REVIEW PAPER

A Review of the Antioxidant Mechanisms of Polyphenol Compounds Related to Iron Binding

Nathan R. Perron · Julia L. Brumaghim

Abstract In this review, primary attention is given to the antioxidant (and prooxidant) activity of polyphenols arising from their interactions with iron both in vitro and in vivo. In addition, an overview of oxidative stress and the Fenton reaction is provided, as well as a discussion of the chemistry of iron binding by catecholate, gallate, and semiquinone ligands along with their stability constants, UV–vis spectra, stoichiometries in solution as a function of pH, rates of iron oxidation by O_2 upon polyphenol binding, and the published crystal structures for iron–polyphenol complexes. Radical scavenging mechanisms of polyphenols unrelated to iron binding, their interactions with copper, and the prooxidant activity of iron–polyphenol complexes are briefly discussed.

Abbreviations

Aβ	Amyloid-beta
EC	(–)-Epicatechin
ECG	(–)-Epicatechin-3-gallate
EGC	(–)-Epigallocatechin
EGCG	(–)-Epigallocatechin-3-gallate
GA	Gallic acid
Q	Quercetin
MEGA	Gallic acid methyl ester
MEPCA	Protocatechuic acid methyl ester
PCA	Protocatechuic acid
PrEGA	Gallic acid propyl ester
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rut	Rutin
TA	Tannic acid
VA	Vanillic acid
EPR	Electron paramagnetic resonance
TEAC	Trolox-equivalent antioxidant activity
ORAC	Oxygen radical absorbance capacity
DPPH	2,2-Diphenylpicrylhydrazyl

N. R. Perron · J. L. Brumaghim (✉)
Department of Chemistry, Clemson University, 481 Hunter
Laboratories, P.O. Box 340973, Clemson, SC 29634-0973, USA
e-mail: brumagh@clemson.edu

Polyphenol Compounds: Sources, Structures, and General Biological Activities

Polyphenol compounds are widely studied for their antioxidant properties, although the term antioxidant has a broad range of meanings. For the purposes of this review, antioxidant activity refers to both the ability of polyphenol compounds to prevent damage from reactive oxygen species (ROS) (such as through radical scavenging) or to prevent generation of these species (by binding iron). As described in the title, the primary focus will be on polyphenol–iron interactions as a mechanism of antioxidant activity. The typical structural characteristic shared by most polyphenols is the three-membered flavan ring system (Fig. 1), yet the combinatorial library of polyphenol compounds is widely diversified, collectively encompassing many thousands of different compounds [1], which are divided into several sub-classes, such as the catechins,

flavonols, flavanols, flavones, anthocyanins, proanthocyanidins, and phenolic acids, just to name a few (Fig. 1). Polyphenols are found in green [2, 3] and black teas [4, 5], coffee [6], fruits [7, 8], fruit juices [9–11], vegetables [12, 13], olive oil [14, 15], red and white wines [16, 17], and chocolate [18], and are found in medium to high milligram quantities per serving for all of these foods (Figs. 2, 3), although measurements of the precise concentrations of polyphenol compounds in each type of food often vary [7, 11, 12, 17–22]. Thus, people with diets rich in fruits and vegetables may consume one or more grams per day of these compounds, based on the recommendation of 5 servings/day of colorful fruits and vegetables by the Centers for Disease Control and Prevention [23]. Because polyphenols are such a large and integral part of the human diet, it is highly desirable to understand their biological functions and modes of activity.

Green tea leaf is particularly abundant in the group of polyphenols collectively referred to as catechins (Fig. 1), which constitute up to 30% of the plant's dry leaf weight [24]. Within just 2 h after consumption of one cup of green or black tea (350–600 ml) [25–28], catechins have been found in concentrations of 0.3–1 μM in human plasma and may even approach 10 μM with higher doses [29]. Flavonols (Fig. 1) such as quercetin are reportedly less bioavailable than catechins; however, they may reach similar plasma concentrations (high nanomolar to low micromolar) in people who eat large amounts of fruits and vegetables or intentionally supplement their diets with polyphenols [30, 31].

