. 1969). Excessive caffeine was used to precipitate TRs rich fraction in each phase after the black tea infusion was partitioned against ethyl acetate (Powell et al. 1993). Besides, Krishnan and Maru (2006) applied a Soxhlet-based solid-liquid extraction method and compared the isolated fractions with that from Brown's liquid-liquid extraction method. As a result, a more refined TRs extraction was got with less solvents and time cost, indicating a better isolation method of TRs.

Same as solvents extraction methods, improvements had also been made on chromatography separation of TRs. Just after TRs were ambiguously defined, chromatography on Sephadex LH-20 column was firstly applied in the separation of black tea factions (Cattell and Nursten 1977). With decades of development, Sephadex LH-20 column is still the most commonly used in the chromatography separation of TRs. In a later study, in order to get better separation of black tea infusion, Toyopearl HW-40F column was selected by Ozawa (1982) for its higher resolving power than that of Sephadex LH-20 column. Furthermore, Solka-Floc cellulose was chosen as an effective choice for the isolation of unresolved polymeric TRs (Bailey, Nursten, and McDowell 1992). Because SI TRs fraction isolated from black tea infusion usually contain large amount of catechins and TFs, recent research prefer to focus on SII fraction (Stodt, Stark, and Engelhardt 2015). For the purpose to get better separated SII TRs fraction, high-speed counter-current chromatography (HPCCC) was applied, considering its effective separation of many smaller particles (Degenhardt et al. 2000).

Recent researches also attempted to combine these basic methods to prepare more refined SII TRs. In order to get a better separation of high molecular weight black tea polyphenols, solvents extraction and chromatography on Toyopeal HW-40F column were combined. Through this method, Fujihara et al. (2007) found a fraction that could increase mitochondrial membrane potential of *Tetrahymena* (a ciliated protozoan) and thus named this fraction mitochondrial activation factors (MAFs). More recently, a method that combines caffeine precipitation and Sephadex LH-20 column chromatography was developed by Wang et al. (2018). In this study, the caffeine precipitate of black tea extract was loaded to Sephadex LH-20 column directly before the sample was eluted by ethanol and aqueous acetone for the isolation of TRs. Compared to other previously published methods, this method avoids using chloroform for decaffeination. Besides, the yield was less in amount but purer in purity, making this method a proper way for the isolation of TRs in further structural analysis and functional studies (Wang et al. 2018).

Comparisons of different methods were done by researchers to decide which one is the best, but with no definite conclusion. For a most recent example, Robert's liquid-liquid extraction, caffeine precipitation method and HPCCC were compared, showing that Roberts' extraction yielded the largest amounts of TRs whilst the caffeine precipitation method demanded the least time. The most purified TRs fraction was obtained through HPCCC, but with less yields and more time cost (Stodt, Stark, and Engelhardt 2015). Actually, each of the listed isolation methods has its own advantages and disadvantages (Table 1), and may be it is advisable for researchers to carefully choose according to their research purposes. For instance, caffeine precipitation requires less time, but also bring more caffeine in the isolated TRs if not being decaffeinated and high dose of caffeine may induce severe symptoms in laboratory animals. Chloroform is the most effective agent to remove caffeine in the preparation of TRs, but usually not appropriate in researches that focus on the biofunctions of TRs because of its toxicity. Solvents extraction methods require less workload, bring good yields with less purity, making them applicable for large amount of rough extraction. Column chromatography was usually used to study the constitution of tea infusion in precedent researches, but combined with solvents extraction, it seems to be a useful technique to isolate specific TRs fractions. In order to fully study TRs, combination between solvent extraction and chromatography on different columns to isolated more specific TRs fractions, or even distinguish some fractions from TRs family, should be a promising research direction.

Research progress on the structure and formation mechanism of TRs

The difficulty faced in isolation also hinders the speculation of structure and formation mechanism of TRs fractions. Since Roberts' pioneering study of TRs, little progress has been achieved on these areas although quite a lot of studies were carried out.

When TRs were defined by Roberts (1958), he also suggested that TFs are possible intermediates in TRs formation and the principal substrates are epigallocatechin (EGC) and epigallocatechin gallate (EGCG). Haslam (2003) further concluded that possible pathways in the formation of TRs largely derived from quinones, TFs, theasinensins and other benztropolones under the catalysis by PPO and POD during the fermentation of black tea. Finger (1994) once provided evidence that PPO and POD function differently during the formation of TRs. In vitro oxidation experiments of crude flavanols mixture showed that products obtained from PPO catalysis contained higher levels of TFs and HPLC resolvable

Table 1. Comparation of different TRs isolation methods.

Isolation method	Time consumption (from dried black tea)	Purity and main composition (determined by HPLC)	Yields (as related to solids of tea infusion)	Reference
Roberts' liquid-liquid extraction	Medium (3–4 days)	Low (separated SI and SII TRs, many non-TRs)	High (∼11.80%)	Roberts, Cartwright, and Oldschool (1957); Stodt, Stark, and Engelhardt (2015)
Chloroform before partition by ethyl acetate	High (>6 days)	Low (separated SI and SII TRs, many non-TRs, no caffeine)	Medium (∼7.07%)	Brown et al. (1969); Krishnan and Maru (2006)
Excessive caffeine after partition by ethyl acetate (caffeine precipitation)	Low (1–2 days)	Low (mainly SII TRs, some non-TRs and caffeine)	Medium (∼8.84%)	Powell et al. (1993); Stodt, Stark, and Engelhardt (2015)
Soxhlet-based solid-liquid extraction	Medium (>3 days)	Low (separated SI and SII TRs, many non-TRs, no caffeine)	High (∼10.33%)	Krishnan and Maru (2006)
Chromatography on Sephadex LH-20 column	Low (1–2 days)	Medium (separated SI and SII TRs, some non-TRs)	Not issued	Cattell and Nursten (1977); Wang et al. (2018)
Chromatography on Toyopearl HW-40F column	Low (1–2 days)	Medium (separated SI and SII TRs, some non-TRs)	Not issued	Ozawa (1982)
Chromatography on Solka-Floc cellulose column	Low (1–2 days)	Medium (theafulvin, an unresolved polymeric TRs, no protein, caffeine and flavonol glycosides)	Not issued	Bailey, Nursten, and McDowell (1992)
High-speed counter current chromatography	High (4–5 days)	High (mainly SII TRs, few non-TRs)	Low (∼2.42%)	Degenhardt et al. (2000); Stodt, Stark, and Engelhardt (2015)
Combination of liquid-liquid extraction and Toyopeal HW-40F column chromatography	High (5–6 days)	High (MAFs, thought to be parts of SII TRs)	Low (∼3.03%)	Fujihara et al. (2007)
Combination of caffeine precipitation and Sephadex LH-20 column chromatography	High (4–5 days)	High (mainly SII TRs, few non-TRs)	High (∼10.37%)	Wang et al. (2018)

