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Review

Antioxidant and prooxidant properties of flavonoids ☆

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ABSTRACT

The interest in possible health benefits of flavonoids has increased owing to their potent antioxidant and free radical scavenging activities observed *in vitro*. Nevertheless, the antioxidant efficacy of flavonoids *in vivo* is less documented and their prooxidant properties have been actually described *in vivo*. Due to their prooxidant properties, they are able to cause oxidative damage by reacting with various biomolecules, such as lipids, proteins and DNA. Hence, the aim of this review is to discuss both the antioxidant and prooxidant effects of flavonoids.

Contents

Abbreviations: CAT, catalase; EpRE, electrophile responsive element; ESR, electron spin resonance; FRAP, ferric reducing antioxidant power; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione S-transferase; LDL, low-density, lipoproteins; NO, nitric oxide; NOS, nitric oxide synthase; •OH, hydroxyl radical; PECAM-1, platelet endothelial cell adhesion molecule-1; ROS, reactive oxygen species; SOD, superoxide dismutase; TEAC, trolox equivalent antioxidant capacity.

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1. Introduction

Flavonoids (the term is derived from the Latin word "flavus", meaning yellow) are ubiquitous plant secondary products that are best known as the characteristic red, blue, and purple anthocyanin pigments of plant tissues [1]. Apart from their physiological roles in the plants, flavonoids are important components of the human diet, although they are not considered as nutrients.

The dietary anomaly called French paradox was first observed in French population and found later also in other Mediterranean populations. Epidemiological studies revealed that flavonoid-rich diet is correlated with the increased longevity and decreased incidence of cardiovascular diseases seen in these populations despite their high intake of fat [2–5]. In addition to their antioxidant properties, flavonoids have been reported to exhibit other multiple biological effects, e.g. antiviral [6], antibacterial [7], anti-inflammatory [8,9], vasodilatory [10], anticancer [3], and anti-ischemic [11–13]. Moreover, they are able to inhibit lipid peroxidation and platelet aggregation and improve increased capillary permeability and fragility [14–16].

Nowadays, interest in their possible health benefits has increased owing most of all to their potent antioxidant and free radical scavenging activities observed *in vitro*. Nevertheless, the antioxidant efficacy of flavonoids *in vivo* is less documented and their prooxidant properties have been actually described *in vivo*. Presented review summarizes antioxidant as well as prooxidant activities of common flavonoids with respect to their structural features.

2. Flavonoids as antioxidants

The term antioxidant is commonly used in scientific literature but it can be defined in multiple ways according to the methods used to measure antioxidant activity. Therefore, Halliwell and Gutteridge [17] proposed a definition of an antioxidant as "any substance that delays, prevents or removes oxidative damage to a target molecule". Physiological role of these compounds, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals [18].

The flavonoid constituents of red wine and red grapes are factors of particular interest [19,20] due to the French paradox: the Southern French have a very low incidence of coronary heart disease despite their high fat diet, low exercise, and smoking habits [4,21]. The exact molecular mechanism underlying this prevention remains poorly defined. One of the features that has been highlighted relates to the high consumption of red wine in the French population and the question as to whether the polyphenolic antioxidants from this dietary source contribute to the protection from coronary heart disease along with the antioxidants in the olive oil and the high intake of antioxidant nutrients from the fresh fruit and vegetable-rich Mediterranean diet [22]. Polyphenolic grape extract of red wine was described to stimulate inhibition receptor PECAM-1 (platelet endothelial cell adhesion molecule-1) thereby inhibiting platelet activation [4]. Similarly, Hertog et al. [23] observed an inverse association of the flavonol intake (mainly quercetin) with the mortality from coronary heart disease in the Zutphen Elderly Study. The flavonol intake also predicted a reduced incidence of first myocardial infarction in elderly men [23,24].

2.1. In vitro antioxidant activity of flavonoids

The antioxidant capacities of many flavonoids are much stronger than those of vitamins C and E [25]. For example, the one-electron reduction potential of epigallocatechin gallate under standard conditions is 550 mV, a value lower than that of glutathione (920 mV) and comparable to that of α-tocopherol (480 mV) [26–29]. Flavonoids can prevent injury caused by free radicals by the following mechanisms:

(1) direct scavenging of reactive oxygen species (ROS),
(2) activation of antioxidant enzymes [30],
(3) metal chelating activity [31],
(4) reduction of α-tocopheryl radicals [32,33],
(5) inhibition of oxidases [33,34],
(6) mitigation of oxidative stress caused by nitric oxide [35],
(7) increase in uric acid levels [36],
(8) increase in antioxidant properties of low molecular antioxidants [37].

2.1.1. Direct scavenging of ROS

Flavonoids are able to scavenge free radicals directly by hydrogen atom donation. Radicals are made inactive according to the following equation, where R• is a free radical and Fl-O• is a flavonoid phenoxyl radical (Fig. 1).

