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Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships

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Abstract

Flavonoids are a class of secondary plant phenolics with significant antioxidant and chelating properties. In the human diet, they are most concentrated in fruits, vegetables, wines, teas and cocoa. Their cardioprotective effects stem from the ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species. Flavonoids occur in foods primarily as glycosides and polymers that are degraded to variable extents in the digestive tract. Although metabolism of these compounds remains elusive, enteric absorption occurs sufficiently to reduce plasma indices of oxidant status. The propensity of a flavonoid to inhibit free-radical mediated events is governed by its chemical structure. Since these compounds are based on the flavan nucleus, the number, positions, and types of substitutions influence radical scavenging and chelating activity. The diversity and multiple mechanisms of flavonoid action, together with the numerous methods of initiation, detection and measurement of oxidative processes *in vitro* and *in vivo* offer plausible explanations for existing discrepancies in structure-activity relationships. Despite some inconsistent lines of evidence, several structure-activity relationships are well established *in vitro.* Multiple hydroxyl groups confer upon the molecule substantial antioxidant, chelating and prooxidant activity. Methoxy groups introduce unfavorable steric effects and increase lipophilicity and membrane partitioning. A double bond and carbonyl function in the heterocycle or polymerization of the nuclear structure increases activity by affording a more stable flavonoid radical through conjugation and electron delocalization. Further investigation of the metabolism of these phytochemicals is justified to extend structure-activity relationships (SAR) to preventive and therapeutic nutritional strategies. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Flavonoid; Polyphenol; Antioxidant; Lipid peroxidation; Structure-activity relationship

1. Introduction

Fruit and vegetable intake is associated with a reduced risk of cancer and cardiovascular disease. While these protective effects have been primarily attributed to beta-carotene and ascorbate, phenolic constituents may also play a role. Flavonoids are a broad class of low molecular weight, secondary plant phenolics characterized by the flavan nucleus. Widely distributed in the leaves, seeds, bark and flowers of plants, over 4,000 flavonoids have been identified to date. In plants, these compounds afford protection against ultraviolet radiation, pathogens, and herbivores [1]. The anthocyanin copigments in flowers attract pollinating insects [1] and are responsible for the characteristic red and blue colors of berries, wines, and certain vegetables—major sources of flavonoids in the human diet [2–10]. Although dietary intake varies considerably among geographic regions and cultures, it is estimated to be 23 mg daily in the Netherlands [11].

Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities. By virtue of their capacity to inhibit LDL oxidation, flavonoids have demonstrated unique cardioprotective effects [12,13]. Flavonoid-rich diets have been shown to reduce myocardial post-ischemic damage in rats [14]. A protective role in the diet of humans has also been indicated in some large, prospective studies. For example, high flavonoid intake predicted lower mortality from coronary heart disease and lower incidence of myocardial infarction in older men [15] and reduced the risk of coronary heart disease by 38% in postmenopausal women [16]. The Zutphen Elderly Study demonstrated an inverse relationship between consumption of catechin, a predominant flavonoid in tea, and ischemic heart disease mortality in a cohort of 806 men [17].

Reactive oxygen species (ROS) are capable of oxidizing

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cellular proteins, nucleic acids and lipids. Lipid peroxidation is a free-radical mediated propagation of oxidative insult to polyunsaturated fatty acids involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants [18]. ROS contribute to cellular aging [19], mutagenesis [20], carcinogenesis [21], and coronary heart disease [22]; possibly through destabilization of membranes [23], DNA damage [20], and oxidation of low-density lipoprotein (LDL). Reactive nitrogen species (RNS) also appear to contribute to the pathology of cardiovascular disease. Peroxynitrite, a potent oxidant generated by the reaction of nitric oxide (NO) with superoxide in the vascular endothelium, induces LDL oxidation [24,25] and proinflammatory cytokine-mediated myocardial dysfunction [26,27]. Nitrotyrosine, a product of protein nitration by RNS, is present in human atherosclerotic lesions [28]. The significance of antioxidants in the diet and their putative value in the intervention and prophylaxis of cardiovascular diseases has been of considerable interest in recent years.

The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer electrons free radicals, chelate metal catalysts [29], activate antioxidant enzymes [30], reduce alpha-tocopherol radicals [31], and inhibit oxidases [32]. Although this multidimensional effect is likely responsible for the consistent overall effectiveness of these compounds in diverse experimental systems, it poses difficulties in delineating structure-activity relationships (SAR). A number of structure-activity studies employ oxidases and/or transition metals as ROS generators, both of which confound the identification of relationships between chemical structure and free radical scavenging. Over the past fifteen years, a considerable number of *in vitro* studies have sought to arrive at a common heirarchy of flavonoids in terms of substitutions and antioxidant activity. These data enable a better understanding of the antioxidant and prooxidant effects of flavonoids, and offer reasonable predictions of the influence of structural modifications that ensue during metabolism. Further advancement of this research may lead to the development of nutritional products and semisynthetic analogs that retain substantial antioxidant capacity but produce minimal adverse effects. In summarizing diverse structure-activity studies conducted to date, this review aims to identify consistent lines of evidence that support specific structural elements as central determinants of free radical scavenging, chelation and prooxidant activity.

