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Review Paper

Flavonoids and mechanisms of their anticancer action

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Abstract: Numerous studies collected over the last decade indicate the beneficial health effects of flavonoids. The diseases that could be potentially cured with flavonoids include cancer, cardiovascular disease, diabetes, and neurodegenerative diseases. Flavonoids are natural compounds found in fruits, vegetables, tea, and plant leaves.They are present in every day diets. All flavonoids possess a similar core structure that consists of benzo-γ-pyrone with an attached benzene ring. The position at which the benzene ring is attached andthe presence of hydroxyl and methyl groups define the structural diversity of the flavonoids and a variety of the exerted biochemical effects. A π-conjugated structure of flavonoids along with a number of benzene ring substituents defines the active participation of flavonoids in redoxreactions and, as a result, the exhibition of antioxidant and pro-oxidant properties. While antioxidant properties appear to be favorable in maintaining good health, it is the pro-oxidant activity of flavonoids that forms the basis of numerous reactions leading to cancer preventive effects. The reactions of flavonoids with metal cations or peroxidases may result in the formation of polyphenol radicals that damage DNA and proteins, thus stimulating apoptosis. Flavonoids may also modify the activity of various enzymes, therefore modulating metabolism and affecting the dynamics of apoptotic cellular death.

The aim of the present review is to summarize recent advances in understanding (1) the biochemical basis of the pro-oxidant properties of flavonoids brought about by metal cations and peroxidases, (2) the interactions of flavonoids with enzymes metabolizing xenobiotics, specifically carcinogens, and (3) the influence of flavonoids on the activity of drug efflux transporters. A special attention is devoted to the relationship between flavonoid structure and its potential pro-oxidant and anticancer activity.

Introduction

Flavonoids are a group of over 6,000 polyphenolic compounds present in fruit, vegetables, and beverages of plant origin. Flavonoids have similar structures derived from benzo-γ-pyrone. Based on their molecular structure, flavonoids can be divided into 6 principal subgroups: flavanones, flavanols, flavanonols, flavonols, flavones, anthocyanidins, and isoflavones. Flavanones (eg, naringenin and

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hesperetin) are mainly contained in citrus fruits. Flavanols (catechin and epicatechin) are the constituents of tea, apples, grapes, chocolate, and red wine. Flavanonols (taxifolin, aromadendrin, and fusetin) have been found in conifers, pine trees, and lacquer trees. Flavonols (quercetin, kaempferol, and myricetin) are present in leafy vegetables such as onions, broccoli, and berries. Flavones (apigenin and luteolin) and anthocyanidins are present in grains, leafy vegetables, and herbs. Isoflavones (daidzein and genistein) are contained in soy-based products. The structures of flavonoid basic subgroups are shown in Figure 1.

Intensive epidemiological research has been performed towards the elucidation of the impact of flavonoid diet on human health.[1,2] A potential role of flavonoids in various diseases such as cardiovascular diseases, diabetes, and cancer has been described in many studies. For example, the consumption of cacao and green tea rich in flavanols such as catechins and epigallocatechin gallate was found to correlate with the progression of endothelial function.[3] This is very beneficial to human health since endothelial dysfunction is associated with the development of diabetes and atherosclerosis. Green tea is identified as the major dietary source of flavonoids in adults and is a prevalent beverage in Asian communities.[4] The intake of fruits and vegetables has been also correlated with a reduced level of markers of oxidative stress and inflammation in addition to cardiovascular diseases. A diet reach in fruits and vegetables was found to reduce these markers not only in adults, but also in adolescent boys and girls.[5]

In vitro studies have also confirmed the health beneficial effects of flavonoids. Thus Slemmer and coworkers performed studies with cranberries and blueberries having a high content of flavonoids.[6] Using endothelial intestinal cells as a model, the researchers showed that berry homogenates (1) suppressed the generation of reactive oxygen species (ROS) by hydrogen peroxide and (2) increased mitochondrial metabolic rates. Blueberries that passed through the gastrointestinal tract increased the level of reduced glutathione (GSH) with respect to the oxidized glutathione (GSSG). Remarkably, the antioxidant potency of cranberries was not suppressed after treatment with digestive enzymes.

It is widely accepted and supported by population studies that the consumption of fruits and vegetables is associated with a reduction in the risk of various types of cancer. Zamora-Roset al. studied a population of 477,312 people from 10 European countries by using lifestyle surveys and a food-composition database on flavonoids. The authors found an inverse correlation between the intake of flavonoids and a risk of gastric cancer in women. A considerable decrease in the risk of gastric cancer was observed in women consuming specific flavonoid subgroups such as flavonols, flavanols, flavones, and anthocyanidins.[7] A strong inverse correlation between the intake of flavonoids and a risk of advanced prostate cancer was detected in the Netherland Cohort study. Specifically, the intake of black tea and flavonoids such as catechin, epicatechin, myricetin, and kaempferol led to the reduction of the risk of stage IV prostate cancer.[8] A case-control study in Scotland involving 1,456 people diagnosed with colorectal cancer and 1,456 controls was performed, which revealed the inverse correlation between some flavonoid subgroups and the risk of colorectal cancer. Flavonol quercetin and flavanols catechin and epicatechin proved active in the

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suppression of the cancer risk.[9] The correlation between flavonoid groups and the risk of breast cancer was studied using a group of women residing in Long Island, New York. The population group consisted of 1,434 incidents and 1,440 controls. A reduced risk of breast cancer caused by the intake of flavonols, flavones, and flavanols was most noticeable in postmenopausal women.[10] A similar conclusion was formulated by Hui et al. who performed a meta-analysis of the correlation between breast cancer and flavonoid intake using the data published before July 2012.[11] A number of researchers investigated how soy consumption impacts the development of breast cancer. Soy food comprises a significant portion of the Asian diet. Isoflavones, major constituents of soy products, were shown to interact with a number of polymorphisms of genes related to breast cancer. It was shown that soy does not interfere with major types of breast cancer therapy. Soy food consumed at a level ingested in Asian countries reduces the risk of breast cancer.[12] An Italian case-control study was carried out that involved 10,000 people diagnosed with various types of cancer and 16,000 controls. An inverse correlation between different groups of flavonoids and cancer risk was identified. Thus the intake of flavanones and flavonols reduced the risk of oral and laryngeal cancers, flavanols reduced the risk of laryngeal cancer, and flavanones inversely correlated with esophageal cancer. Anthocyanidins, flavonols, flavones, and isoflavones caused the reduction of colorectal cancer; flavones and flavonols reduced the risk of breast cancer and renal cancer, while flavonols and isoflavones suppressed the risk of ovarian cancer.[13]

The mechanisms underlying the anticancer action of flavonoids are not comprehensively understood. There is evidence that some subgroups of flavonoids are activated into radicals in the presence of the peroxidase/H_2O_2 oxidation system. A peroxidase oxidized by H_2O_2 detaches one electron from a flavonoid, which turns into a phenoxyl radical. Reactions of flavonoids with metal cations may also result in the formation of flavonoid radicals. These radicals may interact with other small molecules in the cell causing the formation of ROS and the peroxidation of phospholipids, which in turn trigger apoptosis. The induction of apoptosis in cancer cells may explain the anticancer action of flavonoids. There is evidence suggesting that flavonoids induce apoptosis selectively in cancer cells without affecting normal cells.[14] The resulting ROS also alter the redox state of the cell thus modifying proteins' cysteine residues, influencing protein folding states and the binding of transcription factors to DNA. These modifications overall lead to the changes in gene expression. Flavonoids may inhibit the growth of cancer cells by modulating genes involved in the control of the cell cycle.[15,16] The anticancer activity of some isoflavones is associated with their ability to bind estrogenic receptors.[17] This review (1) summarizes the knowledge on the mechanisms of the generation of ROS by flavonoids via their interaction with peroxidases and metals and (2) covers the recent advances in the cancer preventive interactions of flavonoids with metabolizing enzymes.