The *in vitro* flavonoid antioxidant activity depends on the arrangement of functional groups on its core structure. Both the configuration and total number of hydroxyl groups substantially influence the mechanism of the antioxidant activity [33]. The B ring hydroxyl configuration is the most significant determinant of ROS scavenging [39], whereas substitution of the rings A and C has little impact on superoxide anion radical scavenging rate constants [40,41]. The *in vitro* antioxidant activity could be increased by polymerization of flavonoid monomers, e.g. proanthocyanidins (also known as condensed tannins), the polymers of catechins, are excellent *in vitro* antioxidants due to the high number of hydroxyl groups in their molecules. The antioxidant capacity of proanthocyanidins depends on their oligomer chain length and the type of ROS with which they react

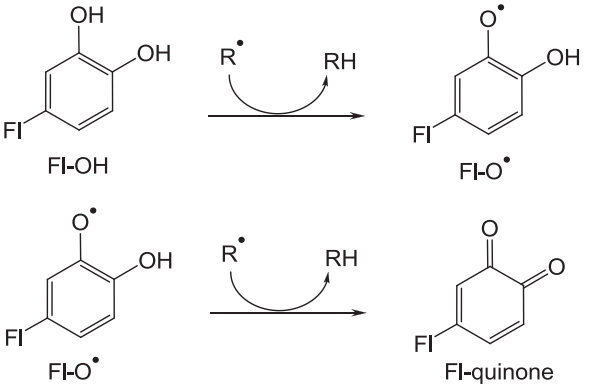

Fig. 1. Scavenging of reactive oxygen species (R•) by flavonoid. The free radical Fl-O• may react with a second radical, acquiring a stable quinone structure [38]. (The figure is presented with the kind permission of Prof. Pietta.)

[42]. The glycosylation of flavonoids reduces their *in vitro* antioxidant activity when compared to the corresponding aglycons [43–47]. Comparison of TEAC values of quercetin (4.42 mM) and rutin (2.02 mM), quercetin-3-*O*-rutinoside, shows that glycosylation of the 3-OH group has strongly suppressive effect on the antioxidant activity [43]. Similar results were observed also for other pairs of flavonoid aglycon and glycoside (e.g. hesperetin–hesperidin, luteolin–luteolin 4′-glucoside; luteolin–luteolin 7-glucoside; baicalein–baicalin; and quercetin–quercitrin) [43,44]. Quercetin glycosylation also significantly reduced its superoxide scavenging ability [48], hypochlorite scavenging activity [49] and power to reduce Fe (III) to Fe(II) (determined by FRAP assay) [50].

The main structural features of flavonoids required for efficient radical scavenging could be summarized as follows [51,52]:

a) an *ortho*-dihydroxy (catechol) structure in the B ring, for electron delocalization (Fig. 2):
b) 2,3-double bond in conjugation with a 4-oxo function in the C ring provides electron delocalization from the B ring (Fig. 3):
c) hydroxyl groups at positions 3 and 5 provide hydrogen bonding to the oxo group (Fig. 4):

According to the previously stated criteria, flavonols quercetin and myricetin should be the most effective radical scavengers in the aqueous phase, which has been confirmed experimentally [43].

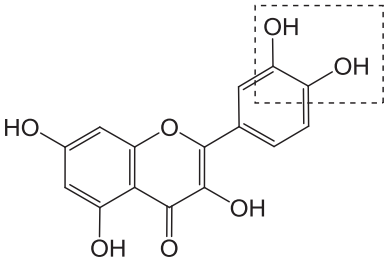

Fig. 2. An *ortho*-dihydroxy (catechol) structure in the B ring [52].

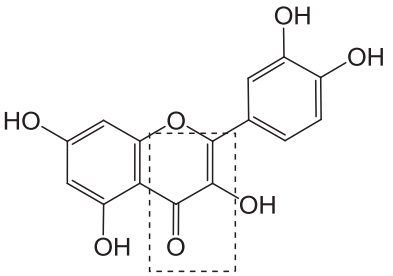

Fig. 3. 2,3-Double bond in conjugation with a 4-oxo function in the C ring [52].

2.1.2. Ability to activate antioxidant enzymes

Other possible mechanism by which flavonoids act is through interaction with various antioxidant enzymes. Furthermore, some effects may be a reset of a combination of radical scavenging and the interaction with enzyme functions [30].