While polyphenols are primarily recognized for their antioxidant functions, they also have many other biological activities, such as anti-histamine [32], anti-inflammatory [33], antibacterial [34], and antiviral activities [35]. Cardiovascular effects such as vasodilation have been observed in tea drinkers [36, 37], and this property has been attributed to the ability of polyphenols to increase endothelial nitric oxide synthase (eNOS) activity by over 400% [36]. They have also been shown to bind many different proteins such as caseins [36], and inhibit telomerase [38], α-amylase, pepsin, trypsin, and lipase [39], among many other enzymes. Furthermore, polyphenols are implicated in the prevention of neurodegenerative diseases [2, 40] and cancer [41]. They also induce apoptosis in cancer cells, implicating them in cancer senescence as well [42–46].

Because a full discussion of the biological activity of polyphenols would be prohibitively long, this review will focus on the antioxidant mechanisms of polyphenols specifically related to iron-binding, with brief mention made of other mechanisms such as radical scavenging, prooxidant activity, and interactions between polyphenols and copper.

Oxidative Stress, DNA Damage, and the Fenton Reaction

Reactive oxygen species and reactive nitrogen species (RNS), such as hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^-$), and others, are major sources of oxidative stress in cells, damaging proteins, lipids, and DNA [47]. Oxidative DNA damage has been implicated as a cause of cancer [48, 49], aging and neurodegenerative diseases such as Alzheimer's and Parkinson's [50–54], cardiovascular diseases such as arteriosclerosis [55, 56], and is the primary cause of cell death and tissue damage resulting from heart attack and stroke [57, 58]. Therefore, prevention of oxidative stress caused by ROS and RNS has important implications for the prevention and treatment of disease.

Fig. 1 Flavan general structure, showing the ring labeling and numbering system. Structures of catechol, gallol, and general structures of catechins, flavonols, flavones, and anthocyanins. R groups are typically H, OH, OCH₃, galloyl esters, or carbohydrate groups, depending on the compound
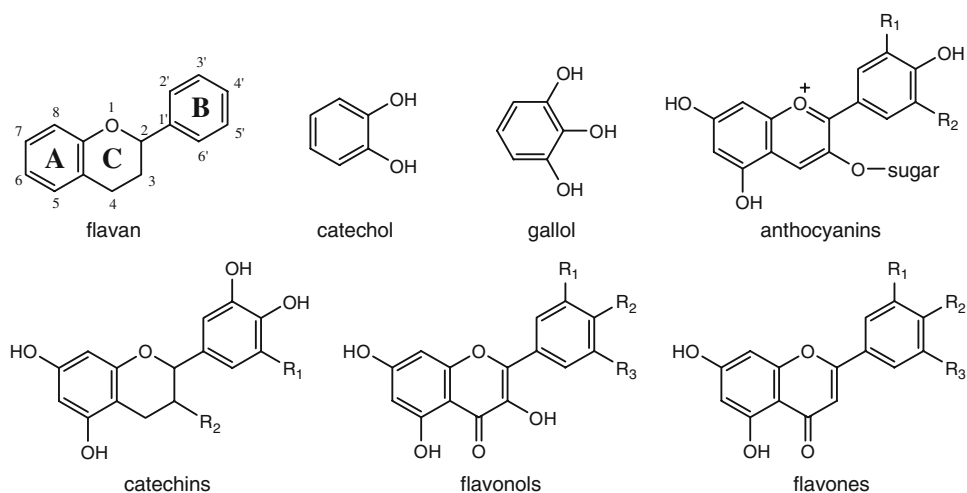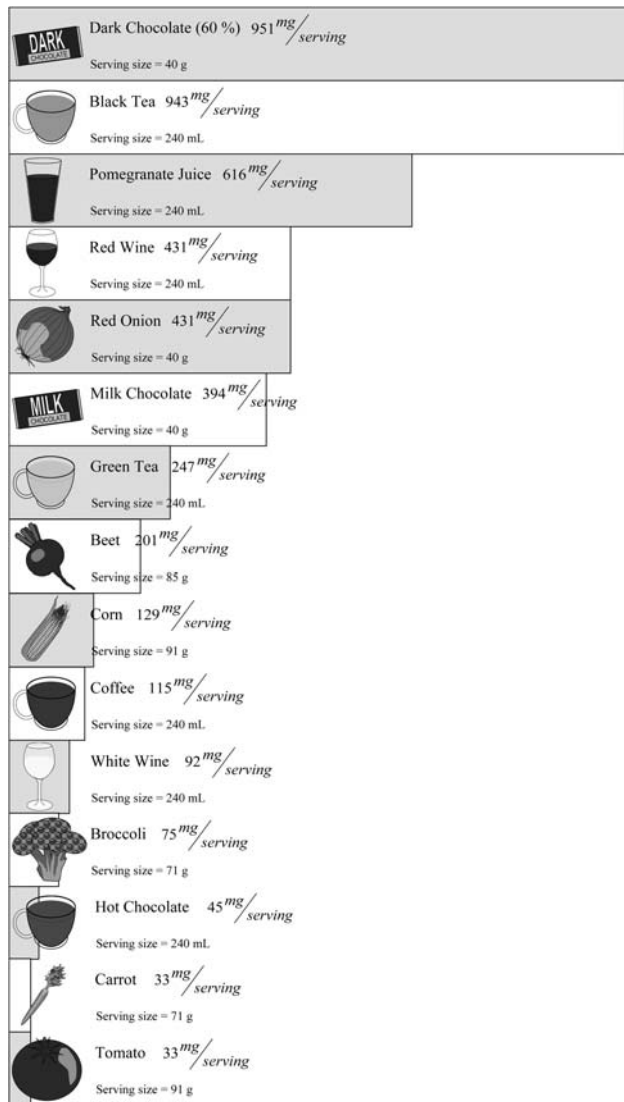
Fig. 2 A chart showing the phenolic content of selected beverages, vegetables, and chocolate in milligrams per serving. Serving size is based on a typical beverage size (240 ml), piece of chocolate (40 g), or serving of vegetables. Values are taken or calculated from data in the references [5, 6, 11, 12, 17, 18]. Reported polyphenol content varies