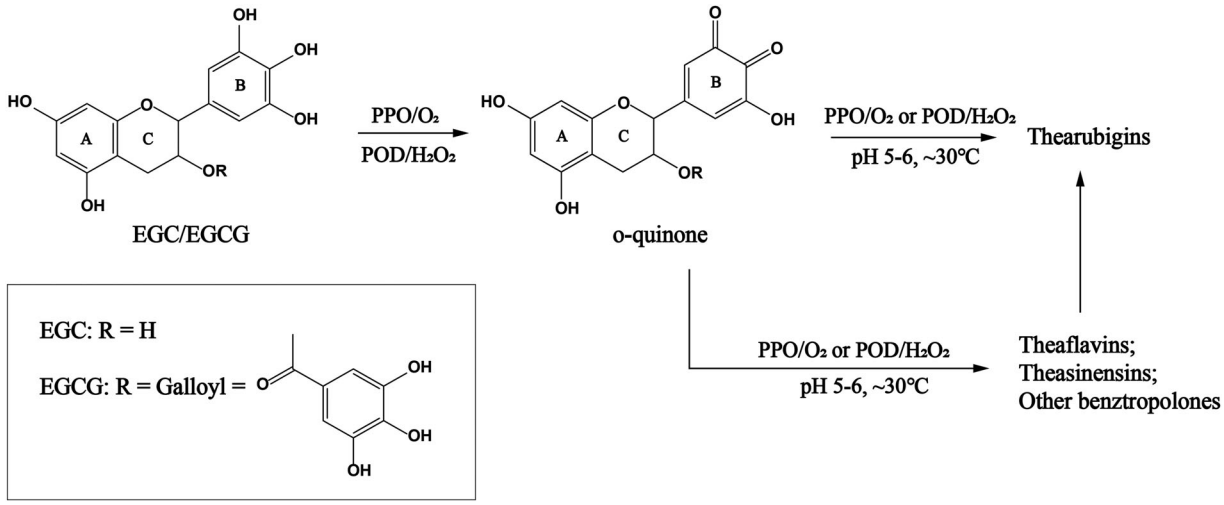

Figure 1. Possible basic formation mechanism of TRs.

TRs compounds, while catalysis through POD resulted in productions of more HPLC unresolved higher molecular weight TRs (Finger 1994). These findings were in accordance with previous research (Millin and Swaine 1981), and basic formation route of TRs could be concluded (Figure 1). However, because most studies on TRs used samples directly isolated from black tea infusion and the fermentation process of black tea is usually not standardized, few studies focus on the detail reaction conditions. Thus, the reaction conditions to form TRs are ambiguous. But basically, PPO, POD and oxygen are indispensable; moderate pH and temperature are required to keep the activation of these enzymes. Compared to temperature, reaction time seems to affect more on the formation of TRs, longer oxidation time resulted in more TFs degradation and TRs formation (Das, Samanta, and Datta 2019; Ngure et al. 2009).

With the basic formation routes being clued out, later studies focused further on the details that happened after the formation of ortho-quinones and dipolymers, in which structural speculation are involved inevitably. Studies on structure speculation of TRs started from the degradation of black tea extract. Through degradation of TRs by acidic hydrolysis, Brown et al. (1969) concluded that TRs are polymeric proanthocynidins because cyanidin and delphinidin were found. However, since there was no evidence showing that proanthocyanidin type structures could be formed from

flavan-3-ols through oxidation, later studies pointed out that those residual structural fragments in acidic hydrolysate of TRs might come from very small amount of these compounds present originally in fresh tea leaves (Haslam 2003). Some more recent studies suggested these compounds may take part in black tea fermentation process but not as main precursors for formation of TRs (Menet et al. 2004; Yassin, Koek, and Kuhnert 2015). Through methylation, degallation and chemical degradation of TRs isolated from solvents extraction and chromatography on Toyopeal HW-40F column, Ozawa et al. (1996) concluded that TRs are heterogeneous polymers of flavan-3-ols and flavan-3-ol gallates that may have bonds at C4, C6, C8, C2', C5' and C6' in the flavan-3-ols units.

Menet et al. (2004) studied the structure of TRs with delayed pulsed ion extraction of ions generated by the matrix-assisted laser desorption ionization (MALDI) technique, on line with a linear time-of-flight (TOF) mass spectrometer (MS). Analysis of MALDI-TOF spectra indicated that some TRs might have similar structures with TFs because the same loss of mass was observed. This result coincided with studies focused on the manufacture of high quality black tea which showed that during the fermentation of black tea, oxidative degradation of TFs usually resulted in higher amounts of TRs and thus suggested that the degradation products of TFs, namely epitheaflavic acids, play important roles in the formation of TRs (Berkowitz, Coggon, and Sanderson 1971; Ngure et al. 2009). According to these studies, possible structures and more detailed formation mechanism for some TRs could be concluded (Figure 2).

Since 2010, a series of attempts to unravel the structure of TRs were done by Kuhnert and his group by various mass spectrometric characterization, leading to a novel theory for the formation mechanism of black tea TRs called oxidative cascade hypothesis (Kuhnert et al. 2010). This hypothesis concluded that further oxidation of catechin oligomers which formed by oxidative coupling is responsible for the oxygen insertion on aromatic moieties and further polymerization between polyhydroxylated species and their quinone counterparts. Their recent study provided significant spectroscopic evidences for the structures of primary oxidation products, such as TFs, theasinensins and their gallate esters, which are main precursors for TRs formation (Yassin, Koek, and Kuhnert 2015). Besides, their study suggested that, compare to chocolate and red wine, the oxidation of black tea polyphenols is mainly taking place on B-rings and the galloyl groups, where the oxidized components undergo further oxidative coupling to form TRs. However, through MS and nuclear magnetic resonance (NMR) experiments, a more recent study pointed out that the reaction between A-rings and B-rings could be another important mechanism for TRs formation and should not be neglected (Uchida, Ogawa, and Yanase 2016).

Even though there is still no conclusive result, studies shown above provided different approaches and valuable ideas to study the structure and formation of TRs. It is quite possible that all these previous findings are important for finally resolving the formation mechanism and establishing more accurate structures of TRs. But before that, further studies are required. For instance, studies focus on condition optimization of TRs production could greatly facilitate the study of structure and formation process.

Research progress on the biofunctions of TRs

Compared to green tea, which contains high amount of catechins to perform many health benefits, TRs are the dominant components in black tea, leading researchers to link the potential health benefits of black tea with TRs. Plenty of studies were carried out to probe into the biofunctions of TRs (Table 2), but how TRs confer these biofunctions is a question that have not been fully answered. Given the complexity of TRs, the knowledge on their bioavailability in mammals is currently limited. It was clinically reported that neither TFs nor their phase II metabolites were detected in urine after intake, which suggested their low bioavailability in human body (Pereira-Caro et al. 2017). Thus, a low bioavailability could also be expected for more polymerized TRs, which means a large portion of black tea TRs will not be digested in small intestine and reach the colon unabsorbed, where they are subsequently broken down into phenolic acids or other small bacterial metabolites by gut microbiota. Meanwhile, the composition of gut microbiota will also be modulated (Liu et al. 2018). Such reciprocal interactions between TRs and gut microbiota may finally result in various health benefits to the host.