Flavonoids are able to induce phase II detoxifying enzymes (e.g. NAD(P)H-quinone oxidoreductase, glutathione S-transferase, and UDP-glucuronosyl transferase), which are the major defense enzymes against electrophilic toxicants and oxidative stress. Regulation of this protective gene expression can be mediated by an electrophile responsive element (EpRE), which is a regulatory sequence of a number of genes encoding these phase II enzymes [53,54]. The ability of flavonoids to activate the EpRE-mediated response correlates with their redox properties. Lee-Hilz et al. [55] observed activation of firefly luciferase reporter gene in Hepa-1c1c7 mouse hepatoma cells upon induction with flavonoids of different structure. The most effective inducers were flavonoids containing a hydroxyl group at the 3-position in the ring C (quercetin and myricetin), whereas flavonoids without this hydroxyl group only (luteolin and galangin) were low luciferase inducers. Therefore, flavonoids with a higher intrinsic potential to generate oxidative stress and redox cycling are the most potent inducers of EpRE-mediated gene expression. It can be concluded that the prooxidant activity of flavonoids can contribute to their health-promoting activity by inducing important detoxifying enzymes, pointing to a beneficial effect of a supposed toxic chemical reaction [55].

Nagata et al. [56] investigated cytoprotective effect of quercetin and catechin against hydrogen peroxide cytotoxicity in cultured rat hepatocytes BL-9, which are cells highly expressing cytosolic glutathione peroxidase (GPx). The

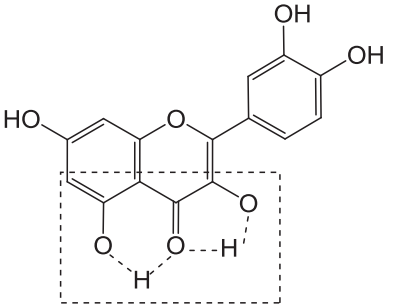

Fig. 4. Hydroxyl groups at positions 3 and 5 [52].

authors described that the protective activity of tested flavonoids was related to the activation of GPx. Martín et al. [57] described activation of survival signalling proteins (protein kinase B and extracellular regulated kinases) and increase in the activities of GPx and glutathione reductase (GR) in human hepatocytes caused by cocoa flavonoids. Leung et al. [58] provided evidence that luteolin-induced human lung carcinoma CH27 cell apoptosis was accompanied by activation of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), but not through the production of ROS and disruption of mitochondrial membrane potential. Therefore, the effects of luteolin on CH27 cell apoptosis were suspected to result from the antioxidant rather than the prooxidant action of this compound. Administration of the flavonoid-rich fraction along with a high fat diet caused a significant increase of SOD, CAT and GPx activities in rat erythrocytes [59]. Similar results were observed also after administration of naringin to hypercholesterolemic volunteers [60].

However, some contradictory observations in this field were obtained, e.g. glutathione S-transferase activity (GST) was significantly induced by apigenin, genistein and tangeretin in the rat heart but not in colon or liver. In red blood cells chrysin, quercetin and genistein significantly decreased the activity of GR, CAT and GPx, whereas SOD was only significantly decreased by genistein [61].

2.1.3. Metal chelating activity

Specific flavonoids are known to chelate iron and copper, thereby removing a causal factor for the development of free radicals. Quercetin was able to prevent oxidative injury induced in the erythrocyte membrane by a number of oxidizing agents (e.g. acrolein and phenylhydrazine), which cause release of iron in its free, redox active form [31]. Pietta [38] proposed that the binding sites for trace metals in the molecule of flavonoids are the catechol moiety in the ring B, the 3-hydroxyl and 4-oxo groups in the heterocyclic ring C, and the 4-oxo and 5-hydroxyl groups between the C and A rings (Fig. 5).

The catechol moiety in the B ring has been shown to be important for Cu^{2+}-chelate formation and thus being the major contributory site of the metal chelation [62]. Quercetin, in particular, is known for its iron-chelating and iron-stabilizing properties. Morin and quercetin were shown to form complexes with Cd(II) and exhibit strong antioxidant activity in the *in vitro* studies. Their sulfonic water-soluble derivatives exert low toxicity and therefore could be potential antidotes in cadmium intoxication [63–65].

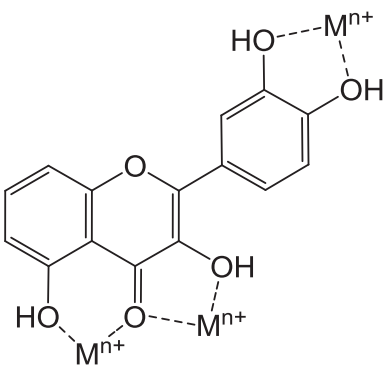

Fig. 5. Binding sites for trace metals [38]. (The figure is presented with the kind permission of Prof. Pietta.)

2.1.4. Reducing α-tocopheryl radicals

The α-tocopherol represents a major antioxidant in cell membranes and in human low-density lipoproteins (LDL) which protects lipoprotein particles against oxidative damage. Hirano et al. [32] suggested that flavonoids can act as hydrogen donors to α-tocopheryl radical, which is a potential prooxidant. Furthermore, by interaction with α-tocopheryl radical, they possess a great potential to delay the oxidation of LDL. Flavonoids (kaempferol, morin, myricetin and quercetin) showed a varying protective activity against depletion of α-tocopherol in LDL, with kaempferol and morin being less effective than myricetin and quercetin [66]. Catechins may be even more effective than ascorbate in regenerating α-tocopherol in micellar solution [67]. Similarly, the addition of green tea catechin extracts (epigallocatechin, epigallocatechin gallate, epicatechin, and epicatechin gallate) demonstrated a gradual regeneration of α-tocopherol in human LDL [68]. These observations are supported by the low value of redox potentials of some flavonoids (e.g. quercetin $E_7 = 0.2$ V or catechin $E_7 = 0.4$ V) [38].