2. Classification and chemical structure

Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings (Fig. 1) and are classified according to substitutions (Fig. 2). Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A- and B- rings. During metabolism, hydroxyl groups are added, methylated,

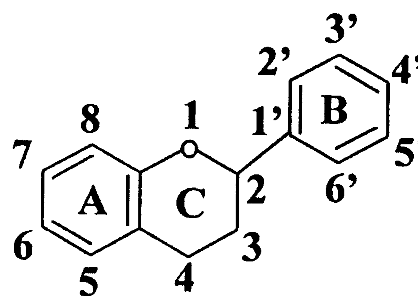


Fig. 1. Nuclear structure of flavonoids. Dietary flavonoids are diverse and vary according to hydroxylation pattern, conjugation between the aromatic rings, glycosidic moieties, and methoxy groups. Polymerization of this nuclear structure yields tannins and other complex species occurring in red wine, grapes and black tea. (From reference [38]).

sulfated or glucuronidated. In food, flavonoids exist primarily as 3-*O*-glycosides and polymers [2]. Several types of higher structure exist, and polymers comprise a substantial fraction of dietary flavonoid intake [33]. Enzymatic oxidation of green tea leaves (*Camellia sinensis*) during fermentation to black tea results in polymerization of flavanols to tannins and other complex compounds [34]. Condensed tannins, or proanthocyanidins, consist of flavanol units. Of these compounds, the procyanidins are most relevant to the human diet; these compounds consist of (+)-catechin and (−)-epicatechin monomers. The $\beta_{4\rightarrow6}$ and $\beta_{4\rightarrow8}$-linked procyanidin dimers, trimers, and oligomers occur in red wines [3], grape seeds [35], apples [36] and cocoa [37]. Proanthocyanidins may reach high molecular weights, consisting of up to 17 flavanol units [33,36]. Esters of gallic acid are known as hydrolyzable tannins or gallotannins. The galloyl moieties of these tannins and of the monomeric catechins in green tea are partly responsible for the chelating [38] and radical scavenging [39] properties of these compounds.

3. Absorption and metabolism

Understanding the biodynamics of flavonoids after oral administration is fundamental to appropriate extrapolation of existing SAR information to preventive nutrition. In addition to structural and physico-chemical attributes of the nascent compound, the absorption, pharmacokinetics, biotransformation, and the relative activities of metabolites are critical determinants of biological effects in organisms. *In vitro* data effectively and consistently demonstrate the antioxidant efficacy of structurally diverse flavonoids under many circumstances of oxidative stress. However, the current understanding of absorption and metabolism in humans is limited to a small number of dietary flavonoids. To integrate new knowledge of SAR into the areas of human nutrition and medicine, future research must elucidate (i) the extent of absorption in relation to structure, (ii) pharmacokinetics in humans, (iii) characterization of flavonoid metabolites, and (iv) SAR and health effects of these metabolites.

Class	General structure	Flavonoid	Substitution Pattern	Dietary Sources	TEAC (mM)
Flavanol		(+)-catechin	3,5,7,3′,4′-OH	Tea (camellia sinensis)[6]	2.4
		(-)-epicatechin	3,5,7,3′,4′-OH	Tea[6]	2.5
		Epigallocatechin gallate	3,5,7,3′,4′,5′-OH,3-gallate	Tea[6]	4.75
Flavone		chrysin	5,7-OH	Fruit skins	1.43
		apigenin	5,7,4′-OH	Parsley, celery	1.45
		rutin	5,7,3′,4′-OH, 3-rutinose	Red wine[5], buckwheat[7] citrus, tomato skin[8]	2.4
		luteolin	5,7,3′,4′-OH	Red pepper[11]	2.1
		luteolin glucosides	5,7,3′-OH, 4′-glucose		1.74
			5,4′-OH, 4′,7-glucose		0.79
Flavonol		kaempferol	3,5,7,4′-OH	Leek, broccoli, endives grapefruit, black tea	1.34
		quercetin	3,5,7,3′,4′-OH	Onion, lettuce, broccoli tomato, tea, red wine berries, olive oil, appleskin	4.7
		myricetin	3,5,7,3′,4′,5′-OH	Cranberry grapes, red wine	3.1
		tamarixetin	3,5,7,3′-OH,4′-OMe		
Flavanone (dihydroflavon		naringin	5,4′-OH,7-rhamnoglucose	Citrus, grapefruit	0.24
		naringenin	5,7,4′-OH	Citrus fruits	1.53
		taxifolin	3,5,7,3′,4′-OH	Citrus fruits	1.9
		eriodictyol	5,7,3′,4′-OH	Lemons[64]	1.8
		hesperidin	3,5,3′-OH,4′-OMe, 7-rutinose	Oranges[9]	1.08
Isoflavone		genistin	5,4′-OH, 7-glucose	Soybean[10]	1.24
		genistein	5,7,4′-OH	Soybean[10]	2.9
		daidzin	4′-OH, 7-glucose	Soybean[10]	1.15
		daidzein	7,4′-OH	Soybean[10]	1.25
Anthocyanidin		apigenidin	5,7,4′-OH	Colored fruits	2.35
		cyanidin	3,5,7,4′-OH,3,5-OMe	Cherry, raspberry, strawberry	4.42

Fig. 2. Classification, structure, food sources, and Trolox equivalent antioxidant activities (TEAC) of dietary flavonoids. Higher TEAC values reflect greater antioxidant capability. A free 3-hydroxyl group and 3′,4′-catechol (dihydroxy) structure, a 2–3 double bond, and a 4-oxo group endow the flavonoid with activity superior to isoforms lacking these features. Glycosidic substitution decreases TEAC. (Adapted from Rice-Evans et al., references [39] and [129]).