Antioxidative effects

Being polymerized polyphenols, the most widely studied biofunction of TRs should be antioxidation that comes with the numerous phenolic hydroxyl groups. Early in 1994, Yoshino et al. (1994) proved that TRs could protect lipid peroxide in the liver tissue suspension of tert-butyl hydroperoxide (t-BHP) induced rat. In another study, TRs showed inhibition effects to hydroxyl radicals and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and could protect HPF-1 cells from oxidative damage (Yang et al. 2007). Results from plasma oxidation assay also indicated a dose-dependent relief from oxidative hemolysis and copper-induced plasma oxidation (Liu and Huang 2015). The antioxidative effects shown by TRs was better than that of glutathione, ascorbic acid, tocopherol and other common antioxidants, but compare to TFs and catechins, these effects are less notable (Liu and Huang 2015; Yang et al. 2007).

It has further been proved that TRs based dietary intervention has the effect to assuage the oxidative stress related maladies in vivo. A recent study found that TRs based dietary intervention resulted a significant improvement in lipid profile, glucose content, renal function and thiobarbituric acid reactive substance (TBARS) with enhancement in insulin, high-density lipoprotein (HDL) and hematological parameters in rats under arginine induced renal malfunction (Imran et al. 2018). These results are consistent with former study which concluded that TRs could inhibit the formation

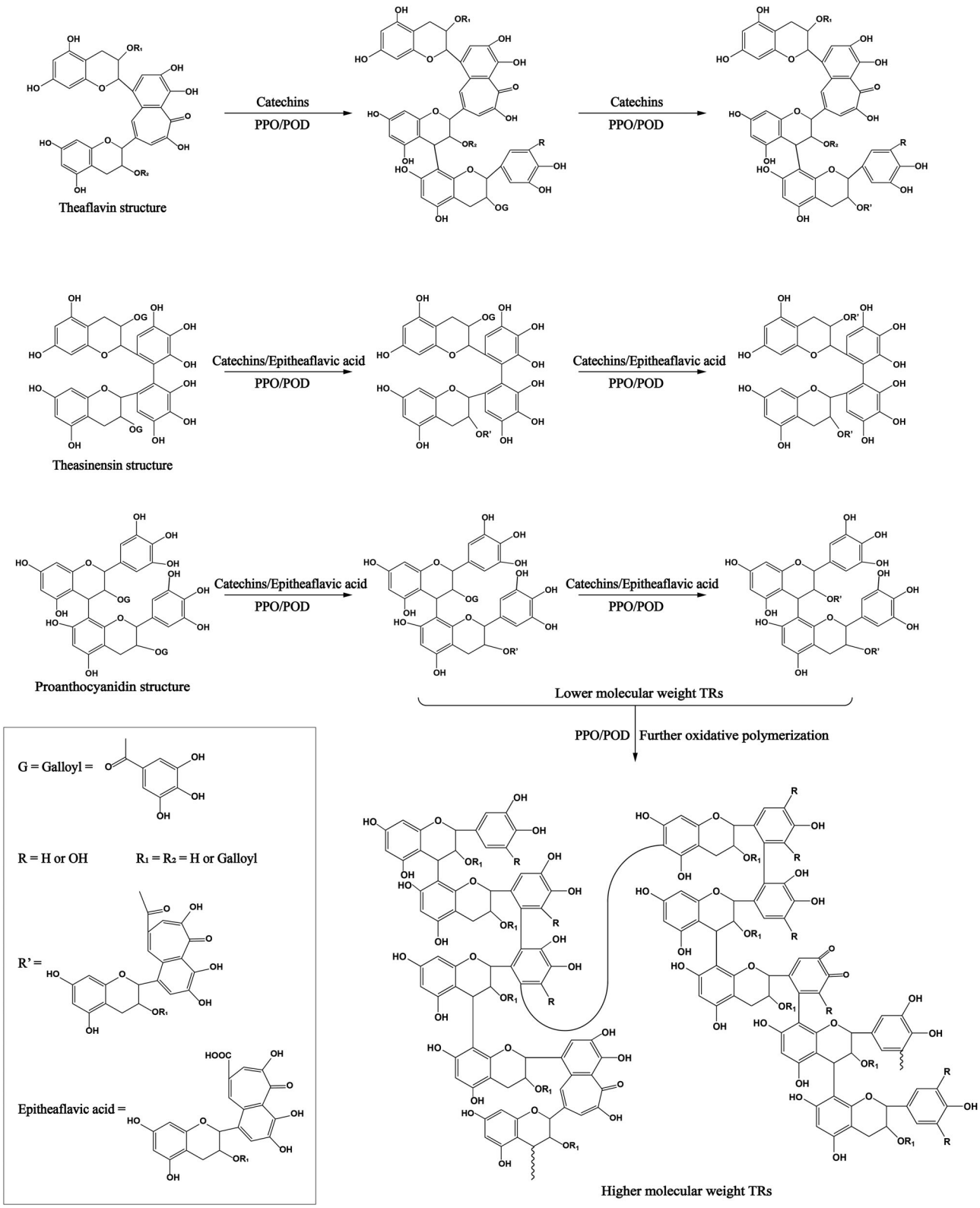

Figure 2. Possible formation mechanism and structures of some TRs.

of TBARS and copper-induced lipid peroxidation of low-density lipoprotein (LDL) and HDL (Liu and Huang 2015). Same as in vitro studies, the antioxidative effects of TRs shown in vivo are less than that of TFs and catechins (Imran et al. 2018).

Possible antioxidant mechanism of TRs in metabolic systems included suppressing the formation and accumulation of intracellular reactive oxygen species (ROS) via preserving the activities of antioxidant enzymes. Besides, inhibition of peroxyl radicals by preventing the oxidation of membrane lipid might also contribute to the antioxidative function of TRs (Liu and Huang 2015).

Many researches tended to link biofunctions of TRs with their antioxidative ability. For a recent example, Murad

Table 2. Possible biofunctions of TRs in cell and animal models (published after 2000).