Fig. 3 A chart showing the phenolic content of selected fruits in milligrams per serving. Serving size is based on a typical serving of fruit. Data from [7]. Reported polyphenol content varies

Radical Scavenging Pathways of Polyphenol Antioxidant Activity

Many mechanisms have been proposed for polyphenol prevention of oxidative stress and ROS/RNS generation both in vitro and in vivo. Radical scavenging by polyphenols is the most widely published mechanism for their antioxidant activity, with over 700 papers since 1995 alone [59]. In this radical scavenging mechanism, polyphenols sacrificially reduce ROS/RNS, such as $^{\bullet}OH$, $O_2^{\bullet -}$, $NO^{\bullet}$, or $OONO^-$ after generation, preventing damage to biomolecules or formation of more reactive ROS [15, 60–68]. Several 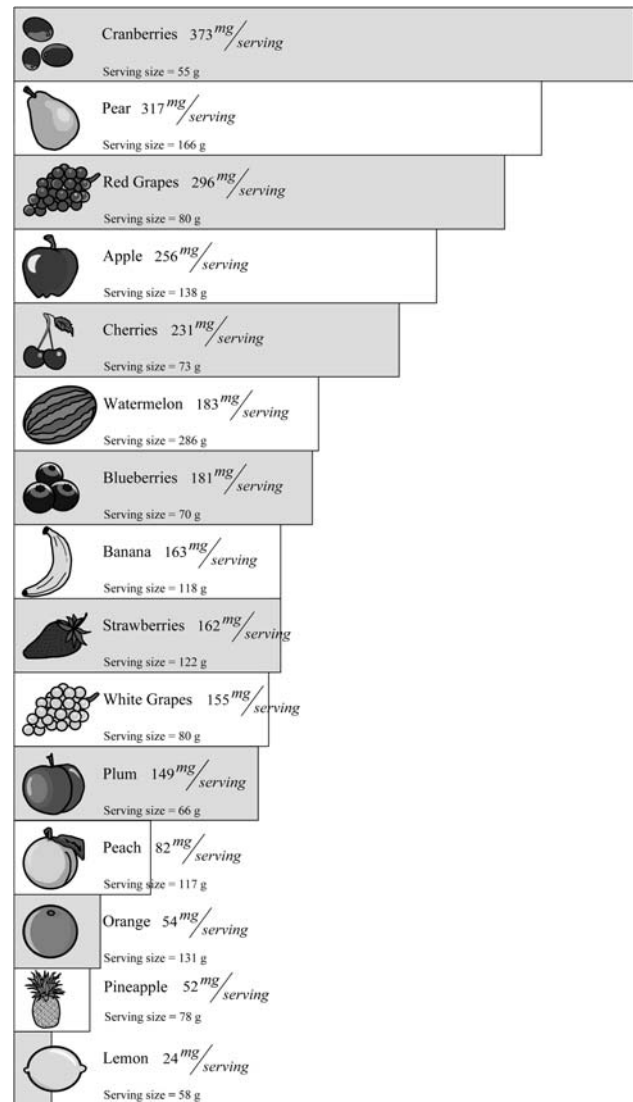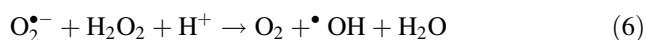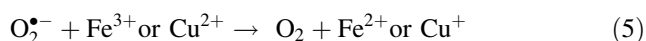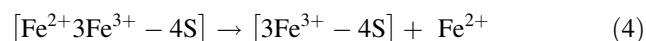assays, such as the trolox-equivalent antioxidant activity (TEAC) and oxygen radical absorbance capacity (ORAC) assays as well as 2,2-diphenylpicrylhydrazyl (DPPH) scavenging, are commonly used to study the radical-scavenging ability of polyphenols [69–72] These assays provide a relative measure of antioxidant activity, but often the radicals scavenged have little relevance to those present in biological systems. In addition, radical scavenging assays do not account for the iron-binding properties of polyphenol antioxidants.