Most dietary flavonoids occur in food as *O*-glycosides [2]. The most common glycosidic unit is glucose, but other examples include glucorhamnose, galactose, arabinose, and rhamnose [38]. Not surprisingly, the β-linkage of these sugars resists hydrolysis by pancreatic enzymes, so it had long been assumed that intestinal microbiota were responsible for beta-hydrolysis of sugar moieties. However, two β-endoglucosidases capable of flavonoid glycoside hydrolysis have since been characterized in the human small intestine, including lactase phlorizin hydrolase [40,41] and a nonspecific cytosolic enzyme believed to deglycosylate flavonoids to allow a site for conjugation [42,43]. Spencer et al [44] reported that luteolin-7-glucoside, kaempferol-3-glucoside and quercetin-3-glucoside are hydrolyzed and absorbed by the small intestine, supporting β-glucosidase activity. During passage across everted intestine, luteolin-3-glucoside also undergoes complete hydrolysis to luteolin aglycone, in addition to its methyl- and sulfate conjugates [45]. In another study, cell-free extracts from human small intestine cleaved flavonoid 4′- and 7-monoglucosides, but did not modify a series of rhamnoglucosides and diglucosides [46], suggesting differences in flavonoid bioavailability according to the location and structure of the sugar moiety. Although some evidence suggests that anthocyanin glycosides are absorbed intact [47], the hydrolytic removal of glucose and rutinose from quercetin is well documented [44,45,48–51]. Though quercetin represents but one of hundreds of dietary flavonoids, it is among the most abundant, potent, and widely studied and provides insight into the absorption and metabolism of these polyphenols. It is not surprising that absorption kinetics vary considerably among foods, owing to the heterogeneity of sugars and other functional groups about the flavan nucleus [52,53]. Absorption may also depend on dosage, vehicle of administration, antecedent diet, sex differences [54], and microbial population of the colon. Separate locations of uptake also suggest different metabolic fates of rutinosides versus glucosides, as the liver may play a larger role in the metabolism of flavonoids absorbed in the small intestine compared to metabolism of compounds taken up by the colon.

For hydrolysis and absorption of some flavonoid glycosides, enteric bacteria are indispensable. The requirement of colonic microflora for hydrolysis of rutinosides may explain the low bioavailability of rutin (quercetin-3-rutinoside) compared to quercetin-3-glucoside in human studies. Following oral administration of rutin, quercetin is gradually recovered in plasma of subjects with an intact colon [55], but is undetectable in plasma of ileostomy patients [50]. In the latter investigation, a glucose moiety increased absorption of quercetin in the small intestine to 52%, compared to 24% for the aglycone and 17% for rutin. Quercetin glucoside, but not rutin, has been reported to interact with epithelial glucose transporters [56], offering a possible explanation for the rapid uptake and bioavailability of glucosides [52].

Due to molecular size, absorption of polymeric flavonoids across the intestinal epithelium requires preliminary degradation to smaller, low molecular weight compounds. Procyanidin dimers and trimers, but not oligomers averaging 7 units, are capable of translocating across the small intestinal epithelium [57]. Thus, it is possible that degree of polymerization is less predictive of antioxidant activity *in vivo* compared to *in vitro,* and the value of the latter research is limited in describing the role of proanthocyanidins in human nutrition. Since these molecules generally consist of (+)-catechin and (−)-epicatechin subunits, it is conceivable that catechins are predominant degradation products. Caecal bacteria [58] and the low gastric pH [59] contribute to this process. The latter investigation demonstrated the hydrolysis of proanthocyanidin oligomers of 3 to 6 units into catechin dimers and free catechins after 3.5 hr in the gastric environment. Beyond 3 catechin units, susceptibility to degradation increased proportionally to the degree of polymerization. Although these observations suggest that catechins are responsible for the health effects of high-molecular weight proanthocyanidins, 3.5 hr exceeds the normal human gastric emptying rate of 30 to 90 min, and the contribution of acid hydrolysis is probably less significant than subsequent metabolic events.

Aside from hydrolysis of flavonoid glycosides, cecal microflora participate in degradation of polymers and scission of monomeric flavonoids to monophenolic acids. Metabolism of quercetin by intestinal bacteria produces 3,4-dihydroxyphenylacetic acid and phloroglucinol via cleavage of the C_3-C_4 bond of the heterocycle [60]. Following oral administration of rutin to rats, phenylacetic acids, 3,4-dihydroxytoluene, and 3-(*m*-hydroxyphenyl)propionic acid are recovered in urine [61]. Tannins are degraded by cultured colon flora to similar aromatic compounds [51]. Deprez and colleagues [52] incubated cultures of human colonic bacteria with ^{14}C-labeled proanthocyanidins, averaging 7 β-linked flavanol units, under anaerobic conditions resembling the enteric environment. After 48 hr of incubation, nearly all of the substrate was degraded into monohydroxylated phenylacetic, phenylpropionic and phenylvaleric acids as determined by gas chromatography coupled to mass spectrometry. These metabolites are similar to the phenolic acid degradation products of catechin [63] and procyanidin dimers [58]. Rice-Evans and coworkers proposed a metabolic scheme for quercetin in which the flavone heterocycle is cleaved to phenolic acids subject to subsequent dehydroxylation, *O*-methylation, or β-oxidation to benzoic acid derivatives [39]. Miyake and colleagues [64] promulgated a metabolic scheme for orally administered eriocitrin, a flavone diglycoside from lemons, after oral administration to rats. The native eriocitrin was undetectable in plasma, but metabolites were conjugated to glucuronic acid or sulfate and were characterized as eriocitrin aglycone, 4′-methoxy eriodictyol (hesperetin), 3′-methoxy eriodictyol (homoeriodictyol), and 3,4-dihydroxyhydrocinnamic acid. Although the relative antioxidative activities of these metabolites were not directly evaluated, the susceptibility of plasma to oxidative damage decreased significantly in their presence.

Using the Trolox (a water-soluble α-tocopherol analog)-equivalent antioxidant capacity (TEAC) assay, Rice-Evans and coworkers [39] compared radical scavenging activities of phenolic acids occurring in higher plants. This method assesses the hydrogen donating ability of flavonoids in the aqueous phase by evaluating 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^{\cdot+}$) scavenging, and activity is expressed as the millimolar concentration of Trolox (TEAC = 1) equivalent to the activity of a 1 mM solution of the experimental compound. All phenolic acids demonstrated lower TEAC values than flavonoids, and hydroxycinnamates were generally more active than most hydroxyphenylacetates and hydroxybenzoates. The quercetin metabolite 3,4-dihydroxyphenylacetic acid (TEAC = 2.19) was substantially weaker than quercetin (TEAC = 4.7) but remained over twice as effective as vitamin E. The putative 4-dehydroxy metabolite of quercetin (TEAC = 0.90) was much less effective than both quercetin and 3,4-hydroxyphenylacetic acid. While this evidence supports that scission of the flavonoid heterocycle produces compounds with lower activity, these metabolites retain a radical scavenging capacity comparable to vitamin E. Structure-activity comparisons suggest that antioxidant activity of phenolic acids depends on the number and orientation of hydroxyl groups relative to the electron-withdrawing CO_2H, CH_2CO_2H, or $(CH)_2CO_2CH$ functional group [39].