2.1.5. Ability to inhibit oxidases

Flavonoids inhibit the enzymes responsible for superoxide ($O_2^{\bullet -}$) production, such as xanthine oxidase [69] and protein kinase C [70]. Quercetin and silybin inhibited xanthine oxidase activity, thereby resulting in decreased oxidative injury [30,71–73]. Cos et al. [34] carried out a study on structure–activity relationship in which luteolin was reported to be the most potent inhibitor of xanthine oxidase. Flavonoids have been also shown to inhibit cyclooxygenase, lipoxygenase, microsomal succinoxidase and NADH oxidase [62,74].

NADPH oxidase is membrane-associated system catalyzing production of $O_2^{\bullet -}$ in activated neutrophils. The mechanism of its activation includes interaction of an agonist with specific receptor on neutrophil membrane, activation of phosholipase C with subsequent formation of second messengers, which activate protein kinase C. This enzyme phosphorylates p47phox subunit of NADPH oxidase, a key component of this enzyme, and thus causes activation of respiratory burst [75,76]. The inhibition of protein kinase C was suggested to be a mechanism of an inhibition of NADPH oxidase by quercetin [70]. Potent flavonoid inhibitors of protein kinase C (e.g. quercetin, fisetin, and luteolin) possess a coplanar flavone structure with free hydroxyl substituents at the 3′, 4′ and 7-positions [77].

2.1.6. Mitigating oxidative stress caused by nitric oxide

Nitric oxide (NO) is important in maintaining the dilation of blood vessels [78] but its high concentrations may result in oxidative damage. NO is produced by the oxidation of L-arginine catalyzed by NO synthases (NOS). Nitric oxide toxicity is mainly mediated by peroxinitrite, which is formed in the reaction of NO with $O_2^{\bullet -}$ [79].

Flavonoids exerted NO production inhibitory activity in several lipopolysaccharide-activated cell lines and cultures

(mouse peritoneal macrophages, RAW 264.7 cells, and J774.2). This effect was probably caused by flavonoid inhibitory effect on expression of inducible NOS but not by the inhibition of its activity [80–82]. Several structural requirements of flavonoids for this activity were elucidated: presence of 2,3-double bond with 4-oxo group and 3,5,4′-trihydroxyl group were crucial. Their activity was even enhanced by methylation of 3-, 5-, or 4′-hydroxyl group and reduced by glycoside moiety and catechol or pyrrogallol arrangement of the B-ring. Thus, apigenin, diosmetin, and luteolin belong among naturally occurring flavones with the most potent inhibitory activity [80–82]. These flavonoids may at the same time increase the activity of endothelial NOS. Several flavonoids, including quercetin, result in a reduction in ischemia–reperfusion injury by interfering with inducible NOS activity [30,71]. Flavonoids also possess ability to directly scavenge molecules of NO [25].

How flavonoids inhibit induction of NOS and NO production is not clearly understood yet, but several explanations are argued. The first possibility may be derived from the antioxidant property of flavonoids by which these compounds scavenge ROS. The second possibility is that flavonoids may act as lipopolysaccharide-signalling molecule inhibitors [83].

Flavonoids are known to scavenge peroxynitrite directly [84]. The most significant determinant of their activity against peroxynitrite is the 3′,4′-catechol arrangement, followed by an unsubstituted 3-hydroxyl group. An apparent positive correlation between number of hydroxyl groups, particularly of the B-ring, and antiradical activity has been described [33,85,86].

2.1.7. Increasing uric acid levels

Interestingly, there are the great discrepancies between plasma or serum total antioxidant capacity and plasma concentrations of flavonoids. Lotito and Frei [36] suppose that the large increase in plasma total antioxidant capacity observed after the consumption of flavonoid-rich foods is not caused by the flavonoids themselves, but is likely the consequence of increased uric acid levels, which is a major contributor to plasma total antioxidant capacity. Cao et al. [87] described the significant increase in plasma or serum urate after consumption of strawberries, spinach or red wine. Similar increase of plasma or serum urate was described after drinking of port wine [88], French Bordeaux [89], tea or coffee [90]. Thus, several studies indicate that the consumption of flavonoid-rich foods may increase plasma urate, although the underlying mechanism still remains unclear. On the other hand, since elevated urate may be a risk factor for some diseases, the alleged ‘antioxidant benefit’ may not be what it seems [91].