Flavonoids and metals

Flavonoids are known to produce radicals upon reactions with transition metals. In a healthy organism, metal ions are essentially sequestered. For example, iron ions are hidden by ferritin and transferrin, while copper ions are captured by ceruloplasmin. However, tissue injuries may result in

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transition metal release. Thus, samples from atherosclerotic lesions were found to contain detectable iron and copper. The concentrations of these ions were determined to be sufficient to catalyze lipid peroxidation.[18] The accumulation of copper may be also caused by Wilson disease. In this disease, the body is unable to eliminate extra copper, and the metal is stored in the liver, eyes, and brain leading to serious organ impairment. In these cases, the reactions of flavonoids with metal ions may play a significant role and must be considered among other flavonoids' reactions in cells.

A number of studies demonstrated that flavonoids may exhibit antioxidant effects via the formation of complexes with transition metal ions. Four flavonoids quercetin, taxifolin, luteolin, and eriodictyol suppressed oxidative stress in retinal cells exposed to an oxidant pair ascorbate/Fe^{2+}. The lipid peroxidation caused by this oxidative stress was also inhibited by the flavonoids.[19] A flavonoid-metal complex formation study performed by de Souza et al. revealed that the flavonoids bound to metal ions are more powerful radical scavengers than the free flavonoids. In this study, four flavonoids quercetin, catechin, galangin, and rutin were employed, which formed complexes with copper, iron, aluminum, and zinc.[20] Kostyug and coworkers studied transition metal complexes of Fe^{2+}, Fe^{3+}, and Cu^{2+} with flavonoids rutin, epicatechin, luteolin, and taxifolin. The authors demonstrated that these complexes are capable of scavenging superoxide and thus exhibit the properties of superoxide dismutase.[21] In a later study, scientists from the same research group showed that the rutin iron (II) complex is a poor Fenton catalyst compared with free iron (II). The authors also examined the activity of the rutin iron (II) complex *in vivo* with respect to its ability to suppress oxidative stress in mice liver. It was found that the complex improved GSH/GSSH ratio in mice after injections of thioacetamide, a compound known to cause pronounced hepatoxicity in animals. The flavonoid complex also precluded the reduction in the activity of selenium independent glutathione peroxidase and glutathione reductase.[22]

The pro-oxidant effects of flavonoids exhibited in the presence of transition metal ions have also been observed by many researchers. Yamanaka and coworkers studied the process of the oxidation of low density lipoproteins (LDL) induced by Cu^{2+} ions and the effect of constituents of green tea epicatechin and epigallocatechin on this oxidation. The oxidation of LDL is a radical chain reaction, which consists of the initiation, propagation, and termination steps. This process is believed to be associated with the development stages of atherosclerosis. The authors showed that in the initiation phase of the reaction, the oxidation of LDL stimulated by copper ions was inhibited by epicatechin and epigallocatechin. However, in the propagation phase, the two catechins boosted the oxidation rate such that the maximum acceleration ratio was about six.[23] Moran and coworkers studied the ability of flavonoids to chelate iron cations and catalyze iron-dependent free radical reactions. The authors found that flavonoids having catechol and pyrogallol groups and exhibiting strong chelating activity towards iron atoms also stimulate the iron-dependent damage to DNA and deoxyribose. The authors speculated that this pro-oxidant activity may be related to the ability of phenolics (1) to interact with O_2^- and OH• radicals produced in the reaction and (2) to efficiently reduce Fe^{3+}.[24] Interestingly, the same flavonoids were found to inhibit the

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peroxidation of lipids and the inactivation of proteins stimulated by iron ions.

The scavenging property of flavonoids manifests itself in the ability of the phenolic compounds to donate a hydrogen atom to reactive species, which results in the reduction of the latter and the formation of phenoxyl radical such as semiquinone. The fate of the phenoxyl radicals depends on their structure and the presence of species affecting the stability of the free radicals. Jacobs and coworkers investigated the reactivity of the oxidation products of a semi-synthetic flavonoid monoHER and compared it with a natural natural flavonoid quercetin. The authors showed that in human blood plasma oxidized quercetin reacts readily with protein thiols forming disulfides and thus potentially generating protein oxidative folding, ROS toxicity, and damage to essential cellular constituents. However, monoHER is reduced by ascorbate before it reacts with thiols.[25] The authors attribute the difference in the reactivity to principally different structures and the energy profiles of the oxidation products. The toxicity of the phenoxyl radicals formed as a result of their antioxidant action also depends on their stability. At normal physiological conditions, phenoxyl radicals are unstable and promptly decompose into non-radical substances. Remarkably, metals such as Zn, Cd, Ca, Mg and Al have been reported to stabilize oxidation products of flavonoids by enhancing their free radical state.[26] Sakihama and Yamasaki used plant and model systems to show that Al^{3+} ions enhanced lipid peroxidation induced by phenolics and thus increased their pro-oxidant activity. This effect of aluminum ions is attributed to the ability of the metal to change the redox balance of phenolic reactions possibly by stabilizing the oxidized forms of phenolics.[27]

Fe^{3+} and Cu^{2+} are capable of catalyzing the conversion of phenolics to semiquinone in the presence of molecular oxygen. These conversions also result in side products such as O_2^- and OH• radicals, which may produce lesions on DNA and other cellular constituents. Sakihama and coworkers correlated the gel-electorophoresis studies of DNA damage caused by three different phytophenols in the presence of Cu(II) with the ESR studies of the OH• radicals formed in the same systems. The three phenolics investigated were dihydrocaffeic acid (DHCA), caffeic acid, and chlorogenic acid (CGA). The authors utilized a spin trap technique, in which OH• radicals oxidize DMSO with the formation of methyl radicals. Methyl radicals form a stable radical with POBN producing a distinctive six-line EPR spectrum. The level of DNA lesions such as double and single strand breaks was the highest for DHCA. In the case of caffeic acid, DNA damage was less pronounced, while CGA produced only a minimal amount of strand breaks in the system containing Cu(II). Remarkably, the intensity of the lines in the EPR spectrum decreased in the same order as the amount of DNA damage, being the highest in the case of DHCA and disappearing with CGA. This experiment confirmed the pro-oxidant activity of the phytophenolics, specifically their potential ability to produce ROS in the presence of transition metal cations and thus cause damage to biomolecules.[26]

Furukawa and coworkers studied the mechanisms and the extent of DNA damage caused by green tea catechins in the presence of Fe(III) and Cu(II) complexes.[28] The authors performed experiments on calf thymus DNA and with cultured cells. The following catechins were examined: (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin

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gallate (ECG), and catechin (Figure 2). In the presence of Cu(II), the extent of DNA damage decreased in the following order: EGC > catechin > EGCG > ECG. In the presence of Fe(III), the amount of DNA damage induced by catechin was very small compared with the other three flavonoids. The order of the damage extent was the following: EGCG ≈ EGC > ECG >> catechin. This experiment demonstrated that the DNA damage activity exhibited by catechins in the presence of transition metal ions is strongly dependent on the structure of the flavonoids. Two out of the four compounds, namely, EGCG and EGC, possess a pyrogallol B ring (three hydroxyl groups), the other two compounds (catechin and ECG) have a catechol B ring (two hydroxyl groups). Only two of them (EGCG and ECG) have a galloyl group in the C ring. The compounds having either a pyrogallol B ring or a galloyl group in the C ring caused significant DNA damage in the presence of both Fe(III) and Cu(II). Catechin, which has a catechol B ring and no galloyl group in the C ring, caused DNA damage in the presence of Cu(II) while in the presence of Fe(III), the damage was very little. Remarkably, the presence of the catechol ring in a molecule or an *o*-dihydroxy element in the B ring of flavonoids is an essential factor that defines an antioxidative or radical scavenging activity of phenolics. The authors also found that in the presence of Fe(III) and EGCG, DNA was affected homogeneously at every nucleotide, and the damage was suppressed by catalase and scavengers of OH• radicals. In the presence of Cu(II), the damage occurred at T and G residues, primarily at 5'-TG-3' and GG sequences. The formation of DNA lesions was inhibited by catalase and bathocuproine, which led to the conclusion that DNA lesions were produced by H_2O_2 and Cu(I). The elevated level of 8-oxodG was also detected in the presence of EGCG and metal ions. The proposed mechanisms of the DNA damage caused by EGCG in the presence of Fe(III) and Cu(II) are shown in Figure 3.[28]

Copper plays an essential role in DNA chemistry as it is present in chromatin and is associated with guanine.[29] It was shown that in the presence of some polyphenols, specifically quercetin, a ternary complex quercetin-Cu(II)-DNA was formed. Quercetin reduced Cu(II) to Cu(I), and the re-oxidation of Cu(I) to Cu(II) resulted in the formation of ROS and DNA degradation. The system involving quercetin and copper was proven to cause DNA degradation in lymphocytes.[30]

Tsai and coworkers studied DNA damage caused by propolis, whose major components are flavonoids chrysin, galangin, and pinocembrin.[31] Interestingly, propolis has long been used as antiseptic. Its protective action has been associated with the antioxidant properties of these flavonoids. In contrast to the common view, the authors showed that the flavonoids do cause oxidative DNA damage and proposed a mechanism, which involved iron cations. According to their model, the components of propolis, act as electron acceptors and donors in a set of reactions. The reactions of Fe(II) with molecular oxygen leads to the formation of superoxide and consequently H_2O_2. The formation of H_2O_2 may occur in a cell-free environment. H_2O_2 molecules may penetrate the membranes and reach nuclei, where they may be converted to OH• radicals via Fenton reactions catalyzed by Fe(III). The generated OH• radicals may produce oxidative damage on nuclear DNA.[31] Remarkably, the authors also showed that cells pre-treated with propolis gain resistance to oxidative stress. This phenomenon may be explained by the fact that the formation of oxidative DNA lesions caused by propolis initiates DNA repair

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processes, so the exposure to oxidative stress following the propolis-treatment results in lower level of DNA damage sites. The model thus explains the antioxidant potency of propolis.

Flavonoids and peroxidases

Peroxidases play an essential role in human metabolism. The redox cycle of a heme-containing peroxidase involves enzyme oxidation by peroxide followed by the reduction with other molecules served as peroxidase substrates. These reduction steps involve electron transfer from the substrates and potentially result inthe formation of free radicals. A number of studies proved that flavonoids may act as electron donors for peroxidases. Thus, Yamasaki and coworkers showed that the reaction of peroxidase with flavonoids serves as a detoxification of plants against hydrogen peroxide.[32] The authors examined the reactions of horseradish peroxidase with quiercetin, kaempferol, and their glycosides in the presence of hydrogen peroxide. The oxidation of the same polyphenols was also examined in a soluble fraction of *S. arboricola* leaf extract. The oxidation rates of the flavonoids were found to be in the following order: quercetin > kaempferol > quercetin glycoside >> kaempferol glycoside. Based on the results of the study, the authors concluded that the electron donating activity of flavonoids requires the presence of 3-OH on the C-ring, the 2,3-double bond together with 4-oxo group, and, most importantly, the presence of 3’-OH and 4’-OH groups on the B ring (catechol structure). The *in vitro* experiments showed that the oxidation of quercetin by peroxidase/H_2O_2 system leads to the formation of dimers and trimmers of the flavonoid. However, in the presence of ascorbate, the generation of these oxidized products of quercetin is inhibited possibly by the reduction of the initial oxidation product, quercetin radical, formed as a result of electron donation to the peroxidase.

There is evidence indicating the ability of some flavonoids to oxidize glutathione (GSH) and NAD(H), which leads to oxygen activation. This process is initiated by the conversion of flavonoids to phenoxyl radicals in the presence of peroxidases. Galati and coworkers showed that apigenin, naringin, and naringenin exhibited significant pro-oxidant activity as they triggered substantial GSH oxidation in the presence of the horseradish peroxidase/H_2O_2 system at physiological pH values. The authors found that the phenoxyl radicals formed in the reaction with peroxidase converted GSH to thyil radicals, which activated oxygen by converting it to the superoxide radical (Figure 4).[33] It was also GSH that inhibited the metabolic destruction of the flavonoids, as it reduced the phenoxyl radicals back to the original molecules. Interestingly, only flavones and flavanones containing a 4’-OH group (phenol B ring) oxidized GSH and consequently activated oxygen in the presence of the peroxidase/H_2O_2 system. Flavonoids with benzene or catechol B rings did not exhibit the ability to oxidize GSH after the oxidation with the peroxidase in the same conditions. The extent of the GSH oxidation was in-line with the redox potential of the PhO•/PhO^- pair. Flavonoids whose redox potential was lower than that of the GS•/GS^- pair were unable to generate superoxide radicals in the presence of peroxidase and GSH. However, the redox potential of apigenin, naringin, and naringenin is greater than that of the PhO•/PhO^- pair, which is consistent with the experimental observation of the increased oxygen uptake.[33] Chan and coworkers demonstrated that apigenin, naringin, naringenin, and apigenin also oxidized NADH in the presence of

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peroxidase with the formation of NAD• radicals, which activated oxygen. The major reaction intermediates formed under the peroxidase catalysis were phenoxyl radicals.[34] In a later study, the same research group employed the spin-trapping method to prove the production of the thyil radical in the reactions involving apigenin, naringenin and the peroxidase/H_2O_2 system. In their experiments, the thyil radicals, generated as intermediates in the conversion of GSH to GSSG, formed adducts with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), which were detected by EPR spectroscopy.[35] Other dietary compounds investigated, namely catechol B ring flavonoids quercetin and luteolin as well as kaempferol also led to GSH transformation in the presence of peroxidase/H_2O_2; however, no thyil radicals were detected. As indicated by MS/MS electrospray spectroscopy and ^{1}H NMR spectroscopy, quercetin, luteolin, and kaempferol formed GSH conjugates, the intermediate oxidation products being *o*-qionone of luteolin or the quinonemethide products of quercetin and kaempferol.[35]

Awed and coworkers performed a detailed analysis of the mechanism of quercetin oxidation by peroxidase/H_2O_2 system and the structures of the products formed in the presence of GSH.[36] Interestingly, in the absence of GSH, the peroxidase oxidation of quercetin resulted in the formation of 20 different products. However, in the presence of GSH only two major compounds were produced. In these compounds, glutathione was attached to the A ring of quercetin at positions 6 and 8 (Figure 5). The authors proposed the mechanism underlying the formation of the glutathionylquercetin adducts. The mechanism involves the formation of a quercetinsemiquinone resulting from the donation of a hydrogen atom by quercetin and further oxidation of semiquinone to *o*-quinone, which transforms to its *p*-quinonemethide isomers (Figure 5). The formation of GSH-quercetin adducts was also demonstrated in a tyrosinase-containing melanoma cell line.[37]

The oxidation of quercetin was also studied in the presence of hemeproteins such as cytochrome c, hemoglobin, myoglobin, and cytochrome b_5.[38] The formation of the oxidized products was observed in the presence of all these proteins except cytochrome b_5, while cytochrome c demonstrated the highest quercetin oxidation rates. The major oxidation product was identified as a quercetin dimer, in which a dioxane linkage is formed between the hydroxygroup of the B ring in one quercetin molecule and the C2-C3 group of another molecule (Figure 6). The oxidation mechanism suggested by the authors involves the formation of radical species, namely the oxygen radical formed on the 4'-OH group of the B ring and the radical centered on the C2 atom of the C ring.