Interactions between flavonoids and cytochromes P450 are complex and are reviewed elsewhere [65]. Hydroxylation of flavonoids by CYP1A isozymes yields 3′4′-dihydroxylated derivatives that retain the flavan nuclear structure [66]. However, flavonoids are known to inhibit various P450 isozymes, including CYP1A [67,68]. Selectivity for P450 isoforms is governed by hydroxyl and methoxy substitution on the 3′ and 4′ position of the B-ring [68]. Since a requisite for microsomal hydroxylation is a maximum of one B-ring hydroxyl group [66], it is conceivable that the increase in OH groups after phase I metabolism of flavonoids with relatively low activity may give rise to transient compounds with greater antioxidant capability, as the

aromatic OH is a critical determinant of hydrogen donation and free radical scavenging by phenolic compounds [69]. Methylation by catechol-*O*-methyltransferase requires a free B-ring OH and mitigates both the antioxidant and prooxidant activity of the flavonoid [70].

Despite a paucity of metabolic and pharmacokinetic data, existing evidence supports that flavonoids are structurally altered *in vivo.* Whether phenolic acids or flavonoid isoforms predominate is unclear. Quercetin is recovered in plasma following oral adminstration to humans, but is minimally detected in urine [55]. Walle and coworkers [71] demonstrated quercetin absorption after an oral dose of 100 mg quercetin aglycone ranges from 36 to 53%. Warden and colleagues [72] reported only 1.68% of 400 mg orally administered tea catechins was recovered in plasma, urine, and feces. Collectively these observations suggest extensive biotransformation by tissues and/or intestinal microbiota. Phase II conjugation in the liver [73] and enterocytes [44, 45,74] gives rise to glucuronides [48,54], sulfates [47,57], methyl conjugates [76] and small quantities of free aglycones [77]. Considering that the reduction potential of the B-ring is lower than that of the A-ring, conjugation at the 3′- or 4′-position is likely to increase the reduction potential such that hydrogen donation is less thermodynamically favorable as opposed to A-ring conjugation [44]. A report by Sanz and coworkers [78] that a 7-glucuronide does not interfere with antioxidant effects in rat liver microsomes is consistent with this premise.

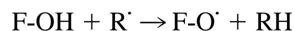
Although peak concentrations of flavonoids typically occur approximately 2 hr after ingestion of a test food [79,80], one study reported peak levels at 24 hr following an oral dose of epicatechin [72]. In healthy volunteers, the half-life of quercetin ranges from 20 to 72 hr [71]. Provided that sufficient dietary intake is sustained, this long half-life is conducive to accumulation in plasma and a concomitant decline in oxidant status [81]. However, chronic intake of high levels may result in a compensatory decrease in absorption, suggesting a steady state mechanism at the gut level. For example, Manach and colleagues [49] reported that in rats fed a high flavonol diet prior to treatment of pharmacological doses of quercetin, absorption was significantly reduced compared to animals previously maintained on low-quercetin diets. Other dietary components, namely proteins and iron, may theoretically impair absorption by forming complexes with polyphenols. Due to the affinity of flavonoid hydroxyl groups for proline residues, the antioxidant capacity of catechin gallates *in vitro* is attenuated by the presence of proteins such as β-casein [82]. This suggests proteins in the food itself, the digestive milieu, and the bloodstream may potentially mask the biological activity of polyhydroxylated flavonoids. However, addition of milk to black tea had no effect on the absorption of quercetin or kaempferol in a group of healthy individuals [83]. The exact mechanisms of flavonoid absorption and metabolism remain uncertain, and appear to depend on the type of flavonoid and other variables. Nonetheless, consumption of these compounds decreases plasma oxidant status in a dose-dependent manner [79,80]. Thiobarbituric acid reactive substances (TBARS) and other plasma indices of oxidative stress used in such studies do not always rule out the possibility that the flavonoid is working indirectly (enzyme interactions, metal chelation) versus directly (radical scavenging), but these lines of evidence demonstrate the bioavailability and antioxidant efficacy of flavonoids in humans.

4. Structural features and antioxidant activity

The antioxidant activity of flavonoids and their metabolites *in vitro* depends upon the arrangement of functional groups about the nuclear structure. The past 15 years of SAR research has generated several consistent lines of evidence supporting the role of specific structural components as requisites for radical scavenging, chelation and oxidant activity. The nutritional application of this information requires extensive investigation of flavonoid metabolism, systematic analysis of foods for flavonoid content and composition, and controlled comparison of antioxidant activity of structural isoforms *in vivo.* Ultimately, SAR may refine current dietary recommendations of fruits, vegetables and other plant foods.

4.1. Hydroxyl groups

The spatial arrangement of substituents is perhaps a greater determinant of antioxidant activity than the flavan backbone alone. Consistent with most polyphenolic antioxidants, both the configuration and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity [84–87]. Free radical scavenging capacity is primarily attributed to the high reactivities of hydroxyl substituents that participate in the following reaction:



$$\text{F-OH} + \text{R}^{\cdot} \rightarrow \text{F-O}^{\cdot} + \text{RH}$$

The B-ring hydroxyl configuration is the most significant determinant of scavenging of ROS [85,87] and RNS [86, 88]. Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical. Among structurally homologous flavones and flavanones, peroxyl and hydroxyl scavenging increases linearly and curvilinearly, respectively, according to the total number of OH groups [84].