2.1.8. Modification of prooxidant properties of low molecular antioxidants

Several authors described prooxidative activity of ß-carotene under certain conditions (e.g. UVA irradiation) and suggested that its combination with an antioxidant may have preventive effect [92,93]. Yeh et al. [37] investigated the interaction of β-carotene with naringin, rutin, and quercetin on DNA damage induced by UVA in C3H10T1/2 cells, mouse embryo fibroblasts. When each flavonoid was combined with β-carotene during preincubation, UVA-induced cellular DNA damage was significantly suppressed and the effects were in the order of naringin ≥ rutin > quercetin. Results of this study suggested that a combination of β-carotene with naringin, rutin or quercetin may increase the safety of β-carotene.

2.2. Metabolism of flavonoids and in vitro antioxidant activity of their metabolites

Although flavonoids display potent antioxidant activity *in vitro*, the bioactive forms of flavonoids *in vivo* are not those forms found in plants (i.e. flavonoid glycosides) due to their extensive biotransformation in the small intestine and hepatic metabolism on absorption. These metabolic changes include extensive phase I de-glycosylation of flavonoid glycosides and phase II metabolism including glucuronidation, sulphation and *O*-methylation of resulting aglycones [94–96]. Also colonic microflora participates in the flavonoid metabolism. Bacterial enzymes may catalyse wide variety of reaction including hydrolysis of glucuronides, sulphates and glycosides, dehydroxilation, demethylation, reduction of double bonds, ring cleavage, and decarboxylation of some phenolic acids [97,98]. The type of ring fission depends on the type of flavonoid (reviewed in [98]). Moreover, flavonoids may undergo at least three forms of intracellular metabolism: oxidative metabolism, P450-related metabolism, and conjugation with thiols, particularly reduced glutathione (GSH) [99–101]. For example, flavonoid quercetin is in the organism metabolised to the extent that little or no free aglycone is present in the plasma. Instead, the major bioavailable forms in animals and humans are represented by quercetin conjugates, namely glucuronides, *O*-methylated glucuronides, and sulphated derivatives [100,102]. The major *in vivo* reported metabolites of this flavonol are 3′-*O*-methyl quercetin, 4′-*O*-methyl quercetin and quercetin 7-glucuronide. After consumption of onions by humans, also metabolites quercetin 3′-sulphate and quercetin 3-glucuronide were found in the plasma [97]. Similarly flavan-3-ols such as catechin and epicatechin are transformed into glucuronide and sulphate conjugates on absorption as well as *O*-methylated derivatives [102–104].

Metabolites of flavonoids (circulating as well as intracellular) have greatly reduced *in vitro* antioxidant potentials compared to the parent compound because of the blocking of free radical scavenging phenolic hydroxyl groups [99,102,105,106]. The *O*-methylated and glucuronidated derivatives of quercetin, (−)-epicatechin, catechin and luteolin had lower antioxidant capacity (determined by TEAC assay) than the corresponding aglycone [102,105,106]. Glucuronidation and *O*-methylation caused also decrease in the ability of quercetin and (−)-epicatechin to inhibit peroxinitrite-induced tyrosine nitration of these compounds compared to the aglycones [102]. There is also evidence that the ability of quercetin, catechin and epicatechin to reduce Fe(III) to Fe(II) determinated by FRAP assay was greatly reduced by methylation in both 3′ and 4′ positions of the ring B [105]. These findings indicate that metabolic modifications occurring *in vivo* may substantially influence the antioxidant activity of dietary flavonoids. Moreover, prooxidant properties of flavonoid metabolites have been actually described *in vivo* [107,108]. Kessler et al. [108] described prooxidative activity of some quercetin metabolites (those with free catechol

moiety, free hydroxyl in position 3 or both) via stimulation of superoxide radical and hydrogen peroxide production. It appears that to avoid prooxidant behaviour of quercetin metabolites, the hydroxyl group in position 3 should be blocked to prevent its autoxidation.

2.3. In vivo antioxidant activity of flavonoids and their metabolites

Procyanidins supplementation in the rat (young and aged) was positively associated to an increased plasma antioxidant activity [109]. Similarly, total flavonoids from *Rosa laevigata* Michx had favourable potency to develop a hypolipidemic and hepatoprotective activities, of which the levels may be mediated, in part, by enhancing the system of antioxidant defence [110].

In considering the possible bioactive mechanisms of action of flavonoids and their *in vivo* metabolites in cell systems, it is important to consider their uptake and possible further metabolism by the cells [111]. For example, (−)-epicatechin and quercetin accumulated mostly as glucuronide and sulfate conjugates in blood plasma after oral administration in rats. No intact quercetin was found in the circulation. However, on the oral administration of these flavonoids, the antioxidative ability of rat plasma was enhanced indicating that conjugated metabolites participate in the antioxidant defence [112].