The role of flavonoids in the development of cancer

Pro-oxidant effects of flavonoids in cancer

The anticancer properties of flavonoids have been traditionally associated with their antioxidant activity, specifically ROS scavenging reactions. However, a number of experimental observations on flavonoids' antitumor action could not be explained solely based on their antioxidant potential. It has been suggested that the pro-oxidant activity of flavonoids constitutes the basis of numerous biochemical processesleading to antitumor effects. The pro-oxidant activity results in the production of ROS, which are necessary for the initiation of apoptosis. ROS also cause apoptotic DNA fragmentation.

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Population studies show that isoflavone genistein is associated with the decreased risk of breast cancer.[12] Moreover, it has been found that genistein exhibits selective cytotoxicity towards cancer cells. It is known that the level of copper in malignant cells is significantly higher than that in normal cells, as copper transporters are overexpressed in cancer cells.[39] Ullah and coworkers investigated the mechanisms of genistein antitumor potency.[40] The author proposed that this isoflavone reacts with intracellular copper, which leads to the generation of ROS,followed by apoptotic cell death. As copper is one of the most redox active metals, and genistein possesses electron donating potential, the reaction of Cu(II) with genistein can easily lead to the formation of Cu(I). The re-oxidation of Cu(I) to Cu(II) in the presence of oxygen is accompanied by the generation of ROS. Remarkably, the growth inhibition of breast cancer cells caused by genistein was suppressed by copper chelators, while iron and zinc chelators proved inactive in influencing cellular proliferation, which emphasizes the crucial role of copper in the pro-oxidant action of genistein. The generation of ROS preceding cellular death is confirmed by the fact that the genistein mediated inhibition of cellular growth was negligible in the presence of ROS scavengers.[40]

Watjen and coworkers studied the antioxidant and pro-oxidant activities of seven flavonoids using H4IIE rat hepatoma cells. Two flavonoids, quercetin and fisetin, were readily taken up by the cells and exhibited the most prominent effects. The antioxidant activity such as protection against DNA strand breaks and apoptosis were detected at concentrations of 10-25 µM. Remarkably, the same flavonoids stimulated DNA fragmentation and caspase activation at concentrations ranging between 50 and 250 µM. Thus, the flavonoids exhibited proapoptotic activity, which could be used in cancer chemotherapy.[41] In an alternative work, the same research group investigated the activity of luteolin in H4IIE rat hepatoma cells.[42] The authors found that luteolin induced caspase activation and DNA damage. It was suggested that the apoptosis was induced by luteolin via a mitochondrial pathway.

A number of studies indicate the importance of pyrogallol-type structure in a B-ring of flavonoids for the induction of DNA damage and apoptosis. Thus Mitsuhashi and coworkers investigated the cytotoxic activity of ten structurally different flavonoids on HEK293T cells.[43] It was revealed that flavonoids possessing a pyrogallol B-ring such as epigallocatechin, epigallocatechin gallate (Figure 2), and gallic acid exhibited the highest cytotoxic effect. DNA-laddering was observed indicating apoptosis. Similar conclusions were made in an earlier work by Saeki et al., who studied apoptosis in human hystiocytic lymphoma U937 cells induced by catechins. The stimulation of apoptosis was most prominent with catechins having pyrogallol moiety, and a 3-*O*-gallate group in cis-position to the B-ring augmented the apoptotic activity.[44]

Interactions of flavonoids with enzymes metabolizing carcinogens

One of the mechanisms underlying the antitumor action of flavonoids is their interference with the activity of metabolizing enzymes, which play an important role in the uptake, digestion, transport, and detoxification of compounds potentially capable of initiating the development of cancer. This interference may occur both at the mRNA level and at the protein level. Moreover, flavonoids may

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share metabolic pathways with other xenobiotics.

Metabolism can be divided into three phases. Enzymes involved in phase I reactions include cytochrome P-450 (CYP) enzymes, non-cytochrome P-450 enzymes, and flavin-containing monooxygenase enzymes. These enzymes, specifically CYP isoforms, catalyze the oxidation of substances and thus introduce polar or reactive groups into the molecules. Atmospheric pollutants polycyclic aromatic hydrocarbons (PAH) represent a well-studied class of mutagens and carcinogens. The mechanisms of their carcinogenicity are based on the formation of PAH reactive metabolites catalyzed by CYP. It was shown on MCF-7 cells that citrus flavanone naringenin inhibited CYP1B1. Specifically, naringenin reduced the expression of CYP1B1 mRNA expression induced by PAH 7,12-dimethylbenz(a)anthracene, which is known to be a powerful carcinogen.[45]

A significant amount of research effort has been directed towards flavonoids' activity against estrogen-mediated cancer, specifically on how flavonoids interfere with the oxidation reactions of estrogen catalyzed by CYP. Studies show that exposure to estrogen is linked to the initiation and the development of breast cancer; and the increase in the hormone dose enhances the risk of tumor development. The mechanisms underlying the estrogen-mediated initiation of cancer are thought to involve the formation of reactive metabolites *o*-quinones and quinone methides. This oxidation is catalyzed by CYP. The resulting metabolites produce alkylation and oxidative damage on DNA leading to mutations and consequently cancer. Moreover, *o*-quinones may bind estrogen receptors and form a highly redox active entity, which affects estrogen sensitive genes.[46] Belous and coworkers presented direct proof of the formation of DNA adducts, which proceeds via CYP-mediated estrogen oxidation reaction.[47] They used recombinant CYP1B1; this isoform is expressed in breast tissue. The hormone 17β-estradiol (E_2) was oxidized in the presence of 2-deoxyguanosine or 2-deoxyadenosine. The following products were detected: 4-hydroxy-E_2(4-OHE_2), quinone 4-hydroxyestradiol-quinone, 4-OHE_2-N7-Gua, and 4-OHE_2-N3-Ade.[47]

It is known that CYP isoforms CYP1A1 and CYP1B1 hydrolyze E_2 to 2-hydroxy-E_2 (2-OHE_2) and 4-OHE_2, respectively (Figure 7A). It was shown that the formation of 4-OHE_2, which predominantly occurs in cancer cells, is suppressed by a natural methoxyflavonoid chrysoeriol. The *in* vitro studies on human breast cancer MCF-7 cells showed that chrysoeriol inhibited the activity of CYP1B1 isoform 5-fold more than the activity of CYP1A1.[48] Structure-reactivity relationship of flavonoids was also investigated in regards to the ability to inhibit enzymatic activity of CYP1B1, CYP1A1, and CYP1B1.[49] The results showed that only compounds with a double bond between 2 and 3 positions on the C-ring, specifically flavones and flavanols (Figure 1), exhibited selective suppressing effects on the activity of CYP1B1. An enhance potential for the reduction of enzymes' activity was detected for methoxyflavonoids such as chrysoeriol and isorhamnetin (Figure 7B). The molecular docking study of the latter two flavones showed that their shapes matches well the active site of CYP1B1, but does not match those of CYP1A1 and CYP1A2 isoforms due to steric hindrance.[49]