A 3′4′-catechol structure in the B-ring strongly enhances lipid peroxidation inhibition [23,89,90]. This arrangement is a salient feature of the most potent scavengers of peroxyl [84,90], superoxide [91], and peroxynitrite radicals [86]. For example, the peroxyl radical scavenging ability of luteolin substantially exceeds kaempferol [92]; both have identical hydroxyl configurations, but kaempferol lacks the B-ring catechol. Peroxynitrite scavenging by catechin is

mainly ascribed to its B-ring catechol [88]. Oxidation of a flavonoid occurs on the B-ring when the catechol is present [92], yielding a fairly stable *ortho*-semiquinone radical [23] through facilitating electron delocalization [93]. Flavones lacking catechol or *o*-trihydroxyl (pyrogallol) systems form relatively unstable radicals and are weak scavengers [85,87, 94].

The significance of other hydroxyl configurations is less clear, but beyond increasing total number of hydroxyl groups, A-ring substitution correlates little with antioxidant activity. A 5-OH may contribute to antioxidant effects [95], and may explain why genestein exhibits a higher TEAC (Fig. 2) and greater peroxynitrite scavenging ability [96]. A 5,7-*m*-dihydroxy arrangement increases TEAC [39], but loss of a free 6-OH group by methylation does not modify inhibition of Fe(II)/ascorbate- and CCl_4-induced lipid peroxidation by flavonoids [78]. Compared to the B-ring hydroxylation pattern, the impact of the A-ring arrangement on antioxidant activity is of questionable significance.

The flavonoid heterocycle contributes to antioxidant activity by (i) the presence of a free 3-OH, and (ii) permitting conjugation between the aromatic rings. The closed C-ring itself may not be critical to the activity of flavonoids, given that chalcones are active antioxidants [97]. Free radical scavenging by flavonoids is highly dependent on the presence of a free 3-OH [87]. Flavonoids with a 3-OH and 3′,4′-catechol are reported to be 10- fold more potent than ebselen, a known RNS scavenger, against peroxynitrite [86]. The superiority of quercetin in inhibiting both metal- and nonmetal-induced oxidative damage is partially ascribed to its free 3-OH substituent [89,93], which is thought to increase the stability of the flavonoid radical. The torsion angle of the B-ring with respect to the rest of the molecule strongly influences free radical scavenging ability. Flavonols and flavanols with a 3-OH are planar, while the flavones and flavanones, lacking this feature, are slightly twisted [92]. Planarity permits conjugation, electron dislocation, and a corresponding increase in flavonoid phenoxyl radical stability [92]. Removal of a 3-OH abrogates coplanarity and conjugation, thereby compromising scavenging ability [92,98].

Quercetin exhibits a TEAC of approximately 4.7, whereas luteolin has a value of 2.1 [39], supporting the role of the 3-OH group in free radical scavenging. Compared to the flavonols quercetin, myricetin, and kaempferol, the flavone luteolin is a very weak scavenger of DPPH (2,2-diphenyl-1-picrylhydrazyl radical) [31]. Substitution of 3-OH by a methyl or glycosyl group completely abolishes the activity of quercetin and kaempferol against β-carotene oxidation in linoleic acid [87]. It is postulated that B-ring hydroxyl groups form hydrogen bonds with the 3-OH, aligning the B-ring with the heterocycle and A-ring. Eliminating this hydrogen bond effects a minor twist of the B-ring, compromising electron delocalization capacity [92]. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is potentiated by the presence of a 3′,4′-catechol [39], explaining the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature.

Compared to the aqueous TEAC assay, experiments of lipid peroxidation provide limited information concerning relationships between structure and antioxidant mechanism. Protection of lipids against oxidative damage can be ascribed to (i) scavenging of hydroxyl, peroxyl, or synthetic radicals, (ii) termination of chain reactions in the lipid phase, involving peroxyl radicals and hydroperoxides, (iii) chelation of divalent cations used to initiate oxidative events *in vitro,* and/or (iv) interactions with other initiators, such as ascorbate, which may reduce and recycle the flavonoid radical or vice-versa. Despite the disparity among methods of assessing activity, there is broad agreement that hydroxyl groups endow flavonoids with substantial radical scavenging ability.

4.2. *O-methylation*

The differences in antioxidant activity between polyhydroxylated and polymethoxylated flavonoids are most likely due to differences in both hydrophobicity and molecular planarity. Quercetin is a potent peroxyl radical scavenger, followed by *O*-methylated and *O*-glycosylated derivatives [90]. Suppression of antioxidant activity by *O*-methylation [87,90,93] may reflect steric effects that perturb planarity. Although the ratio of methoxy to hydroxyl substituents does not necessarily predict the scavenging ability of a flavonoid, the B-ring is particularly sensitive to the position of the methoxy group. Alternating a 6′-OH/4′-OMe configuration to 6′-OMe/4′-OH completely abolishes the scavenging of DPPH by inducing coplanarity [97].

DPPH is not a naturally occurring radical, and is relatively stable compared to the highly reactive superoxide and hydroxyl species primarily responsible for oxidative damage in biological systems. Because the multidimensional effects of flavonoids confound the correlation of chemical structure with a particular mechanism, it is not unexpected that some *in vitro* experiments generate data that are inconsistent with outcomes from simpler assays of aqueous radicals. For example, the half-maximal inhibitory concentration (IC_{50}) of gardenin D (5,3′-OH/6,7,8,4′-OMe-flavone) against CCl_4-induced microsomal lipid peroxidation is considerably lower than (+)catechin [95], but the latter exhibits greater TEAC values than *O*-methylated flavonoids [39,99]. The results of the former study may reflect other physiologically relevant parameters of antioxidant activity, such as the lipophilicity and membrane partitioning ability afforded by methoxy groups [92,100].

Steric obstruction of the 3′4′-catechol structure by 4′-*O*-methylation significantly compromises antioxidant capability. For example, 4′-*O*-methylation of quercetin to tamarixetin decreases percentage inhibition of ferrous sulfate-induced lipid peroxidation from 98.0% to −2.6% [90]. Kaempferol-3′,4′-dimethylether exhibits approximately half the peroxyl scavenging activity of kaempferol [84].