Biofunctions	Study types	Experimental models	Isolation methods of TRs	Possible mechanism	Reference
Antioxidation					
Protection from oxidative damage	In vitro	HPF-1 cells under H_2O_2-induced oxidative damage	Solvents extraction	Free radicals↓	Yang et al. (2007)
Protection from plasma oxidation	In vitro	Erythrocyte hemolysis assay, plasma oxidation assay, cellular antioxidant activity assay	Solvents extraction	Free radicals↓	Liu and Huang (2015)
Protection from hepatic and renal injury	In vivo	Mice under acetaminophen-induced hepatic and renal injury	Solvents extraction after decaffeination by chloroform	Free radicals↓	Murad et al. (2016)
Protection from lipid peroxidation	In vivo	Rats under arginine-induced renal malfunction	Solvents extraction	Oxidative stress↓	Imran et al. (2018)
Antimutation					
Protection form genetic damage	In vivo	Rats under 1,2-dimethylhydrazine induction	Solvents extraction after decaffeination by chloroform	Free radicals↓ Oxidative stress↓	Lodovici et al. (2000)
Protection form genetic damage	In vivo	Mice under cyclophosphamide and dimethylbenz(a)anthracene induction	Solvents extraction after decaffeination by chloroform	Chromosome aberrations↓ Sister chromatid exchanges↓	Gupta et al. (2001)
Revertant colonies decreased	In vitro	Ames Salmonella assay	Solvents extraction after decaffeination by chloroform	Cytochrome P450 dependent bioactivation of the carcinogens↓	Gupta et al. (2002)
Protection form genetic damage	In vitro. In vivo	Ames Salmonella assay. Mice under benzo[a]pyrene induction	Solvents extraction after decaffeination by chloroform	Free radicals↓ Cytochrome P450 dependent bioactivation of the carcinogens↓ Chromosome aberrations↓ Sister chromatid exchanges↓	Halder et al. (2005)
Protection form genetic damage	In vitro	Human lymphocytes	Solvents extraction after decaffeination by chloroform	Chromosomal aberrations↓ Micronuclei formation↓	Halder et al. (2006)
Protection form genetic damage	In vitro. In vivo	Ames Salmonella assay. Mice under dimethylbenz(a)anthracene induction	Solvents extraction	Free radicals↓ Cytochrome P450-dependent bioactivation of the carcinogens↓	Charehsaz et al. (2017)
Anticancer					
Inhibition of cancer cell proliferation	In vitro	Human monoblastic leukemia cells (U937). Stomach cancer cells (MKN-45)	Solvents extraction after decaffeination by chloroform	Cell growth↓ Apoptosis induction↑	Hayakawa et al. (2001)
Tumor volume and multiplicity decreased	In vivo	Rats under dimethylhydrazine-induced colorectal tumorigensis	Solvents extraction after decaffeination by chloroform	Cellular proliferation through Wnt/β-catenin pathway↓	Patel, Ingle, and Maru (2008)
Phase II enzymes in liver and lung increased	In vitro In vivo	Human hepatoma cells (HepG2). Mice under benzo[a]pyrene induction	Solvents extraction after decaffeination by chloroform	Antioxidant-responsive element↑ Activation of Nrf2 pathway↑	Patel, Ingle, and Maru (2008)
Inhibition of cancer cell proliferation	In vitro	Human malignant melanoma cells (A375). Human epidermoid carcinoma cells (A431)	Solvents extraction after decaffeination by chloroform	Cellular proliferation↓ Apoptosis induction↑ Bax:Bcl2 ratio↑ Expression of p21 and p53↑	Halder et al. (2008)
Inhibition of cancer cell proliferation	In vitro	Human malignant melanoma cells (A375)	Solvents extraction after decaffeination by chloroform	Free radicals↓ Apoptosis induction↑ Activation of JNK and p38 MAPK↑	Bhattacharya et al. (2009)
Inhibition of cancer cell proliferation	In vitro	Ames Salmonella assay. Human leukemic cells (U937)	Solvents extraction and Sephadex LH-20 coulumn	Apoptosis induction↑	Bhattacharya, Mukhopadhyay, and Giri (2011)
Inhibition of cancer cell proliferation	In vitro	Colon cancer cells (HCT116). Lung cancer cells (HT460)	Solvents extraction after decaffeination by chloroform	Cell viability↓ Cell cycle arrest Apoptosis induction↑	Imran et al. (2019)
Mitochondrial activation					
Respiratory activation	In vitro	Tetrahymena	Solvents extraction and Toyopearl HW-40F coulumn	Mitochondrial membrane potential↑	Fujihara et al. (2007)
Motility improvement	In vitro	Sea Urchin Sperm	Solvents extraction and Toyopearl HW-40F coulumn	Mitochondrial respiration↑	Kikuchi et al. (2012)

(*continued*)

Table 2. Continued.

Biofunctions	Study types	Experimental models	Isolation methods of TRs	Possible mechanism	Reference
Skeletal and muscle health					
Endurance improvement	In vivo	Mice under endurance training	Solvents extraction and Toyopearl HW-40F coulumn	AMPK and GLUT4 mRNA expression↑ Fast-to-slow transition in plantaris skeletal muscle↑	Eguchi et al. (2013)
Muscle hypertrophy	In vivo	Mice under conditions of functional overload	Solvents extraction and Toyopearl HW-40F coulumn	mTOR signaling↑ Protein synthesis in muscle↑	Aoki et al. (2017)
Ameliorating osteoporosis	In vivo	Ovariectomized rats	Purchased	Osteoclast differentiation↓	Liang et al. (2018)
Better recovery form muscle atrophy	In vivo	Mice under hindlimb suspension	Solvents extraction and Toyopearl HW-40F coulumn	mTOR signaling↑ Protein synthesis in muscle↑	Aoki et al. (2019)
Gastrointestinal health					
Improve gastrointestinal transit	In vitro. In vivo	Mice. Guinea pig ileum	Solvents extraction after decaffeination by chloroform	Prostaglandin↑ Endogenous NO↑	Chaudhuri et al. (2000)
Improve gastric emptying and small intestinal transit	In vivo	Mice under sildenafil-induced gut motility delay	Solvents extraction after decaffeination by chloroform	Endogenous NO↑	Murad and Abdallah (2014)
Other activities					
Ameliorates mucosal injury	In vivo	Mice under trinitrobenzene sulfonic acid-induced colitis	Solvents extraction and Sephadex LH-20 coulumn	NO and O_2^-↓ Neutrophil infiltration↓ Lipid peroxidation↓ Serine protease activity↓ Nuclear factor kappa B↓	Maity et al. (2003)
Hepatic cholesterol-lowering	In vivo	Rats under high fat diet	Solvents extraction and Sephadex LH-20 coulumn	Fecal steroid excretion↑	Miyata et al. (2011)
Reduction in lung inflammation	In vivo	Neonatal rats under lipopolysaccharide-induced acute lung injury	High-speed counter-current chromatography	Cytokine levels in alveolar cavities↓ Oxygen species↓ Activation of Nrf2 pathway↑	Wang et al. (2019)

et al. (2016) found TRs could protect against acetaminophen induced hepatotoxicity and nephrotoxicity in mice and presumed the effects came from antioxidative and free radical scavenging activity. But as mentioned above, considering the antioxidative ability of TRs is not so good as that of TFs or catechins, how could TRs perform the biofunctions that TFs and catechins do not have? Thus, it may be inaccurate to attribute these functions to antioxidation. Further studies are still needed to probe into the underlying mechanisms and to make more precise conclusions.

Antimutagenic and anticancer effects

Large amount of literatures reported that drinking black tea could reduce the incidence of varies kind of cancers (Naveed et al. 2018). Being the most abundant components of black tea, researches on TRs have been done to verify their anticancer and antimutagenic activity.