Shimada and coworkers analyzed structure-activity relationship of flavonoids for the inhibition of CYP1A1, 1A2, 1B1, 2C9, and

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3A4. 33 structurally different flavonoids were employed in the study. The authors used 7-ehoxyresorufin *O*-deethylation activity assay as well as molecular docking analysis. It was revealed that the introduction of hydroxyl group and/or methoxy group into the flavonoid structure enhanced its activity towards CYP1B1 inhibition. The study also indicated that the enzyme inhibition activity of flavonoids depends strongly on the number and positions of hydroxyl and methoxy groups, while the mechanisms of the interactions between the compounds and the active sites of enzymes depend strongly on the identity of a CYP isoform.[50]

Phase II metabolizing enzymes play a crucial role in the detoxification of endogenous compounds, carcinogens, nutrients, and drugs. Specifically, they transform the compounds into forms that are less toxic, more water-soluble, and more readily excretable from the organism. This is accomplished by the conjugation of metabolites with charged groups such as GSH, glycine, sulfate, and glucoronic acid. Phase II enzymes are primarily transferases and include sulfotransferases, N-acetyltransferases, glutathione S-transferases (GST), UDP-gluconosyltransferases (UGT), and methyltransferases.[51] Phase II enzymes also involve NAD(P)H:quinone oxidoreductase-1 (NQO-1), heme oxygenase-1 (HO-1), and gamma-glutamylcysteine ligase (GCL). One of the mechanisms found to be essential in the anticancer function of flavonoids is the activation of metabolizing enzymes of phase II. Sangeetha and coworkers studied the effect of silibinin against 1,2-di-methylhydrazine (DMH) induced colon carcinogenesis in rats. Silibinin is the major constituent of the extract from medicinal plant milk thistle. The treatment of rats with DMH led to the activation of phase I enzymes and the inhibition of phase II enzyme, which consequently induced colonic cancer. In rats exposed to both DMH and silibinin, the modulatory effect on enzymes was detected. The modifications of the activities of enzymes of both phase I and phase II caused by silibinin facilitated the detoxification of DMH and the chemoprevention of colon cancer.[52] Another compound 3',4'-dimethylnobiletin, a metabolite of a citrus flavonoid nobiletin, was shown to stimulate the expression of phase II detoxification genes in PC12 cells.[53] A citrus flavononenaringin was shown to induce mRNA expression of phase II enzymes in rats. The enzymes include GST P1, NQO-1, HO-1, and GCL. The stimulation of the expression of these genes was accomplished via the activation of nuclear factor-erythroid 2-related factor 2 (Nrf2).[54] It was demonstrated that isoflavone genistein, found primarily in soy food products, stimulates the expression of GGL catalytic subunits and HO-1 in Caco-2 cells. The induction of these enzymes was observed at both mRNA and proteinlevels.[55] Genistein was also demonstrated to significantly induce human dehydroepiandrosterone sulfotransferase (hSULT2A1), which catalyzes the sulfonation of hydrosteroids and xenobiotics, in Hep G2 and Caco-2 cells.[56] Earlier research advances on the impact of flavonoids on the activity of phase I and phase II metabolic enzymes have been reviewed by Galati and O'Brein[57], Cermak and Wolffram[58], Egert and Rimbach[59], and Jiang and Hu[60].

The inhibition by flavonoids of drug efflux transporters

Drug efflux transporters belong to a class of phase III metabolizing enzymes. These enzymes play a central role in drug absorption, distribution, and excretion. The

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examples of the phase II enzymes are P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and the organic anion transporting polypeptide 2 (OATP2). P-gp and MRP sbelong to ATP-binding cassette (ABC) transporter superfamily, since they use the energy released in the hydrolysis of ATP to transport substrate across the membrane.[61] The presence of P-gp and MRPs in human tumors results in the efflux of anticancer drugs and therefore therapeutic failure. It is believed that the mechanism of action of compounds modifying the activity of P-gp is based on their competition with drugs for the binding site on the protein.

Studies show that a number of flavonoids are able to change the pharmacokinetics of administered drugs via the modulation of the activity of efflux transporters. Lee and coworkers studied the effect of wogonin on etoposide-induced apoptosis in normal and cancer cells. Wogonin is amethoxyflavone found in *Scutellariabaicalensis*, a flowering plant species. Etoposide is an anticancer drug that interferes with the action of topoisomerase enzymes and thus prevents the normal control of the changes in DNA structure during cell cycle. Interestingly, wogonin inhibited the activity of P-gp, increased the concentration of etoposide, and stimulated apoptosis in cancer cells. However, the same experiment performed in normal cells revealed the opposite result, namely that wogonin inhibited etoposide-induced apoptosis. Thus, wogonin can be used in cancer therapy to overcome multidrug resistance.[62] The potential of another methoxyflavone, oroxylin A, to inhibit the activity of P-gp has been also investigated.[63] Two compounds, vinblastine and paclitaxel, were used to induce cellular cytotoxicity. Vinblastine is an antitumor drug used to treat lymphoma, lung cancer, breast cancer, and other types of cancer. Paclitaxel is also employed in cancer chemotherapy; its action is based on the inhibition of mitosis.Both *in vivo* and *in vitro* studies indicated that oroxylin A inhibited P-gp-mediated drug efflux and thus enhanced cellular susceptibility to vinblastine and paclitaxel.[63]

Molnar et al. studied the effect of flavonoids on the accumulation of rhodamine 123 in breast cancer cells expressing MRP1 and lacking P-gp (HTP26) and in mouse lymphoma cells transfected with *MDR1* gene (L1210).[64] Remarkably, the authors revealed a strong structure-activity correlation of flavonoids in the modulation of the activity of drug efflux transporters. The P-gp-mediated efflux was suppressed by chrysin, rotenone, formononetin, and amorphigenin. MPR-1 was inhibited by chrysin, kaemferol, epigallocatechin, afrormosin, formononetin, amorphogenin, rotenone, and robinin. Most flavonoids proved more effective in the inhibition of P-gp than MPR1. The authors also revealed that flavonoids may bind multiple sites on P-gp. However, only targeting an ATP-binding site and thus affecting the ATPase activity of P-gp results in the inhibition carboxyfluorescein efflux.[64] Farabegoli et al. studied the effect of the green tea constituent EGCG on breast carcinoma cells treated with tamoxifen.[65] Tamoxifen is an anticancer drug used in patients with early breast cancer. This drug blocks estrogen receptor pathway and thus prevents cancer development. A significant fraction of patients treated with tamoxifen exhibit resistance to the medicine. The studies showed that EGCG inhibited the activity of P-gp to 53% compared to control cells. A significant suppression of the activity of breast cancer resistance protein (BCRP) was also detected under treatment with EGCG.[65] Tran et al. studied the effect of 30 flavonoids on the accumulation of

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anticancer drugs and the ability of the same flavonoids to modulate the P-gp ATPase activity.[66] It was revealed that different flavonoids have different influences on the bioavailability of drugs. A group of flavonoids that enhanced the activity of daunorubicin-stimulated P-gp ATPase also decreased the accumulation of the drug whereas flavonoids that suppressed the vinblastine-stimulated ATPase activity of P-gp enhanced the bioavailability of the drug in leukemia cells that overexpressed P-gp. The authors explain the observed effects by multiple binding modes, which change depending on a given flavonoid.[66]

Conclusion

The excessive abundance of flavonoids in every day food as well as the observation of their potential antitumor properties have attracted the attention of thousands of scientists into the mechanisms underlying flavonoids' health beneficial effects.Since apoptosis is the key process in the regulation of the proliferation and growth of cancer cells, the ability of flavonoids to influence apoptosis has become a topic of numerous research works. Biochemical events such as the formation of reactive oxygen species (ROS), lipid oxidation, and DNA fragmentation are known to stimulate apoptosis. The ability of flavonoids to form ROS and cause DNA fragmentation in the presence of metal cations or peroxidases has been proclaimed to be one of the mechanisms leading to apoptosis and consequently chemopreventive action. Flavonoids' influence on the activity of metabolizing enzymes of phase I and phase II constitute another mechanistic pathway contributing to the anticancer action of these polyphenols. The binding of flavonoids to the specific sites of drug efflux transporters may alter the bioavailability of antitumor drugs thus modulating the efficacy of chemotherapy. Since polyphenolic compounds have been shown to both stimulate and suppress the activity of metabolizing enzymes and drug efflux transporters, it is crucial to examine the differential effects of flavonoids on enzymes as well as their potential toxicity in developing combination therapies for cancer patients.