It is clear that polyphenols have many different biological activities; among them are enzyme regulation and antioxidant behavior. Radical scavenging is a probable mechanism for reduction of oxidative stress by these compounds, but as it does not involve iron binding, it is therefore outside the scope of this review.

The Role of Iron in ROS Generation

Hydroxyl radical, the most reactive ROS known, abstracts a hydrogen atom from biological substrates at diffusion-limited rates [67]. Multiple pathways generate $^\bullet$OH, including the decomposition of peroxynitrous acid [73], or the metal-mediated reduction of peroxides. Formation of biological peroxides, such as H_2O_2, is a process that occurs naturally during cellular respiration [74], and cell signaling mechanisms often involve ROS or RNS such as H_2O_2, $O_2^{\bullet-}$, and $NO^\bullet$. These species can also form more potent oxidants if not closely regulated, leading to cellular damage and oxidative stress [75–80]. For example, $O_2^{\bullet-}$ and $NO^\bullet$ can react to form $OONO^-$ [81, 82], which can then decompose into $^\bullet$OH [73]. H_2O_2 is commonly reduced in vivo by either Fe^{2+} or Cu^+, resulting in the formation of $^\bullet$OH via Fenton-type reactions (reaction 1). Superoxide forms H_2O_2 upon protonation in aqueous solution (reaction 2) [83]. DNA damage is observed directly from $^\bullet$OH [84–86], and indirectly from $O_2^{\bullet-}$ oxidation of [4Fe–4S] iron–sulfur clusters (reaction 3) to form H_2O_2 [87]. In addition to H_2O_2 formation, $O_2^{\bullet-}$ also releases Fe^{2+} from enzymes, such as ferritin [88] and the [4Fe–4S]-containing dehydratases by reducing Fe^{3+}, generating an unstable iron–sulfur cluster, and releasing Fe^{2+} (reaction 4) [83, 87, 89]. Superoxide can also reduce aqueous Fe^{3+} or Cu^{2+} (reaction 5), making these metal ions available to react with H_2O_2, although the rate of iron reduction is slow (10 h is the proposed half-time for this reaction in vivo), and it is generally assumed that more abundant cellular reductants such as NADH commonly reduce cellular Fe^{3+} (Fig. 4) [67]. The Haber–Weiss reaction was once thought to be a source of cellular $^\bullet$OH (reaction 6) [90, 91], but it has since been determined that this reaction does not occur in vivo [91, 92].