Administration of TRs could significantly inhibit oxidative DNA damage in 1,2-dimethylhydrazine (DMH) induced rat colon mucosa (Lodovici et al. 2000). Further research found TRs could also inhibit DMH induced colorectal tumorigenesis and the protective effects might result from a decrease of tumor volume and multiplicity through β-catenin regulation (Patel, Ingle, and Maru 2008). Besides, in vitro studies including studies on human lymphocyte cultures (Halder et al. 2006), human epidermoid carcinoma cells (A431) (Halder et al. 2008), human malignant melanoma cells (A375) (Bhattacharya et al. 2009; Halder et al. 2008), human leukemic cells (U937) (Bhattacharya, Mukhopadhyay, and Giri 2011), human stomach cancer cells (MKN45) (Hayakawa et al. 2001), colon cancer cells (HCT116), lung cancer cells (HT460) (Imran et al. 2019) and Ames Salmonella assay on different salmonella strains (Gupta et al. 2002; Halder et al. 2005; Charehsaz et al. 2017) all clearly demonstrated the antimutagenic and anticancer effects of TRs. Flow cytometric and confocal microscopic studies indicated that induction of apoptosis could be an important mechanism behind these effects (Bhattacharya, Mukhopadhyay, and Giri 2011). Further possible mechanisms of cell apoptosis include increase of Bax:Bcl2 ratio, expression of p19, p21, p27 and p53, inhibition of the cell survival protein Akt phosphorylation and generation of intracellular ROS (Halder et al. 2008; Halder, Gupta, and Gomes 2012). TRs also shown anticlastogenic effects that came from inhibition of promutagen activation, the detoxification of mutagens and carcinogens and induction of DNA repairment (Charehsaz et al. 2017; Gupta et al. 2001; Halder et al. 2006).

Although few in vivo studies on this area could be found, the available documents indicate antimutagenic and anticlastogenic effects of TRs in bone marrow cells of mice. In these

studies, TRs effectively protected the DNA of mice cells from chromosome aberrations and sister chromatid exchanges that could be induced by carcinogens such as cyclophosphamide, dimethylbenz[a]anthracene (DMBA) and benzo[a]pyrene (B[a]P) (Gupta et al. 2001; Halder et al. 2005).

In a short summary, most conclusions on the antimutagenic and anticancer activities of TRs were draw from in vitro studies. The absence of in vivo and further clinical evidences may partly result from the difficulty to isolate large amount of TRs fraction that is appropriate for animal models form black tea infusion. Thus, in order to facilitate the study of biofunctions of TRs on human body, effective isolation methods are needed urgently.

Effects on mitochondrial activation

As mentioned previously, through the application of Toyopearl HW-40F column, a fraction called MAFs was isolated from black tea infusion. Being polymerized TRs that have molecular weight locating in 9000 Da to 18,000 Da, MAFs are the first respiratory activators to be reported, and their functions about energy metabolism were focused since then (Fujihara et al. 2007). In 2012, an in vitro study indicated MAFs could increase the motility of sea urchin sperm through the activation of mitochondrial respiration (Kikuchi et al. 2012). Further in vivo studies also proved the biofunctions of MAFs on energy metabolism in skeletal muscle of mice (Aoki et al. 2017; Eguchi et al. 2013).

Through administration of MAFs to mice with or without endurance exercise, Eguchi et al. (2013) pointed out that better aerobic training effects could be obtained by combination with MAFs intake. A more recent study showed that MAFs rich black tea extraction combined with functional overload promoted muscle hypertrophy in mice (Aoki et al. 2017). Their latest study also pointed out that extraction could promote muscle mass recovery from atrophy but could not prevent atrophy caused by hindlimb suspension in mice (Aoki et al. 2019). These functions may come from the activation of intracellular signaling pathways that involve 5′ adenosine monophosphate-activated protein kinase (AMPK) and Akt/mammalian target of rapamycin (mTOR). Possible signaling cascade related are shown in Figure 3 (Aoki et al. 2017; Eguchi et al. 2013).

AMPK is a key factor in the so-called fast-to-slow transition in muscle fiber. Aerobic training could activate AMPK thus finally result in an improvement of endurance capacity. On the other hand, activation of Akt/mTOR could increase protein synthesis, resulting in hypertrophy of muscle during muscle training. Nevertheless, the activation of AMPK usually leads to inhibition of mTOR, meaning less muscle protein production in endurance training. Thus, the studies above are noteworthy as MAFs from black tea could improve the training effects in both endurance training and overload training, making it a possible supplement in physical training and muscle atrophy recovery. However, MAFs fractions used in these two studies were produced from different isolation methods and different in purity (Aoki et al. 2017). Whether these effects came from MAFs or other compounds in black tea extraction remained uncertain. Further studies are still required to verify the training promoting function of MAFs and to clarify the underlying mechanism.

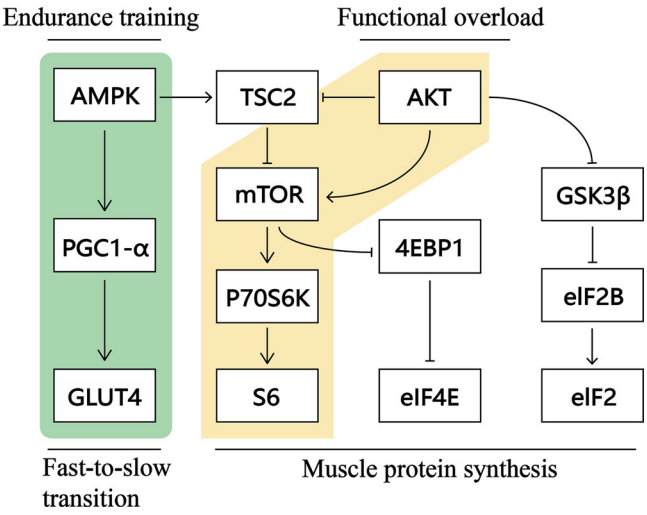

Figure 3. Possible signaling cascade related in the promotion of training effects.

Effects on gastrointestinal motility

Early in 2000, the gastro kinetic effects of black tea extract were studied (Chaudhuri et al. 2000) and researchers pointed out that TRs fraction could accelerate gastrointestinal transit both in vitro and in vivo. Besides, prostaglandin and nitric oxide might play a role in the gastrointestinal motility promotion. This function was reconfirmed by the study of Murad and Abdallah (2014), in which they suggested that TRs dose-dependently improved the sildenafil induced delay in gastric emptying and small intestinal transit in mice. This improvement could be partially blocked by L-NAME also indicated a possible role of nitric oxide. Thus, TRs may be considered effective in remission of dyspepsia. As summarized by Liu et al. (2018), interaction between black tea polyphenols and gut microbiota may play important roles in the potential biofunctions of black tea. It is possible that this interaction could also contribute to the effects on gastrointestinal motility.

Effects on skeletal health

Das et al. (2005) once found that black tea extraction could relive osteoporotic damages in ovariectomized rats through an increase in serum estradiol level. Consistent observations were reported in a recent study. Dietary intervention with TRs significantly improved the levels of maximum bending force, cortical bone thickness and biomarker of bone resorption in ovariectomized rats without significant change on body weight. Furthermore, in vitro studies indicated that TRs can inhibit osteoclastogenesis and diminish the expression levels of the related genes and proteins (Liang et al. 2018). These potential effects on skeletal health indicated

that TRs supplement may be a promising therapy to treat osteoporosis caused by menopause in human females.