Multiple A-ring methoxy groups also reverse the positive effect of a B-ring catechol, as inhibition of formation of the oxidation product malondialdehyde (MDA) by flavones with A-ring *ortho*-dimethoxy or trimethoxy structures is not enhanced by this element [23]. This information suggests that isorhamnetin, the 3′-methoxy metabolite of quercetin detected in humans [55], is a less effective antioxidant than the parent compound. *O*-methylation enhances antioxidant activity in some microsomal systems [95], but microsomal peroxidation assays permit multiple mechanisms of antioxidant activity, such as (i) recycling endogenous microsomal α-tocopherol, (ii) undergoing biotransformation by NADPH-activated cytochrome P450 to a more or less effective derivative, (iii) chelating iron, or (iv) phenoxyl radical reduction and recycling by ascorbate. These cooperative actions may explain greater activity of flavonoids in these systems. Moreover, given that each mechanism may be influenced by structure, it is not unexpected that some of these investigations report outcomes that do not parallel data from simpler, sensitive assays such as the TEAC method and the ORAC (total oxyradical absorbance capacity) assay developed by Cao et al. [101]. The latter employs hydroxyl and peroxyl radicals representative of ROS in the cellular environment. Synthetic radicals used in some methods do not always partition sufficiently into a membrane to which a relatively lipophilic polymethoxylated flavonoid has localized [93]. Based on the foregoing, it is rational to conclude that the influence of *O*-methylation depends on the method of evaluation, the type of radical used, and whether the oxidizable substrate is a lipid structure in which lipophilicity may contribute to total antioxidant activity.

4.3. The 2–3 double bond and 4-oxo function

A distinguishing feature among the general flavonoid structural classes in Fig. 2 is the presence or absence of an unsaturated 2–3 bond in conjugation with a 4-oxo function. Aside from the 3′,4′- catechol, 3-OH and overall hydroxylation pattern of quercetin, several studies have sought to determine the significance of 2–3 unsaturation and a 4-carbonyl group. Experiments of catechins and anthocyanidins suggest that these may be dispensable provided that other structural criteria are fulfilled. For example, the TEAC of quercetin (4.7) and cyanidin (4.44) differ by a narrow margin of 0.36 Trolox equivalents [39]. In a systematic study of 33 flavonoids, Burda and Oleszek [87] identified no consistent correlation between 2–3 unsaturation and antioxidant activity in a methanol solution of DPPH. However, comparison of quercetin with taxifolin suggests that in flavonoids fulfilling other structural criteria, the 4-oxo and double bond distinguishes the better antioxidant. Quercetin is a more potent inhibitor of ferrous sulfate-induced MDA formation than taxifolin [89]; both structures have a 4-oxo group, but taxifolin is saturated between carbons 2 and 3. Flavonoids with a 2–3 double bond in conjugation with a 4-carbonyl group exhibit lower IC_{50} values (indicative of stronger antioxidant activity) in a microsomal system compared to those with saturated heterocycles [95].

The majority of research supports that flavonoids lacking one or both features are less potent antioxidants than those with both elements. Conjugation between the A- and B-rings permits a resonance effect of the aromatic nucleus that lends stability to the flavonoid radical [98] and is therefore critical in optimizing the phenoxyl radical-stabilizing effect of a 3′,4′-catechol [39]. The premise that flavanols are more effective free radical scavengers than flavones [39,102] may be ascribed to the greater number of hydroxyl groups and 3-OH in the former. Hydroxyl and peroxyl scavenging capacities of genestein and 6-OH-flavone, respectively, are lower than the activity of vitamin E as determined by ORAC [84], but both structures lack a 3′4′-catechol, 3-OH, and multiple B-ring OH groups. The TEAC of quercetin (4.7) is almost twice that of (+)-catechin (2.4), illustrating the significance of both 2–3 unsaturation and a carbonyl at position 4 [39,102]. Apigenin (TEAC = 1.45) and naringenin (TEAC = 1.53) exhibit a much smaller difference in $ABTS^{\cdot+}$ scavenging [39], so 2,3-unsaturation may be less important than the 4-oxo itself. Although other structural elements must essentially be considered, free radical scavenging by flavonoids is variably enhanced by the presence of both elements.

4.4. Carbohydrate moieties

Aglycones are more potent antioxidants than their corresponding glycosides [89,94]. Daidzein and genestein aglycones exhibit greater TEAC values (1.25 and 2.9) than their 7-glycosides (1.15 and 1.24, respectively) [39]. Genistin is also inferior to its aglycone, genestein, in attenuating peroxynitrite-induced oxidation of LDL [96]. Plumb and coworkers [103] reported that the antioxidant properties of flavonol glycosides from tea decreased as the number of glycosidic moieties increased. Aside from mere presence and total number, the position and structure of the sugar play an important role. Ioku and colleagues [104] evaluated the effect of quercetin and several of its glycosides in a liposomal phospholipid suspension exposed to aqueous ROS, where inhibition of hydroperoxidation of methyl linoleate was measured to determine peroxyl radical scavenging activity. Luteolin and quercetin aglycones significantly exceeded their 3-,4′- and 7-*O*-glucosides in retarding the accumulation of hydroperoxides in membrane bilayers, but a 4′-sugar was more suppressive than 3- or 7-substitution. Since C-glycosylation in the A-ring also decreases activity [23], this negative effect may stem from the properties of the sugar itself, rather than displacement of a free OH. As in *O*-methylation, steric effects imparted by 4′-glycosylation exact a particularly suppressive influence through blocking the B-ring catechol.