$$Fe^{2+} \text{or } Cu^+ + H_2O_2 + H^+ \rightarrow Fe^{3+} \text{or } Cu^{2+} +^\bullet OH + H_2O \tag{1}$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{2}$$

$$\left[2Fe^{2+}2Fe^{3+} - 4S\right] + O_2^{\bullet-} + 2H^+ \rightarrow \left[Fe^{2+}3Fe^{3+} - 4S\right] + H_2O_2 \tag{3}$$

$$\left[Fe^{2+}3Fe^{3+} - 4S\right] \rightarrow \left[3Fe^{3+} - 4S\right] + Fe^{2+} \tag{4}$$

$$O_2^{\bullet-} + Fe^{3+} \text{or } Cu^{2+} \rightarrow O_2 + Fe^{2+} \text{or } Cu^+ \tag{5}$$

$$O_2^{\bullet-} + H_2O_2 + H^+ \rightarrow O_2 +^\bullet OH + H_2O \tag{6}$$
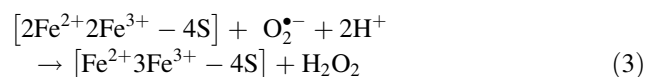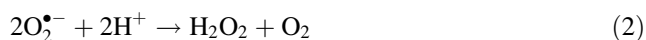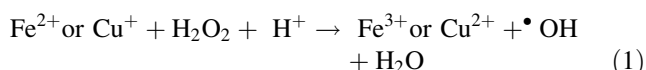
Radical-induced damage to DNA occurs at both the phosphate backbone (strand breakage) and nucleotide bases, and both of these sites of damage are widely used to quantitatively determine the extent of oxidative DNA damage [93–101]. Because the DNA backbone is composed of negatively charged phosphate groups, as well as electron-rich nucleotide bases, metal ions such as Na^+,

Mg^{2+}, Fe^{2+}, Fe^{3+}, Cu^+, Cu^{2+}, etc. localize via electrostatic interaction near the phosphate backbone and transition metal ions such as iron and copper can covalently bind to the nucleotide bases of DNA. Metal ion localization stabilizes DNA by balancing the charge of the oxygen atoms of the phosphate backbone [84] or coordination to electron pairs donated by nitrogen atoms of the bases, particularly at guanine-rich sequences [102–104].

When H_2O_2 is also present as a result of oxidative stress, redox active metal ions such as Fe^{2+} or Cu^+ that are localized or bound to the DNA react with H_2O_2 to form highly reactive $^\bullet$OH in immediate proximity to DNA. Hydroxyl radical then abstracts the $4'$ hydrogen atom from the deoxyribose sugar backbone, leaving a DNA radical adduct that rearranges, ultimately cleaving the phosphodiester backbone and resulting in strand scission [84, 85, 102, 104]. Alternatively, $^\bullet$OH may damage the nucleotide bases themselves, resulting in oxidized base products such as 8-oxo-guanine and fragmented or ring-opened derivatives [105–107]. DNA damage of both types (strand breakage or base damage) can ultimately result in genetic mutations, cancer, or cell death [105].

Both Imlay et al. and Mello-Filho et al. have determined that iron-mediated oxidative DNA damage by $^\bullet$OH is the primary cause of cell death under oxidative stress conditions for both prokaryotes [108], and eukaryotes (including humans) [109, 110]. Iron-mediated DNA damage is primarily thought to originate from solvated iron that is not bound to proteins (such as hemoglobin, transferrin, or ferritin in eukaryotes [111], or the ferritin-like *dpr* protein and ferric uptake regulatory (*fur*) protein of prokaryotes) [112], which would otherwise prevent the iron from participating in the Fenton reaction. In *Escherichia coli*, the concentration of non-protein-bound iron is 10–30 μM [113], and it is believed to be coordinated to low molecular weight intracellular ligands such as ascorbate or citrate [114, 115]. However, if iron homeostasis is not maintained, the intracellular concentration of non-protein-bound iron may increase to between 80 and 320 μM [88, 113], causing a much greater susceptibility to oxidative DNA damage [88, 116]. Whole-cell electron paramagnetic resonance (EPR) indicates that most of this non-protein-bound iron in *E. coli* exists as Fenton-active Fe^{2+} [113]. Oxidative stress also causes release of iron from proteins (reactions 3 and 4), resulting in increased non-protein-bound iron concentrations [67, 88, 89, 117]. An increase in intracellular non-protein-bound iron concentration is associated with oxidative stress in humans and is also implicated in both Alzheimer's and Parkinson's diseases [118, 119], and cardiovascular disease [120]. In addition, even slightly elevated iron levels have been linked to increased cancer incidence in humans [48].