Prospective

Although without fully elucidated mechanisms, both in vitro and in vivo studies have shown the various pharmacological functions of TRs. In this review, we summarized several most studied and recently found biofunctions, to show some possible prospects for further utilization of TRs. However, whether they can perform these expected biofunctions in human beings remains an important question to be answered in future clinical studies. Besides, because desirable isolation of specific TRs fractions seems impossible for now, different isolation methods were chosen and usually mixtures of black tea extract were used in previous studies, leading to a difference in the purity of TRs which made the results less persuasive. Furthermore, considering the possible low bioavailability of TRs, research on the interaction between gut microbiota and high molecular weight polyphenols seems to be a better approach to speculate the underlying mechanisms.

Plenty of studies have been done to look into the structure and formation mechanism of TRs. Unfortunately, no consistent conclusion could be drawn for now. But from all the previous studies, maybe we can assume that TRs is polymerized from catechin oligomers such as TFs, theasinensins and their gallate esters through links mainly in A-B rings and B-B rings, during which the formation of quinones play an important role. The use of various mass spectrometry techniques is effective to speculate the structure and formation of TRs, while better isolation method also matters a lot. When it comes to the isolation of TRs, it seems that combination of different methods according to the purpose is the most effective way for now, better isolation methods are still required.

In a brief conclusion, the practical direction for future study on TRs might lie in the following areas: (1) condition optimization and standardization to produce TRs directly from the oxidation of catechins; (2) combination of different isolation methods to prepare more classified TRs factions, e.g. by molecular weight or solubility; (3) more in vivo and clinical studies to confirm the possible biofunctions in human beings; (4) study of the interaction between intestinal microorganism and TRs to clarify the functional mechanism. We believe there are bright prospects lying ahead the application of TRs, but there is still a long way to go before they can be fully understood and utilized by us.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Aoki, Y., T. Ozawa, O. Numata, and T. Takemasa. 2019. High-molecular-weight polyphenol-rich fraction of black tea does not prevent atrophy by unloading, but promotes soleus muscle mass recovery from atrophy in mice. *Nutrients* 11 (9):2131. doi: 10.3390/nu11092131.

Aoki, Y., T. Ozawa, T. Takemasa, and O. Numata. 2017. Black tea high-molecular-weight polyphenol-rich fraction promotes hypertrophy during functional overload in mice. *Molecules* 22 (4):548. doi: 10.3390/molecules22040548.

Bailey, R. G., H. E. Nursten, and I. McDowell. 1992. Isolation and analysis of a polymeric thearubigin fraction from tea. *Journal of the Science of Food and Agriculture* 59 (3):365–75. doi: 10.1002/jsfa.2740590314.

Bailey, R. G., H. E. Nursten, and I. McDowell. 1994. Isolation and high-performance liquid chromatographic analysis of thearubigin fractions from black tea. *Journal of Chromatography A* 662 (1):101–12. doi: 10.1016/0021-9673(94)85300-2.

Berkowitz, J. E., P. Coggon, and G. W. Sanderson. 1971. Formation of epitheaflavic acid and its transformation to thearubigins during tea fermentation. *Phytochemistry* 10 (10):2271–8. doi: 10.1016/S0031-9422(00)89866-0.

Bhattacharya, U., B. Halder, S. Mukhopadhyay, and A. K. Giri. 2009. Role of oxidation-triggered activation of JNK and p38 MAPK in black tea polyphenols induced apoptotic death of A375 cells. *Cancer Science* 100 (10):1971–8. doi: 10.1111/j.1349-7006.2009.01251.x.

Bhattacharya, U., S. Mukhopadhyay, and A. K. Giri. 2011. Comparative antimutagenic and anticancer activity of three fractions of black tea polyphenols thearubigins. *Nutrition and Cancer* 63 (7):1122–32. doi: 10.1080/01635581.2011.605985.

Brown, A. G., W. B. Eyton, A. Holmes, and W. D. Ollis. 1969. The identification of the thearubigins as polymeric proanthocyanidins. *Phytochemistry* 8 (12):2333–40. doi: 10.1016/S0031-9422(00)88151-0.

Cattell, D. J., and H. E. Nursten. 1977. Separation of thearubigins on Sephadex LH-20. *Phytochemistry* 16 (8):1269–72. doi: 10.1016/S0031-9422(00)94372-3.

Charehsaz, M., H. Sipahi, A. K. Giri, and A. Aydin. 2017. Antimutagenic and anticlastogenic effects of Turkish Black Tea on TA98 and TA100 strains of *Salmonella typhimurium* (in vitro) and mice (in vivo). *Pharmaceutical Biology* 55 (1):1202–6. doi: 10.1080/13880209.2017.1282969.

Chaudhuri, L., S. Basu, P. Seth, T. Chaudhuri, S. E. Besra, J. R. Vedasiromoni, and D. K. Ganguly. 2000. Prokinetic effect of black tea on gastrointestinal motility. *Life Sciences* 66 (9):847–54. doi: 10.1016/S0024-3205(99)00657-8.

Das, A. S., D. Das, M. Mukherjee, S. Mukherjee, and C. Mitra. 2005. Phytoestrogenic effects of black tea extract (*Camellia sinensis*) in an oophorectomized rat (*Rattus norvegicus*) model of osteoporosis. *Life Sciences* 77 (24):3049–57. doi: 10.1016/j.lfs.2005.02.035.

Das, S., T. Samanta, and A. K. Datta. 2019. Analysis and modeling of major polyphenols during oxidation in production of black tea. *Journal of Food Processing and Preservation* 43 (12):e14283. doi: 10.1111/jfpp.14283.

Degenhardt, A., U. H. Engelhardt, A.-S. Wendt, and P. Winterhalter. 2000. Isolation of black tea pigments using high-speed countercurrent chromatography and studies on properties of black tea polymers. *Journal of Agricultural and Food Chemistry* 48 (11):5200–5. doi: 10.1021/jf000757+.

Delius, J., O. Frank, and T. Hofmann. 2017. Label-free quantitative 1H NMR spectroscopy to study low-affinity ligand-protein interactions in solution: A contribution to the mechanism of polyphenol-mediated astringency. *PLoS One* 12 (9):e0184487. doi: 10.1371/journal.pone.0184487.

Drynan, J. W., M. N. Clifford, J. Obuchowicz, and N. Kuhnert. 2010. The chemistry of low molecular weight black tea polyphenols. *Natural Product Reports* 27 (3):417–62. doi: 10.1039/b912523j.

Eguchi, T., C. Kumagai, T. Fujihara, T. Takemasa, T. Ozawa, and O. Numata. 2013. Black tea high-molecular-weight polyphenol stimulates exercise training-induced improvement of endurance capacity in mouse via the link between AMPK and GLUT4. *PLoS One* 8 (7): e69480. doi: 10.1371/journal.pone.0069480.