Interestingly, the metabolic pattern of quercetin depends on the way of administration. When rats were fed by the chow supplemented with 0.02% quercetin over 3 weeks, major plasma metabolites were 3′-*O*-methyl quercetin (isorhamnetin) glucuronide and sulphate conjugates, the most plausible conjugation positions being at the 3-, 5- and 7-hydroxyl positions. Isorhamnetin conjugates were methylated at the 3′-OH position, which decreased the high antioxidant activity of quercetin and its metabolites and their contribution to plasma antioxidant potential. On the other hand, after a single high-dose administration, where the major metabolites were quercetin conjugates at 5- and 7-hydroxyl positions, a significantly increased plasma antioxidant activity was observed [113]. Justino et al. [114] reported that plasma antioxidant status was significantly higher in rats to which quercetin was administrated, suggesting that quercetin metabolites can retain some antioxidant activity when the *o*-catechol group does not undergo conjugation reactions.

Nevertheless, describing antioxidant properties of flavonoids and their metabolites *in vivo* with respect to their structural features is very challenging. Only limited data is available for human *in vivo* studies, where pure flavonoid was administered. Instead, flavonoid-rich foods (e.g. cocoa and/or chocolate [115,116], onion [117]) or beverages (e.g. coffee and/or green tea [118], pomegranate juice [119]) were administered in many studies in this field, and such foods contain also other constituents that might be able to influence oxidative damage and thus over- or under-estimate real benefit of the flavonoids. Moreover, results of these studies are ambiguous, some of them showed antioxidant effects, others no effect and yet others some mild prooxidant effects (for details see [120]).

3. Flavonoids as prooxidants

As well as many of so-called antioxidants, also flavonoids can act, under certain circumstances, as prooxidants and, hence, promote the oxidation of other compounds.

3.1. Direct prooxidant function of flavonoids

Prooxidant activity is thought to be directly proportional to the total number of hydroxyl groups in a flavonoid molecule [84]. Series of mono- and dihydroxyflavonoids demonstrated no detectable prooxidant activity, while multiple hydroxyl groups, especially in the B-ring, significantly increased production of hydroxyl radicals in Fenton reaction [33,69]. The latter compounds include baicalein containing a pyrogallol structure in the A-ring, which has also been reported to promote hydrogen peroxide production [33,121] from which highly reactive hydroxyl radicals may be generated via Fenton reaction [33,122]. There is also evidence that the 2,3-double bond and 4-oxo arrangement of flavones may promote the formation of ROS induced by divalent copper in the presence of oxygen [48]. It is possible that flavonoid prooxidant function could be a toll of their other beneficial functions. For example, epigallocatechin gallate promotes apoptosis and has bactericidal activity, which is attributed to its ability to reduce O_2 to yield H_2O_2 [29,123,124].

The possible prooxidant effects of flavonoids may be important *in vivo* if free transition metal ions are involved in oxidation processes. Flavonoids are capable of Cu(II) reduction to Cu(I) and thus enable formation of initiating radicals [84]. In the healthy human body, metal ions appear largely sequestered in forms unable to catalyze free radical reactions (e.g. in ferritin or caeruloplasmin) [17]. However, injury to tissues may release iron or copper, and catalytic metal ions have been measured in atherosclerotic lesions [125]. In these cases the potential for flavonoids to act as prooxidants cannot be ignored [52].

Flavonoid prooxidant properties seem to be concentration-dependent. For example, Yen et al. [126] monitored prooxidant properties of quercetin, naringenin, hesperetin and morin in human lymphocytes. Whereas H_2O_2 concentration could not be detected when flavanones naringenin and hesperin were added in the concentration range of 0–200 μM, flavonols quercetin and morin increased H_2O_2 concentration in the concentration range of 25–200 μM and 125–200 μM, respectively. The generation of superoxide anion radical and products of lipid peroxidation (measured as thiobarbituric acid reacting substances) increased with increasing concentration of each of these flavonoids. Moreover, these compounds were able to induce DNA strand breakage in concentration-dependent manner as was determined by sensitive comet assay. This effect could be explained by enhancing hydroxyl radical formation by all four tested flavonoids. The reported prooxidant activity was related to the structural characteristics of these flavonoids, when the compound with the most pronounced prooxidative activity was flavonol quercetin, while flavanones hesperetin and naringenin exerted lower effect [126].

In rat liver microsomes, gossypol, quercetin and myricetin powerfully inhibited iron-induced lipid peroxidation at low micromolar concentrations ($IC_{50} \leq 1.5$ μM). However, all three compounds at 100 μM concentration greatly enhanced

hydroxyl radical formation up to eight-fold [127]. Similarly, protection of human leucocytes against superoxide-induced oxidative DNA damage by quercetin was ambiguous. Incubation concentrations of quercetin (1–50 μM) reduced levels of oxidative DNA damage, while at 100 μM the amount of damage was increased. These results were supported by ESR-findings on quercetin in solution, also showing a prooxidant effect at 100 μM [128].