In the diet, flavonoid glycosidic moieties occur most frequently at the 3- or 7- position, but an A-ring sugar results in a greater diminution of activity than 3-glycosyla-

tion in the heterocycle. *O*-glycosylation at carbon 7, but not carbon 3, weakens the antioxidant effect of flavonoids in rat mitochondria [23], but no difference between 3- and 7-glucosides of quercetin was detected against hydroperoxide in phospholipid bilayers [104]. It is plausible that the relative influence of 3- and 7-glycosylation is ruled by other structural considerations. It is also important to acknowledge that a glycosyl substituent, regardless of position and structure, seldom confers an antioxidant advantage over the aglycone. Like methylation, *O*-glycosylation interferes with the coplanarity of the B-ring with the rest of the flavonoid and the ability to delocalize electrons [92,98]. Though glycosides are usually weaker antioxidants than aglycones [87, 105], bioavailability is sometimes enhanced by a glucose moiety [52].

Rutinose is a unique case in that addition of this disaccharide to quercetin to form rutin does not consistently decrease antioxidant ability. Rutin is only marginally weaker than quercetin in attenuating Fe(II)-induced MDA formation in liposomes [93] and ascorbic acid-induced lipid peroxidation in rat mitochondria [89]. In addition, Mora and colleagues [23] reported only a minor difference in IC_{50} of ferrous sulfate-induced oxidative damage by rutin (19.5 uM) compared to quercetin (17.6 uM). However, the TEAC of quercetin is nearly twice that of rutin [102]. The reason why the suppressive effect of rutinose is less pronounced in the aforementioned experiments is unclear and may involve differences in methodology.

Whether the sugar moiety is glucose, rhamnose, or rutinose is also relevant. For example, compared to rutinose, a rhamnose moiety on quercetin significantly reduces scavenging of radicals generated by stimulated human neutrophils [106]. Aside from occupying free OH groups necessary for hydrogen abstraction and radical scavenging, any sugar substituent is capable of (i) diminishing coplanarity of the B-ring relative to the rest of the flavonoid, and/or (ii) lending hydrophilicity and altering access to lipid peroxyl and alkoxyl radicals during propagation of LPO in membranes. In light of the aforementioned evidence that glycosidic bonds are often cleaved at the gut level, the influence of sugar moieties on antioxidant properties is of questionable significance in humans. Based on cumulative evidence, removal of the glycosidic substituent by enteric enzymes or bacteria is likely to increase the activity of dietary flavonoids *in vivo.*

4.5. Degree of polymerization

Polymeric flavonoids present a complex extension of SAR that is poorly understood. Tannins comprise a substantial fraction of daily flavonoid intake among Western cultures in the form of black tea [34], red wine [3] and cocoa [37]. Grape seed extract, currently marketed as a dietary antioxidant supplement, consists primarily of oligomeric and dimeric catechins [35]. Due to the relative complexity and diversity of tannins, less is known regarding structure-activity relationships. Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion, but the activities of dimers and trimers differ little [107]. Tetramers exhibit greater activity against peroxynitrite- [99] and superoxide- [107] mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimers and tetramers [107]. It appears that to a point, increasing degree of polymerization enhances the effectiveness of procyanidins against a variety of radical species. Extensive conjugation between 3-OH and B-ring catechol groups, together with abundant $\beta_{4\rightarrow8}$ linkages, endow a polymer with significant radical scavenging properties by increasing the stability of its radical [108].

The clinical efficacy of dimeric, trimeric and oligomeric procyanidins is supported by controlled human trials. In a randomized, placebo-controlled crossover study, 20 human volunteers receiving a standardized diet were supplemented with 300 mg grape procyanidins daily over 4 days. By day 5, total serum antioxidant activity had increased by 13% [109]. Red wine, containing predominantly procyanidin dimers [3], has been reported to increase serum antioxidant activity [110] and may contribute to the French Paradox by reducing LDL oxidation and atheroma formation [111]. From this evidence, it is conceivable that flavonoid dimers play a unique, protective role in the human diet. Unlike larger procyanidins, dimers and trimers are more resistant to acid hydrolysis in the stomach [59] and may be absorbed intact without scission of the β-linkage [57]. Though it is rational to attribute the health effects of red wine to procyanidins, further research is necessary to elucidate SAR of other polyphenolic constituents of wine. A reproducible heirarchy of structure-activity relationships of procyanidins and other tannins has yet to materialize.

5. Flavonoid inhibition of RNS

Inhibition of RNS by flavonoids involves the same basic SAR as with inhibition of ROS. Flavonoids are known to directly scavenge peroxynitrite [86] and its progenitor, superoxide [91]. Haenen and colleagues [86] reported that the most significant determinant of activity against peroxynitrite is the 3′,4′-catechol arrangement, followed by an unsubstituted 3-OH. In this study, there was an apparent positive correlation between number of hydroxyl groups, particularly of the B-ring, and antiradical activity. *O*-Methylation and/or glycosylation [86,96] substantially reduces activity against RNS. Tetrameric procyanidins are more potent than epicatechin monomers, dimers, trimers, and other oligomers in inhibiting peroxynitrite-mediated oxidation and nitration *in vitro* [99]. The contribution of RNS inhibition to the cardioprotective influences of dietary flavonoids deserves further study.

6. Structural features of metal-chelating flavonoids

The chelating properties of flavonoids and tannins contribute to their antioxidant activity. By removing and neutralizing iron ions from iron-loaded hepatocytes, flavonoids inhibit oxidative damage [112]. Chelation of a divalent cation does not necessarily render the flavonoid inactive, as the complex retains ROS scavenging activity [93,113]. The clinical utility of this knowledge is promising in cases of oxidative stress associated with iron overload, which has been demonstrated in iron-overloaded rats [114]. In these animals, supplemental rutin resulted in significant reductions in peroxidation of liver microsomes and oxygen radical production by phagocytes, while minimal effects were found among animals with normal iron status.