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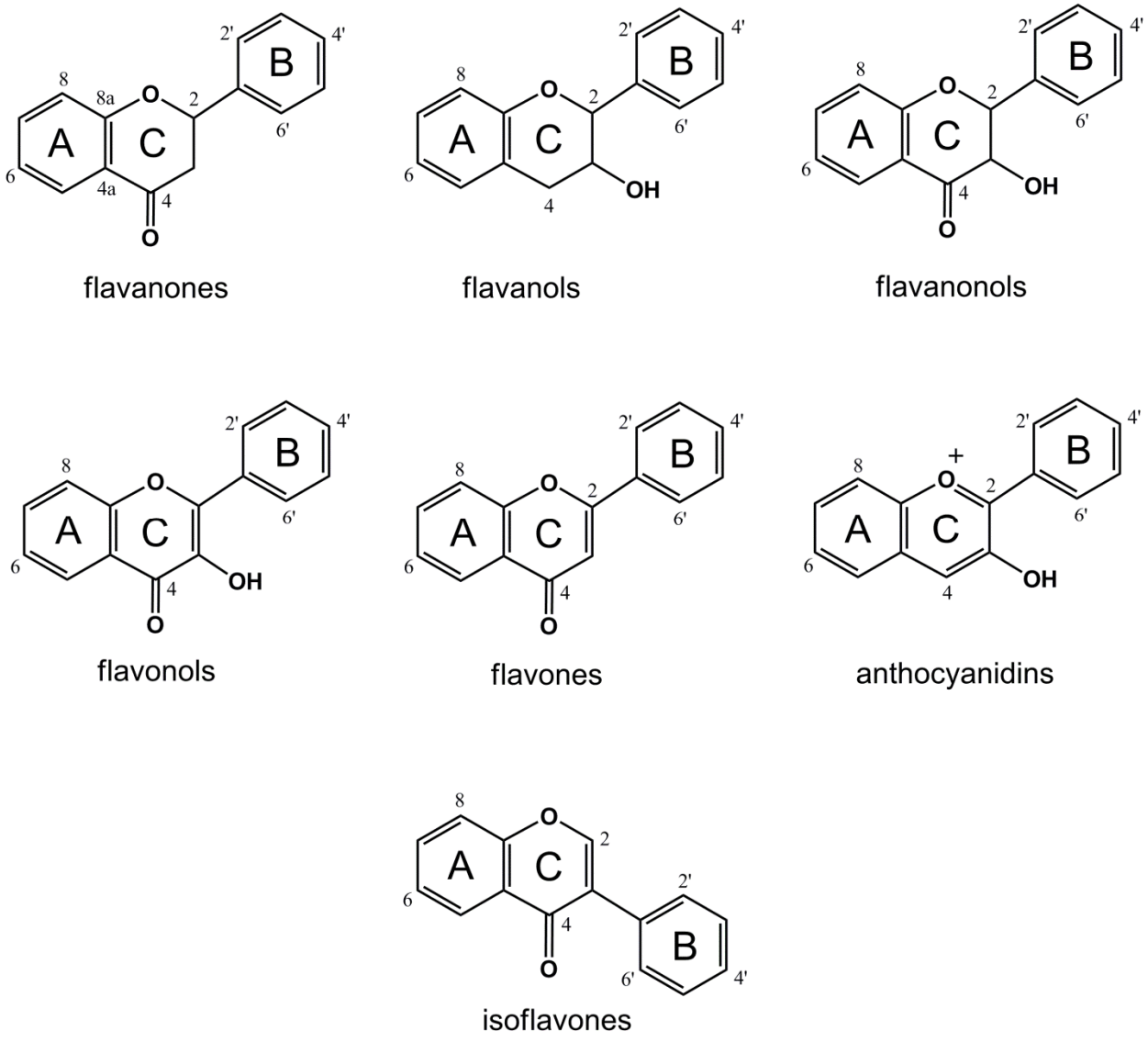

Figure1. The structures of the six basic flavonoid subgroups.

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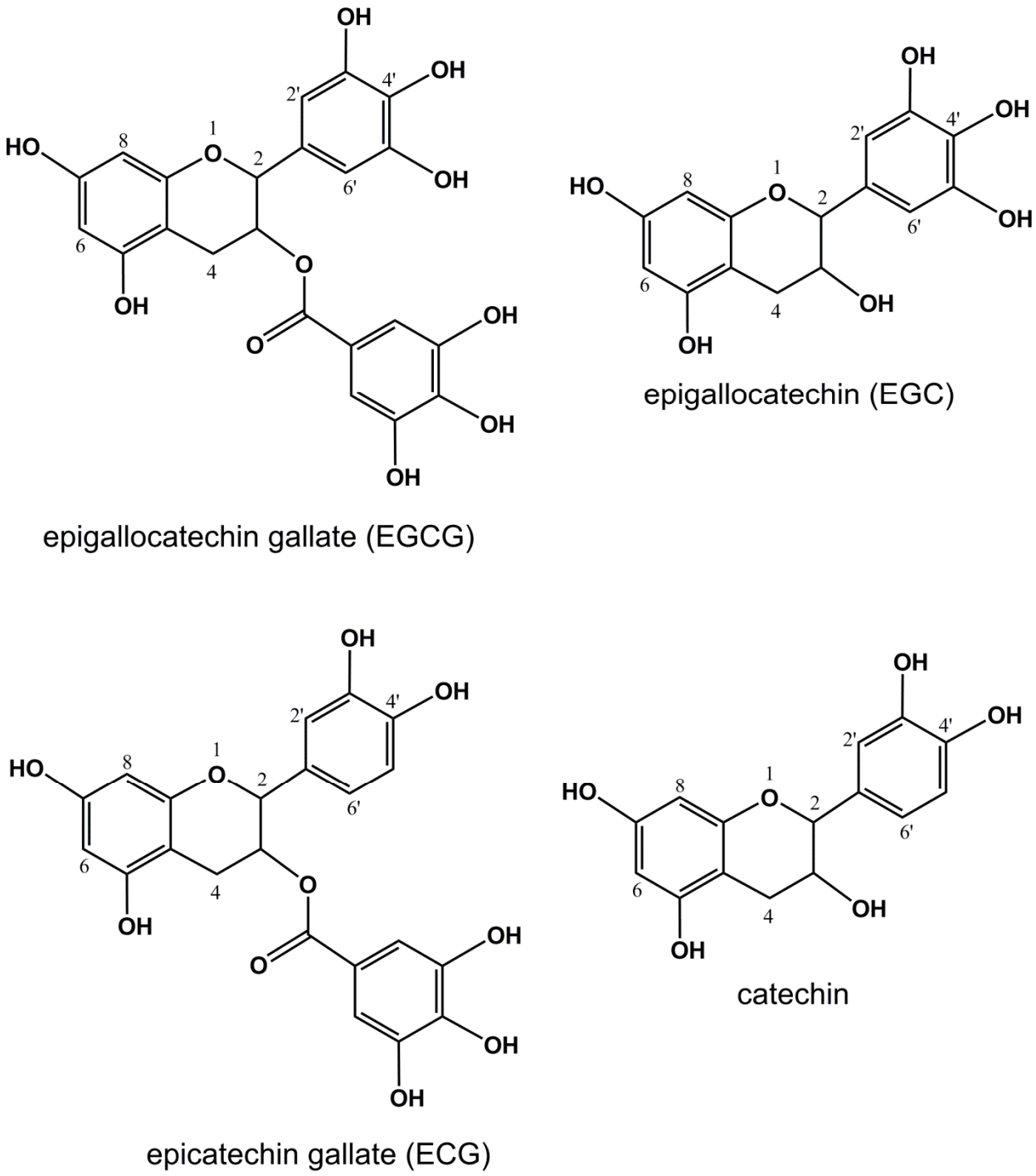