3.2. Oxidation by flavonoid phenoxyl radicals

According to the "classical" definition, antioxidant is a molecule: (1) that could donate electrons or hydrogen atoms, (2) yields an antioxidant-derived radical that (3) is efficiently quenched by other electron or hydrogen sources to prevent cellular damage, and (4) whose properties are spatially and temporally correlated with oxidative stress events [129–131].

The end products of ROS scavenging by flavonoids are flavonoid phenoxyl radicals (Fl-O•) with a lifetime of 200 μs [132]. They are highly reactive and subjected to further oxidation, yielding, among other possible products, the more stable flavonoid quinones (Fig. 1). Flavonoid quinones are still reactive but they can be stabilized by conjugation with nucleophiles, such as GSH, cysteine or nucleic acids [131,133–135]. This reaction is responsible for one of the prooxidant effects of flavonoids [38,136]. The prooxidant properties of the flavonoids apigenin, naringenin, and naringin have been described by showing that their phenoxyl radicals rapidly oxidize NADH, resulting in extensive oxygen uptake and $O_2^{\bullet -}$ formation [137–139]. Another reaction, which may be responsible for undesired prooxidant properties of flavonoids, could be the interaction of Fl-O• with oxygen in the presence of high levels of transition metals (Fig. 6), generating quinones and $O_2^{\bullet -}$ [38,41].

The source of phenoxyl radicals could be an autoxidation as well. Canada et al. [140] found that the rate of autoxidation for both quercetin and myricetin was highly pH-dependent with no autoxidation detected for quercetin at physiological pH. The rate of quercetin autoxidation was substantially increased both by an addition of iron and by an addition of iron followed by SOD. The addition of iron increased the rate of autoxidation of myricetin as well. On the other hand, neither kaempferol, a monohydroxylated flavonol, nor rutin, a glycosylated quercetin, showed any ability to autoxidize. Autoxidation of quercetin accompanied by rapid accumulation of H_2O_2 was observed also in the presence of copper ions at neutral pH [141].

3.3. Inhibition of mitochondrial respiration

Some flavonoids are able to cause a substrate-independent cyanide insensitive respiratory burst in isolated mitochondria and undergo autoxidation, which is associated with the production of ROS [142,143]. Moreover, the ability of several flavonoids (robinetin, rhamnetin, eupatorin, baicalein, 7,8-dihydroxyflavon, and norwogonin) to inhibit beef heart mitochondrial succinoxidase and NADH-oxidase was tested [143]. Flavonoids with adjacent tri-hydroxyl (robinetin, baicalein) or *para*-dihydroxyl groups (norwogonin) exhibited a substantial rate of autoxidation, which was accelerated by the addition of cyanide. Flavonoids possessing a catechol configuration (rhamnetin, 7,8-dihydroxyflavone) exhibited a slow rate of autoxidation in buffer that was stimulated by the addition of cyanide.

Flavonols with catechol group in the ring B (e.g. quercetin and fisetin) are capable of producing *o*-semiquinones and to oxidize NADH in a variety of mammalian cells. Quercetin and fisetin caused decrease in a mitochondrial NADH to NAD^+ ratio in isolated liver cells in a dose-dependent manner; half-maximal reduction occurred at concentrations of 32.6 μM and 37.01 μM, respectively. Changes in NADH to NAD^+ ratio were accompanied by reduction in ketogenesis, stimulation of citric acid cycle and uncoupling effect on oxidative phosphorylation (probably caused by flavonoid effect on mitochondrial respiration and direct inhibition of ATP-synthase). Furthermore, low quercetin concentrations (25 μM) stimulated oxygen uptake, while its high concentrations (50–300 μM) inhibited oxygen uptake and stimulated citric acid cycle. Both flavonols are probably able to participate in the oxidation of NADH (both cytosolic and mitochondrial) in mammalian cells, shifting the cellular conditions to a more oxidized state (prooxidant activity) [144,145].

3.4. Oxidation by peroxidases

Alternative mechanism for flavonoid prooxidant toxicity involves the numerous peroxidases that catalyze the oxidation of polyphenols. Intracellular phenoxyl radicals (redox-cycling phenols) formed by myeloperoxidase in neutrophiles also induce lipid peroxidation and co-oxidize GSH to form thiol radicals with concomitant oxygen activation [139,146]. The prooxidant properties of the flavonoids apigenin, naringenin and naringin have been described by showing that