Finger, A. 1994. In-vitro studies on the effect of polyphenol oxidase and peroxidase on the formation of polyphenolic black tea constituents. *Journal of the Science of Food and Agriculture* 66 (3):293–305. doi: 10.1002/jsfa.2740660306.

Fujihara, T., A. Nakagawa-Izumi, T. Ozawa, and O. Numata. 2007. High-molecular-weight polyphenols from oolong tea and black tea: Purification, some properties, and role in increasing mitochondrial membrane potential. *Bioscience, Biotechnology, and Biochemistry* 71 (3):711–9. doi: 10.1271/bbb.60562.

Gupta, S., T. Chaudhuri, D. K. Ganguly, and A. K. Giri. 2001. Anticlastogenic effects of black tea (World blend) and its two active polyphenols theaflavins and thearubigins in vivo in Swiss albino mice. *Life Sciences* 69 (23):2735–44. doi: 10.1016/S0024-3205(01)01348-0.

Gupta, S., T. Chaudhuri, P. Seth, D. K. Ganguly, and A. K. Giri. 2002. Antimutagenic effects of black tea (World Blend) and its two active polyphenols theaflavins and thearubigins in Salmonella assays. *Phytotherapy Research: PTR* 16 (7):655–61. doi: 10.1002/ptr.1038.

Halder, B., U. Bhattacharya, S. Mukhopadhyay, and A. K. Giri. 2008. Molecular mechanism of black tea polyphenols induced apoptosis in human skin cancer cells: Involvement of Bax translocation and mitochondria mediated death cascade. *Carcinogenesis* 29 (1):129–38. doi: 10.1093/carcin/bgm233.

Halder, B., S. D. Gupta, and A. Gomes. 2012. Black tea polyphenols induce human leukemic cell cycle arrest by inhibiting Akt signaling: Possible involvement of Hsp90, Wnt/β-catenin signaling and FOXO1. *The FEBS Journal* 279 (16):2876–91. doi: 10.1111/j.1742-4658.2012.08668.x.

Halder, B., S. Pramanick, S. Mukhopadhyay, and A. K. Giri. 2005. Inhibition of benzo[a]pyrene induced mutagenicity and genotoxicity by black tea polyphenols theaflavins and thearubigins in multiple test systems. *Food and Chemical Toxicology* 43 (4):591–7. doi: 10.1016/j.fct.2005.01.002.

Halder, B., S. Pramanick, S. Mukhopadhyay, and A. K. Giri. 2006. Anticlastogenic effects of black tea polyphenols theaflavins and thearubigins in human lymphocytes in vitro. *Toxicology In Vitro* 20 (5): 608–13. doi: 10.1016/j.tiv.2005.10.010.

Haslam, E. 2003. Thoughts on thearubigins. *Phytochemistry* 64 (1): 61–73. doi: 10.1016/S0031-9422(03)00355-8.

Hayakawa, S., T. Kimura, K. Saeki, Y. Koyama, Y. Aoyagi, T. Noro, Y. Nakamura, and M. Isemura. 2001. Apoptosis-inducing activity of high molecular weight fractions of tea extracts. *Bioscience, Biotechnology, and Biochemistry* 65 (2):459–62. doi: 10.1271/bbb.65.459.

Imran, A., M. U. Arshad, M. S. Arshad, M. Imran, F. Saeed, and M. Sohaib. 2018. Lipid peroxidation diminishing perspective of isolated theaflavins and thearubigins from black tea in arginine induced renal malfunctional rats. *Lipids in Health and Disease* 17 (1):157. doi: 10.1186/s12944-018-0808-3.

Imran, A., M. S. Butt, H. Xiao, M. Imran, A. Rauf, M. S. Mubarak, and M. F. Ramadan. 2019. Inhibitory effect of black tea (*Camellia sinensis*) theaflavins and thearubigins against HCT 116 colon cancer cells and HT 460 lung cancer cells. *Journal of Food Biochemistry* 43 (5): e12822. doi: 10.1111/jfbc.12822.

Kikuchi, A., K. Shiba, T. Ozawa, K. Nakano, K. Inaba, and O. Numata. 2012. Black tea high-molecular-weight polyphenol increases the motility of sea urchin sperm by activating mitochondrial respiration. *Bioscience, Biotechnology, and Biochemistry* 76 (12):2321–4. doi: 10.1271/bbb.120493.

Krishnan, R., and G. B. Maru. 2006. Isolation and analyses of polymeric polyphenol fractions from black tea. *Food Chemistry* 94 (3): 331–40. doi: 10.1016/j.foodchem.2004.11.039.

Kuhnert, N., J. W. Drynan, J. Obuchowicz, M. N. Clifford, and M. Witt. 2010. Mass spectrometric characterization of black tea thearubigins leading to an oxidative cascade hypothesis for thearubigin formation. *Rapid Communications in Mass Spectrometry* 24 (23): 3387–404. doi: 10.1002/rcm.4778.

Liang, Q., M. Lv, X. Zhang, J. Hu, Y. Wu, Y. Huang, X. Wang, and J. Sheng. 2018. Effect of black tea extract and thearubigins on osteoporosis in rats and osteoclast formation in vitro. *Frontiers in Physiology* 9:1225. doi: 10.3389/fphys.2018.01225.

Liu, S. M., and H. H. Huang. 2015. Assessments of antioxidant effect of black tea extract and its rationals by erythrocyte haemolysis assay, plasma oxidation assay and cellular antioxidant activity (CAA) assay. *Journal of Functional Foods* 18:1095–105. doi: 10.1016/j.jff.2014.08.023.

Liu, Z., M. E. Bruins, L. Ni, and J. P. Vincken. 2018. Green and black tea phenolics: bioavailability, transformation by colonic microbiota, and modulation of colonic microbiota. *Journal of Agricultural and Food Chemistry* 66 (32):8469–77. doi: 10.1021/acs.jafc.8b02233.

Lodovici, M., C. Casalini, C. De Filippo, E. Copeland, X. Xu, M. Clifford, and P. Dolara. 2000. Inhibition of 1,2-dimethylhydrazine-induced oxidative DNA damage in rat colon mucosa by black tea complex polyphenols. *Food and Chemical Toxicology* 38 (12):1085–8. doi: 10.1016/S0278-6915(00)00109-5.

Maity, S., A. Ukil, S. Karmakar, N. Datta, T. Chaudhuri, J. R. Vedasiromoni, D. K. Ganguly, and P. K. Das. 2003. Thearubigin, the major polyphenol of black tea, ameliorates mucosal injury in trinitrobenzene sulfonic acid-induced colitis. *European Journal of Pharmacology* 470 (1-2):103–12. doi: 10.1016/s0014-2999(03)01760-6.

Miyata, Y., T. Tanaka, K. Tamaya, T. Matsui, S. Tamaru, and K. Tanaka. 2011. Cholesterol-lowering effect of black tea polyphenols, theaflavins, theasinensin A and thearubigins, in rats fed high fat diet. *Food Science and Technology Research* 17 (6):585–588. doi: 10.3136/fstr.17.585.