Figure 2. The structures of flavanols catechin, ECG, EGCG, and EGC.

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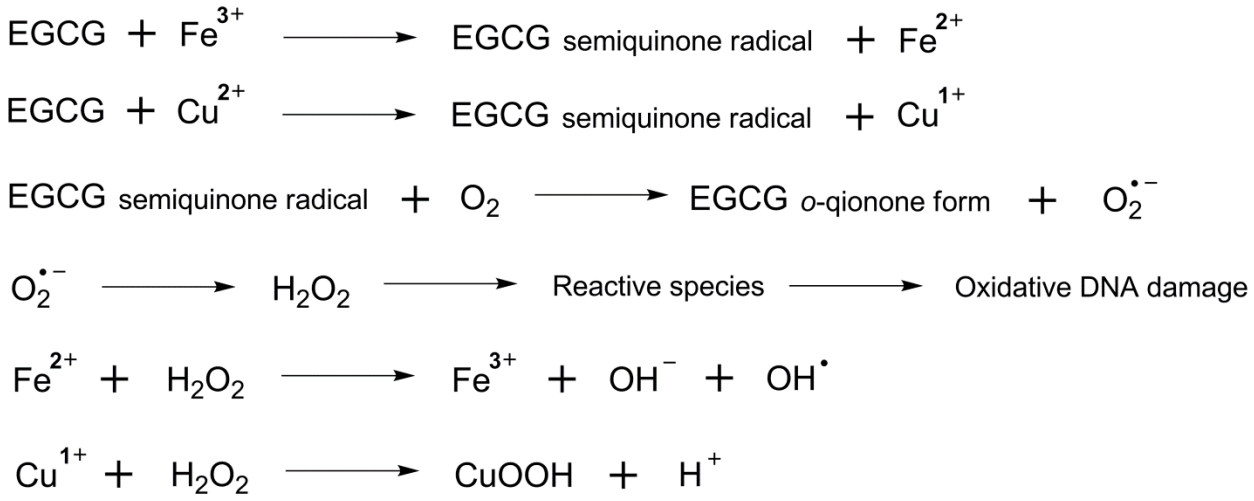
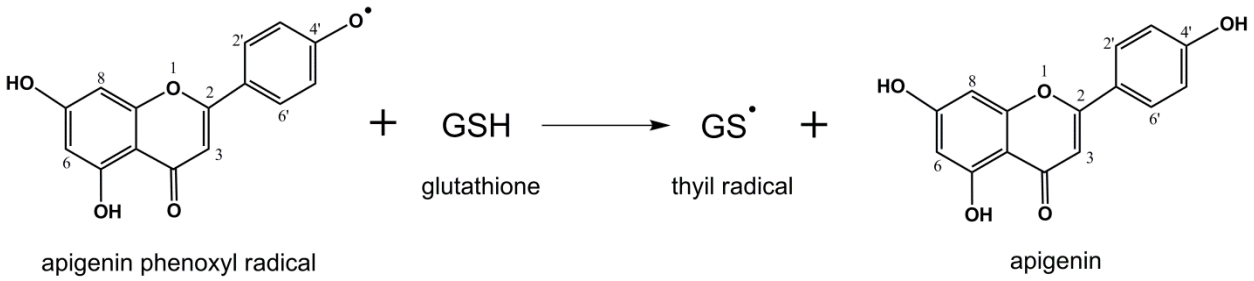


Figure 3. The reaction scheme demonstrating intermediate steps leading towards DNA damage initiated by iron- and copper-mediated oxidation of EGCG.



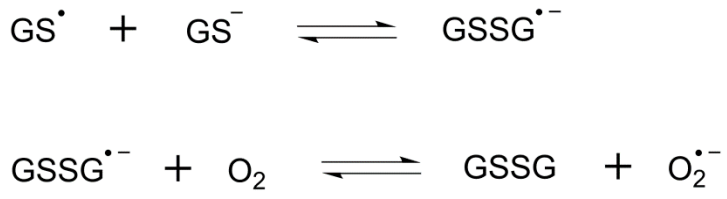


Figure 4. The reaction scheme showing the formation of superoxide radical from apigenin radical via the oxidation of GSH to thyil radicals.

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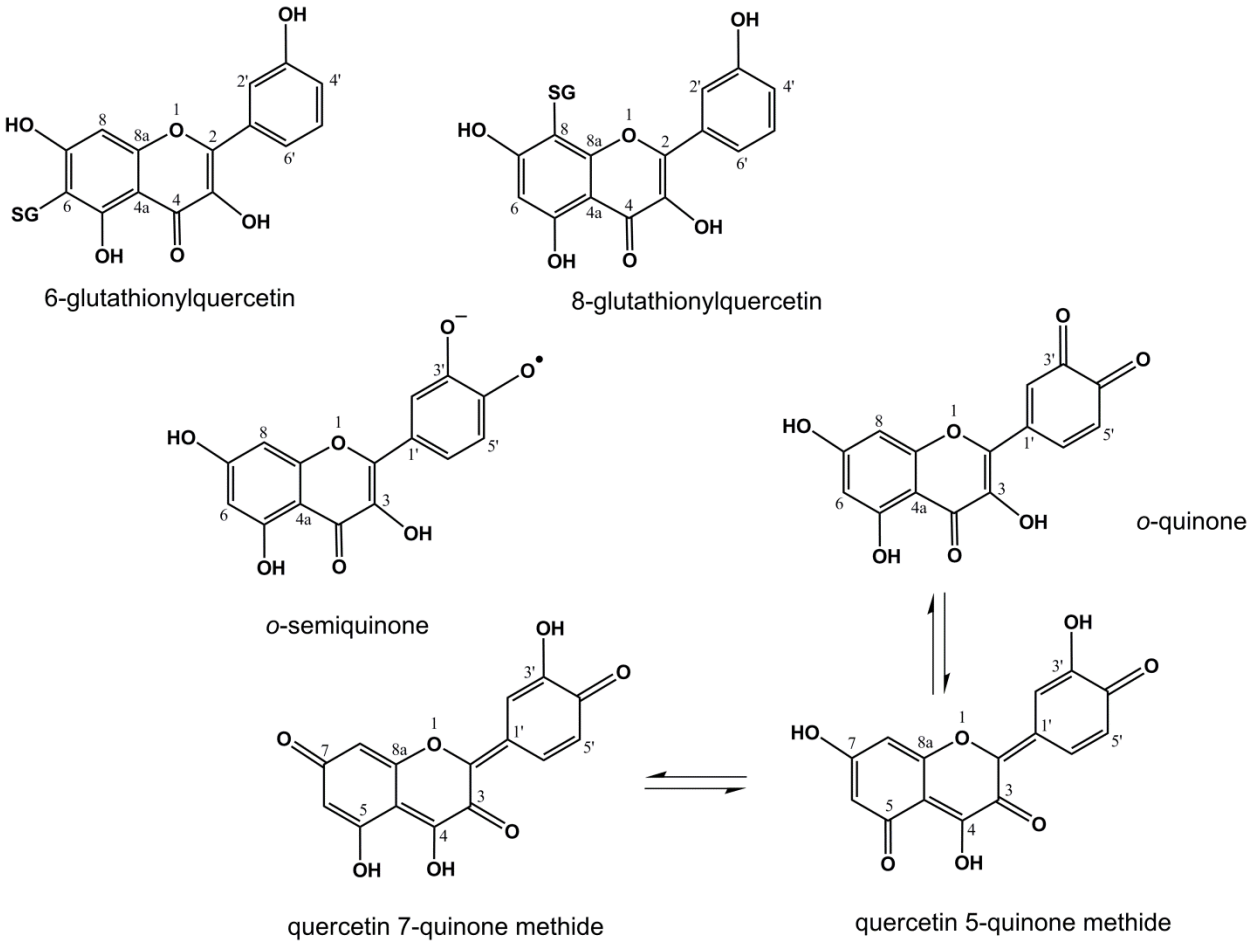