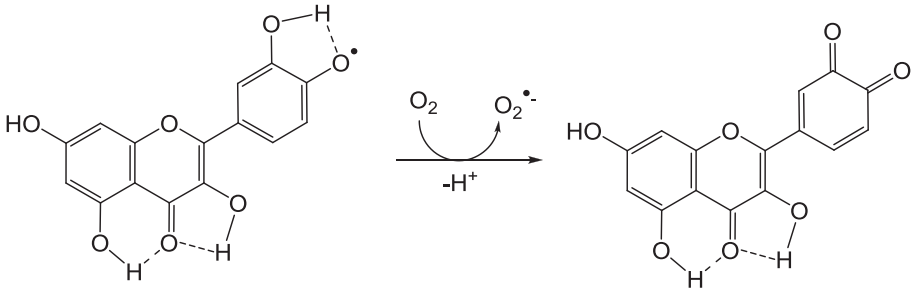

Fig. 6. Prooxidant activity of flavonoids [41]. (The figure is presented with the kind permission of Dr. Amić.)

their phenoxyl radicals rapidly co-oxidize NADH, resulting in extensive oxygen uptake and $O_2^{\bullet -}$ formation [137–139].

3.5. Oxidation of low molecular antioxidants

The catechol ring-containing flavonoids (catechol, luteolin, eriodictyol and quercetin) were found to oxidize ascorbate, NADH or GSH. In general, it was found that their effectiveness of catalyzing the oxidation increased as their redox potential decreased. Thus, the more readily oxidizable flavonoids were the most effective. This is likely because the lower redox potential catechols were more readily oxidized by peroxidase/H_2O_2 than were the higher redox potential phenols [139]. Nevertheless, antioxidants do not act lonely *in vivo*; oxidized antioxidants can be recycled in interplay with other antioxidants such as ascorbate and glutathione, called antioxidant networking [147–150]. For example, ascorbate can regenerate oxidized vitamin E and GSH can regenerate oxidized ascorbate depending on the redox potentials of these antioxidants. When flavonoid becomes oxidized, an *o*-quinone can be formed. This *o*-quinone can be recycled by GSH or ascorbate, which becomes oxidized during this reaction giving dehydroascorbate [149]. Such synergistic antioxidant mechanism even potentiates scavenging properties of individual compounds [150]. Quercetin, myricetin and kaempferol induced a concentration-dependent decrease of both the nuclear GSH content and GST activity in a model system of isolated rat liver nuclei. Myricetin, which has the maximum number of hydroxyl groups, was the most active among tested flavonoids. The impairment of the nuclear antioxidant defence GSH and GST by the polyphenolic flavonoids can lead to oxidative DNA damage, which may be responsible for their mutagenicity [151].

Flavonoids can influence negatively the absorption of low molecular antioxidants as well, e.g. lutein absorption can be impaired by naringenin [152].

3.6. Direct DNA damage

In the presence of reactive nitrogen species, flavonoids with A- or B-ring pyrogallol configurations induce DNA single-strand breakage [153]. Yamashita et al. [154] proposed the mechanism of site-specific DNA damage caused by quercetin. Its catechol group in the ring A or B is oxidized by a Cu(II) ion bound to DNA, which can generate ROS responsible for DNA damage [41]. By contrast, kaempferol and luteolin induced little DNA damage even in the presence of Cu(II). El Amrani et al. [155] studied the oxidative DNA cleavage induced by Fe(III)-flavonoid complex [41]. The proposed mechanism of Fe(II)-flavonoid complex, which then binds to DNA and generates ROS, is presented in Fig. 7.

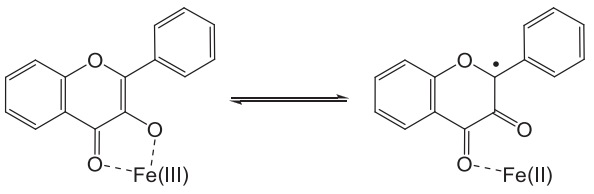

Fig. 7. Proposed route for generation of Fe(II)–flavonoid complex from Fe(III)–flavonoid complex [41]. (The figure is presented with the kind permission of Dr. Amić.)

4. Conclusions

It seems that prooxidant or antioxidant properties of a particular flavonoid depend most of all on its concentration. However, it is misrepresenting to view their prooxidant properties only as toxic ones. Their prooxidant properties could be associated with a cell signalling by which flavonoids contribute to the co-ordination of cell functions.

In addition, flavonoids are typical xenobiotics for animals and humans, metabolized as such and rapidly removed from the circulation [90] and it is tempting to speculate that the capacity of flavonoids to induce detoxifying enzymes is a major mechanism by which flavonoids protect organism against mutagens and carcinogens, i.e., act as cancer chemo-preventive agents [36].

Thus, flavonoids cannot only be considered purely as antioxidants, since under certain reaction conditions they can also display prooxidant activity. This unexpected behaviour could explain, in part, the observed toxicity of some flavonoids *in vivo* [108]. However, in practice prooxidant effects can also be beneficial, since, by imposing a mild degree of oxidative stress, the levels of antioxidant defences and biotransformation enzymes might be raised, leading to overall cytoprotection [120]. The extent to which are flavonoids able to act as anti- or prooxidants *in vivo* is still poorly understood and this topic clearly requires further studies.

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