Hydroxyl radicals are the most reactive and detrimental ROS in biological systems. Free ferrous iron is quite sensitive to oxygen and gives rise to ferric iron and superoxide, thereby generating hydrogen peroxide [115]. Reaction of ferrous iron with hydrogen peroxide generates the hydroxyl radical, which may subsequently oxidize surrounding biomolecules. In this process, known as the Fenton reaction, hydroxyl radical production is directly related to the concentration of copper or iron [116,117]. In pathological states involving iron overload or impaired sequestering of iron by transport or storage proteins, Fenton chemistry is an important generator of ROS *in vivo* [115]. Both quercetin and rutin are highly effective chelators of transition metals [118], suggesting little difference between aglycones and glycosides in the ability to complex metals. Fenton-induced oxidation is strongly inhibited by flavonoids with 3′,4′-catechol, 4-oxo, and 5-OH arrangements [119]. Chelating complexes with divalent cations may form between the 5-OH and 4-oxo group, or between the 3′- and 4′-OH [119]. By virtue of both metal-chelating properties and radical scavenging ability, polyhydroxylated flavonoids may offer considerable benefit as inhibitors of the Fenton reaction *in vivo.* That these polyphenols are often more effective inhibitors of metal-induced oxidation compared to non-metal-induced oxidation lends support to the role of metal chelation in flavonoid inhibition of free radical damage, which may be more significant than previously thought [93].

The well-established propensity of polyhydroxyflavonoids to complex redox-active metals poses a confounding factor in discerning relationships between structure and scavenging activity in lipid peroxidation assays. To rule out chelation, a nonmetal initiator such as CCl_4 may be used, or the iron contamination of flavonoids may be assessed [89]. Azo-radical-induced peroxidation restricts antioxidant behavior to peroxyl scavenging, and thereby eliminates chelation as a contributing mechanism [93]. From a physiological perspective, both chelation and free radical scavenging decrease oxidant status. However, given the high reducing power of tannins and the affinity of polyhydroxyflavonoids for iron, chronic pharmacological doses might exacerbate iron deficiency. Additional investigation is required to delineate SAR of chelating flavonoids and to evaluate adverse effects of flavonoids in human subjects with clinical iron deficiency.

7. Prooxidant activity

While the antioxidant properties of flavonoids support a positive role in human nutrition and disease prevention, some focus has involved the prooxidant activity of these compounds *in vitro.* Concentrated extracts of flavonoid-rich plants such as propolis, pine bark, green tea leaves, soy isoflavones and grape seed are widely marketed as nutraceuticals, targeting the aging population and individuals with cardiovascular disease, cancer and chronic inflammatory conditions. Thus, reports of mutagenicity related to flavonoid-mediated oxidative damage [120,121] raise obvious concerns.

Prooxidant activity is thought to be directly proportional to the total number of hydroxyl groups [84]. In a report by Hanasaki and colleagues [122], a 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 a Fenton system. The latter compounds included myricetin and baicelein, both of which have a pyrogallol structure in the A-ring, which has also been reported to promote hydrogen peroxide production [123] from which Fenton reaction may generate highly reactive hydroxyl radicals [115]. This prooxidant effect is responsible for the cytotoxic and proapoptotic effects of flavonoids isolated from various herbal medicines [124,125]. In the presence of RNS, flavonoids with A- or B- ring pyrogallol configurations induce DNA single-strand breakage [126]. There is also evidence that the unsaturated 2,3-bond and 4-oxo arrangement of flavones may promote the formation of ROS induced by divalent copper in the presence of oxygen [84]. Collectively, this information suggests that some of the same structural attributes that optimize antioxidant capacity may also exacerbate oxidative stress and damage to functional and structural cellular molecules. However, considering the role of flavonoid radical stability in prooxidant behavior postulated by Bors and colleagues [127], structural advantages to radical stability that increase antioxidant activity, such as a 3′4′-catechol, 3-OH, and conjugation between the A- and B-rings, may modulate adverse oxidative effects of flavonoids.

Glycosylation and methylation of OH groups attenuate the prooxidant behavior of flavonoids [84]. Adverse oxidative effects of flavonoids are mitigated *in vivo* by catechol-*O*-methyltransferase (COMT) [70] and other hepatic methyltransferases, but evidence that flavonoids directly inhibit COMT via ligand binding [128] complicates this hypothesis. Divalent iron or copper cations increase production of free radicals in a concentration-dependent manner [116, 117], but data generated *in vitro* using divalent copper are

less representative of the physiological environment in which copper is relatively unavailable. While the presence of iron may accelerate prooxidant effects *in vivo,* chelation of iron by the flavonoid may theoretically modify this process. In addition, high ascorbate concentrations attenuate generation of ROS by flavonoids *in vitro* [89], and it is postulated that vitamin C status modulates the prooxidant tendency of these compounds *in vivo.* Further work is necessary to delineate the contribution of chelation and the relevance of iron and ascorbate status on oxidative stress imposed by flavonoids *in vivo.* Although the tendency of flavonoids with multiple hydroxyl groups to promote cellular injury by ROS contraindicates the use of quercetin and similar polyphenols in pharmacological doses, metabolic alterations of structure may attenuate the reactivity of quercetin and similar compounds *in vivo.*

8. Summary and conclusions

Over a decade of SAR research has afforded a basic understanding of flavonoid pharmacokinetics, absorption and metabolism in relation to their chemical structure. Evidence that flavonoid metabolites may be more or less active than the native compound has significant implications in the preventive and therapeutic utility of these compounds. Although existing knowledge of absorption and metabolism is confined to a select group of dietary flavonoids, sufficient evidence supports that most of these compounds are absorbed sufficiently to exact a marked decrease in various parameters of plasma oxidant status.

The structural heterogeneity of flavonoids, their multiple mechanisms of action, and the diverse experimental methods used to evaluate their antioxidant activity pose challenges in assembling a collective hierarchy of SAR. Appropriate application of SAR to human nutrition requires systematic comparisons of homologs that differ in a single structural attribute. Although SAR of flavonoid metabolites warrants further research, modifications that ensue during metabolism are known to include hydroxylation, *O*-methylation, cleavage of the heterocycle, deglycosylation, and scission of polymeric species into monomeric units. Structure-activity relationships among naturally occurring flavonoids thereby offer preliminary insight into the impact of these metabolic alterations on various mechanisms of antioxidant activity.

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