Fig. 4 Reduction of Fe^{3+} by cellular reductants (NADH shown) recycles iron for reaction with H_2O_2 in the Fenton reaction, leading to DNA damage
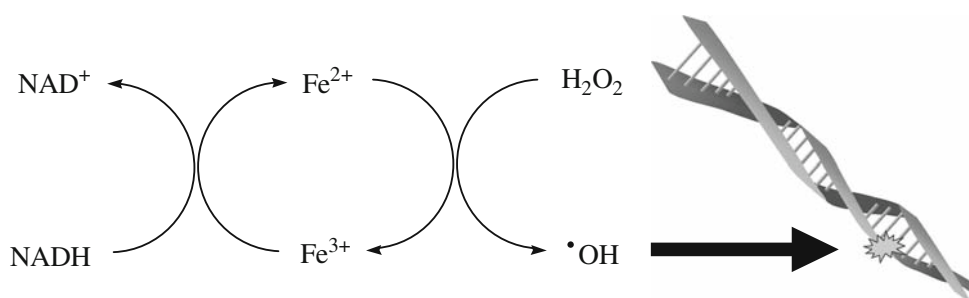
Damage to both nuclear and mitochondrial DNA occurs in cancer and other diseases linked to iron mis-regulation [121, 122]. Although nuclear DNA is packaged with histone proteins in chromatin, several studies have shown that oxidative damage to nuclear DNA occurs even in the presence of histone proteins; in fact, histone proteins can increase metal-mediated oxidative DNA damage because redox-active metal ions are associated with these proteins [123–126]. Mitochondrial DNA is particularly at risk for oxidative damage due to its proximity to respiratory processes that produce $O_2^{\bullet-}$, H_2O_2, and other ROS [127–129]. In fact, oxidative damage to mitochondrial DNA may actually be a more relevant cause of cell death than nuclear DNA damage because of this higher risk of damage [130, 131].

Because iron is a primary cause of ROS generation in vivo and because it plays such a pivotal role in contributing to oxidative stress, DNA damage, and cell death, iron has been the target of many antioxidant therapies. Due to their ability to coordinate iron, polyphenols are one large class of antioxidants that has been extensively examined for treatment and prevention of conditions associated with iron-generated ROS and oxidative stress.

Iron Binding by Catecholate, Gallate, and Semiquinone Ligands

Stability Constants for Iron–Polyphenol Complexes

It is well known that catechol and gallol (Fig. 1) and the many functionalized derivatives thereof (including most polyphenol compounds) are effective metal chelators. When deprotonated, as is required for metal binding, catechol and gallol functionalities are referred to as catecholate and gallate groups, respectively. Metal ions that prefer octahedral geometry, such as Fe^{2+} and Fe^{3+}, can coordinate up to three catecholate or gallate groups (Fig. 5). Because of this, it might be expected that polyphenols with catechol or gallol groups would always bind iron in a 3:1 fashion (Fig. 5). However, since polyphenol compounds are so structurally varied and the complexes

formed are pH dependent, they often exhibit variable coordination modes. Despite pK_a values in the range of 7–9 for the most acidic phenolic hydrogen, polyphenols are easily deprotonated at or below physiological pH in the presence of iron and form very stable complexes [132]. Because iron binding has been proposed as a mechanism for polyphenol antioxidant activity, stability constants for polyphenol–iron interactions provide insight into their antioxidant behavior. For the purposes of this review, "stability constant" is defined as the equilibrium constant, K, of one or more polyphenols binding to an iron ion in aqueous solution. Multiple equilibrium constants (K_1, K_2, and K_3) arise when several polyphenol ligands are bound to one iron center. The product of the individual equilibrium constants is referred to as the overall stability constant of the complex, β.