Figure 5. The structures of the quercetin adducts and the intermediates of the oxidation reaction mediated by peroxidase/H_2O_2 system. **A.** The structures of the GS-quercetin adducts formed in the presence of GSH under peroxidase oxidation. **B.** The structures of the reactive intermediates: *o*-semiquinone, *o*-quinone, and its *p*-quinonemethide isomers.

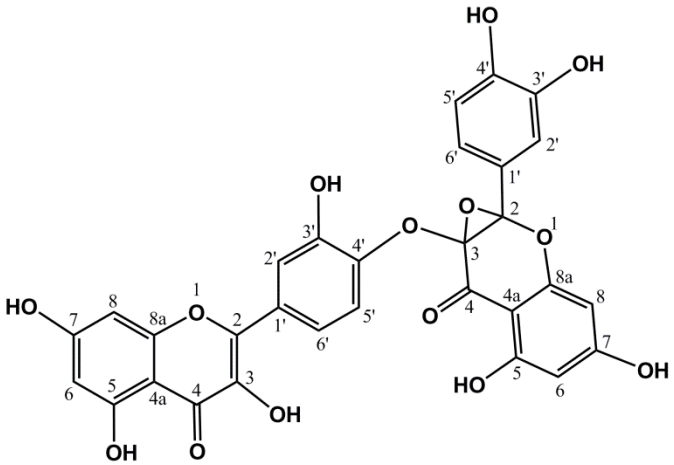

Figure 6. The structure of quercetin dimer formed in the cytochrome c-mediated oxidation of quercetin.

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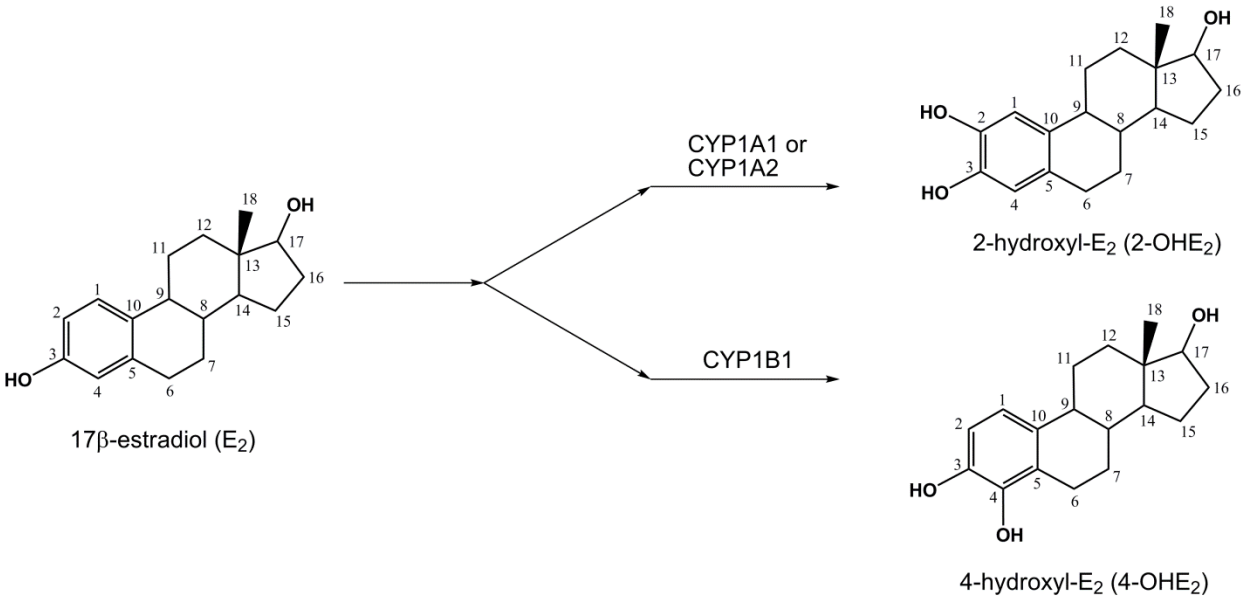

Figure 7A. The structure of hormone 17β-estradiol and its metabolites produced under catalysis by CYP isoforms.

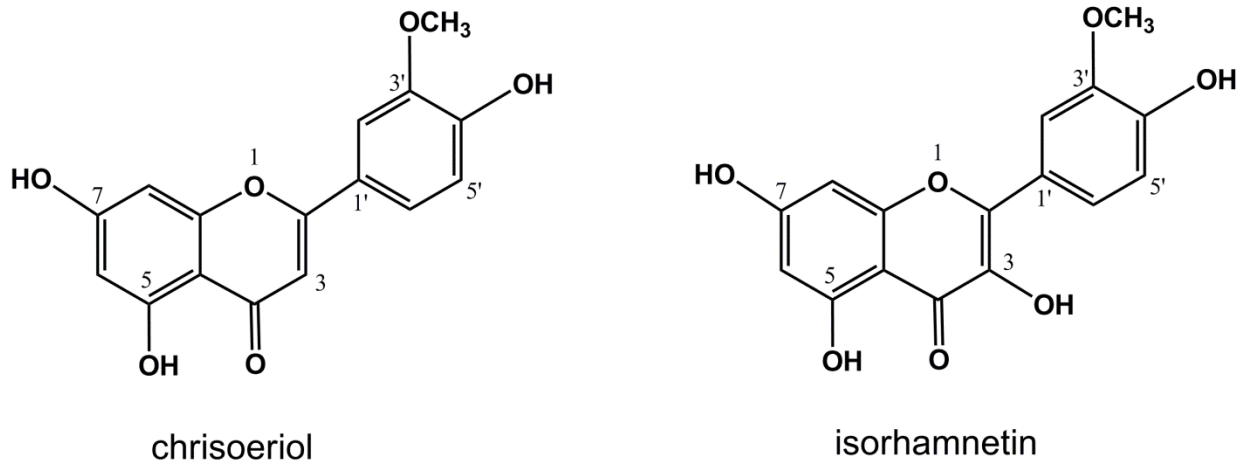

Figure 7B. The structures of the flavonoids exhibited the highest potency in the inhibition of the catalytic activity of CYP1B1.

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