Menet, M.-C., S. Sang, C. S. Yang, C.-T. Ho, and R. T. Rosen. 2004. Analysis of theaflavins and thearubigins from black tea extract by MALDI-TOF mass spectrometry. *Journal of Agricultural and Food Chemistry* 52 (9):2455–61. doi: 10.1021/jf035427e.

Millin, D. J., and D. Swaine. 1981. Fermentation of tea in aqueous suspension. *Journal of the Science of Food and Agriculture* 32 (9): 905–19. doi: 10.1002/jsfa.2740320909.

Murad, H. A., and H. M. Abdallah. 2014. Black tea extract and its thearubigins relieve the sildenafil-induced delayed gut motility in mice: A possible role of nitric oxide. *Phytotherapy Research* 28 (11): 1687–91. doi: 10.1002/ptr.5183.

Murad, H. A., H. Habib, Y. Kamel, S. Alsayed, M. Shakweer, and M. Elshal. 2016. Thearubigins protect against acetaminophen-induced hepatic and renal injury in mice: Biochemical, histopathological, immunohistochemical, and flow cytometry study. *Drug and Chemical Toxicology* 39 (2):190–8. doi: 10.3109/01480545.2015.1070170.

Muthumani, T., and R. S. S. Kumar. 2007. Influence of fermentation time on the development of compounds responsible for quality in black tea. *Food Chemistry* 101 (1):98–102. doi: 10.1016/j.foodchem.2006.01.008.

Naveed, M., J. BiBi, A. A. Kamboh, I. Suheryani, I. Kakar, S. A. Fazlani, X. FangFang, S. A. Kalhoro, L. Yunjuan, M. U. Kakar, et al. 2018. Pharmacological values and therapeutic properties of black tea (*Camellia sinensis*): A comprehensive overview. *Biomedicine & Pharmacotherapy* 100:521–31. doi: 10.1016/j.biopha.2018.02.048.

Ngure, F. M., J. K. Wanyoko, S. M. Mahungu, and A. A. Shitandi. 2009. Catechins depletion patterns in relation to theaflavin and thearubigins formation. *Food Chemistry* 115 (1):8–14. doi: 10.1016/j.foodchem.2008.10.006.

Owuor, P. O., M. Obanda, H. E. Nyirenda, N. I. K. Mphangwe, L. P. Wright, and Z. Apostolides. 2006. The relationship between some chemical parameters and sensory evaluations for plain black tea (*Camellia sinensis*) produced in Kenya and comparison with similar teas from Malawi and South Africa. *Food Chemistry* 97 (4):644–53. doi: 10.1016/j.foodchem.2005.04.027.

Ozawa, T. 1982. Separation of the components in black tea infusion by chromatography on Toyopearl. *Agricultural and Biological Chemistry* 46 (4):1079–81. doi: 10.1271/bbb1961.46.1079.

Ozawa, T., M. Kataoka, K. Morikawa, and O. Negishi. 1996. Elucidation of the partial structure of polymeric thearubigins from black tea by chemical degradation. *Bioscience, Biotechnology, and Biochemistry* 60 (12):2023–7. doi: 10.1271/bbb.60.2023.

Patel, R., A. Ingle, and G. B. Maru. 2008. Polymeric black tea polyphenols inhibit 1,2-dimethylhydrazine induced colorectal carcinogenesis by inhibiting cell proliferation via Wnt/beta-catenin pathway. *Toxicology and Applied Pharmacology* 227 (1):136–46. doi: 10.1016/j.taap.2007.10.009.

Pereira-Caro, G., J. M. Moreno-Rojas, N. Brindani, D. Del Rio, M. E. J. Lean, Y. Hara, and A. Crozier. 2017. Bioavailability of black tea theaflavins: Absorption, metabolism, and colonic catabolism. *Journal of Agricultural and Food Chemistry* 65 (26):5365–74. doi: 10.1021/acs.jafc.7b01707.

Powell, C., M. N. Clifford, S. C. Opie, M. A. Ford, A. Robertson, and C. L. Gibson. 1993. Tea cream formation: The contribution of black tea phenolic pigments determined by HPLC. *Journal of the Science of Food and Agriculture* 63 (1):77–86. doi: 10.1002/jsfa.2740630113.

Roberts, E. A. H. 1958. The phenolic substances of manufactured tea. II. Their origin as enzymic oxidation products in fermentation. *Journal of the Science of Food and Agriculture* 9 (4):212–6. doi: 10.1002/jsfa.2740090405.

Roberts, E. A. H., R. A. Cartwright, and M. Oldschool. 1957. The phenolic substances of manufactured tea. I. Fractionation and paper chromatography of water-soluble substances. *Journal of the Science of Food and Agriculture* 8 (2):72–80. doi: 10.1002/jsfa.2740080203.

Sanlier, N., B. B. Gokcen, and M. Altuğ. 2018. Tea consumption and disease correlations. *Trends in Food Science & Technology* 78: 95–106. doi: 10.1016/j.tifs.2018.05.026.

Stodt, U. W., J. Stark, and U. H. Engelhardt. 2015. Comparison of three strategies for the isolation of black tea thearubigins with a focus on countercurrent chromatography. *Journal of Food Composition and Analysis* 43:160–8. doi: 10.1016/j.jfca.2015.07.002.

Uchida, K., K. Ogawa, and E. Yanase. 2016. Structure determination of novel oxidation products from epicatechin: thearubigin-like molecules. *Molecules (Basel, Switzerland)* 21 (3):273. doi: 10.3390/molecules21030273.

Wang, W., S. Zhang, L. Lv, and S. Sang. 2018. A new method to prepare and redefine black tea thearubigins. *Journal of Chromatography A* 1563:82–8. doi: 10.1016/j.chroma.2018.05.060.

Wang, X., P. He, S. Yi, and C. Wang. 2019. Thearubigin regulates the production of Nrf2 and alleviates LPS-induced acute lung injury in neonatal rats. *3 Biotech* 9 (12):451. doi: 10.1007/s13205-019-1986-z.

Yang, Z. Y., Y. Y. Tu, H. L. Xia, G. L. Jie, X. M. Chen, and P. M. He. 2007. Suppression of free-radicals and protection against H2O2-induced oxidative damage in HPF-1 cell by oxidized phenolic compounds present in black tea. *Food Chemistry* 105 (4):1349–56. doi: 10.1016/j.foodchem.2007.05.006.

Yassin, G. H., J. H. Koek, and N. Kuhnert. 2015. Model system-based mechanistic studies of black tea thearubigin formation. *Food Chemistry* 180:272–9. doi: 10.1016/j.foodchem.2015.01.108.

Yoshino, K., Y. Hara, M. Sano, and I. Tomita. 1994. Antioxidative effects of black tea theaflavins and thearubigin on lipid peroxidation of rat liver homogenates induced by tert-butyl hydroperoxide. *Biological & Pharmaceutical Bulletin* 17 (1):146–9. doi: 10.1248/bpb.17.146.