Deprotonated polyphenol ligands behave as hard Lewis bases, and give rise to particularly large metal-binding stability constants with hard Lewis acids, such as Fe^{3+} [133]. In particular, catecholate complexes of Fe^{3+} often have extremely large stability constants (log $\beta \sim 40$–49) when three catecholate groups are bound to one iron center [134, 135]. Despite the importance of iron binding by polyphenols, only a handful of stability constants are reported for catecholate and gallate complexes of Fe^{3+}, other than siderophore complexes (both naturally occurring and synthetic). Fe^{2+}, in contrast to Fe^{3+}, is a borderline Lewis acid, and does not bind as strongly to the hard oxygen atoms of polyphenol ligands. The stability constant for Fe^{2+} monocatecholate is 7.95, much lower than the
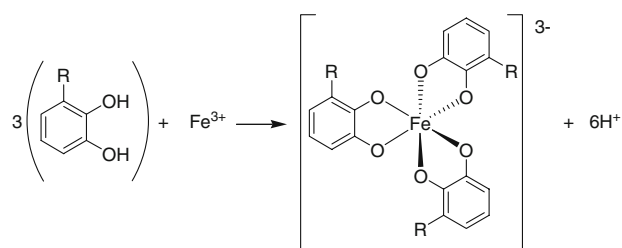

Fig. 5 Expected octahedral coordination geometry of general iron–polyphenol complexes. Gallols, R=OH; catechols, R=H. Coordination requires deprotonation of the polyphenol ligands

Fe^{3+} monocatecholate complex (20.01) [136]. In addition to catechol [136, 137], stability constants for the Fe^{2+} complexes of quercetin and 1,2-dihydroxynaphthalene-4-sulfonate have been reported [138, 139], as well as Fe^{2+} complexes of gallic acid and *n*-propyl gallate (Fig. 6) [137, 140], with limited data for the latter two compounds (Table 1). The scarcity of Fe^{2+}-polyphenol stability constants is most likely due to the fact that performing these measurements requires oxygen-free conditions to prevent oxidation of Fe^{2+} to Fe^{3+}. Log K and β values for catecholate and gallate complexes with iron are given in Table 1, and structures for selected ligands are shown in Fig. 6.

Because polyphenol ligands strongly stabilize Fe^{3+} over Fe^{2+}, catecholate and gallate complexes of Fe^{2+} rapidly oxidize in the presence of O_2 to give Fe^{3+}-polyphenol complexes, a process commonly referred to as autooxidation (Fig. 7a) [141–145]. Typically, Fe^{2+} oxidation occurs slowly in the presence of O_2, but binding of polyphenol ligands to Fe^{2+} lowers the reduction potential of iron [146] and enhances the rate of iron oxidation [137, 143, 147].

This iron oxidation rate varies for polyphenol complexes, with gallate complexes having faster oxidation rates than catecholate complexes [148]. This oxidation of Fe^{2+} to Fe^{3+} upon binding to polyphenol ligands is facilitated by the greater stability of the harder Fe^{3+} metal ion interactions with the hard oxygen ligands of the polyphenol moieties as well as the strongly electron-donating properties of the oxygen ligands that stabilize the higher iron oxidation state. Fe^{2+} autooxidation is not unique to polyphenol ligands; however, this phenomenon has been noted in the presence of various anions such as hydroxide [149], pyrophosphate and phosphate [150], chloride [151], sulfate [152], and perchlorate [153], with the rate of Fe^{2+} autooxidation dependent on the counterion of the Fe^{2+} salt.

Reduction of Fe^{3+} by Polyphenol Ligands

Upon binding of a catecholate or gallate ligand to Fe^{3+}, the polyphenol can reduce the iron to Fe^{2+}. The polyphenol is oxidized to a semiquinone during this process (Fig. 7b) [132, 154–158]. At low pH, the semiquinone ligand is

Fig. 6 Structures of polyphenol compounds for which iron-binding stability constants have been measured
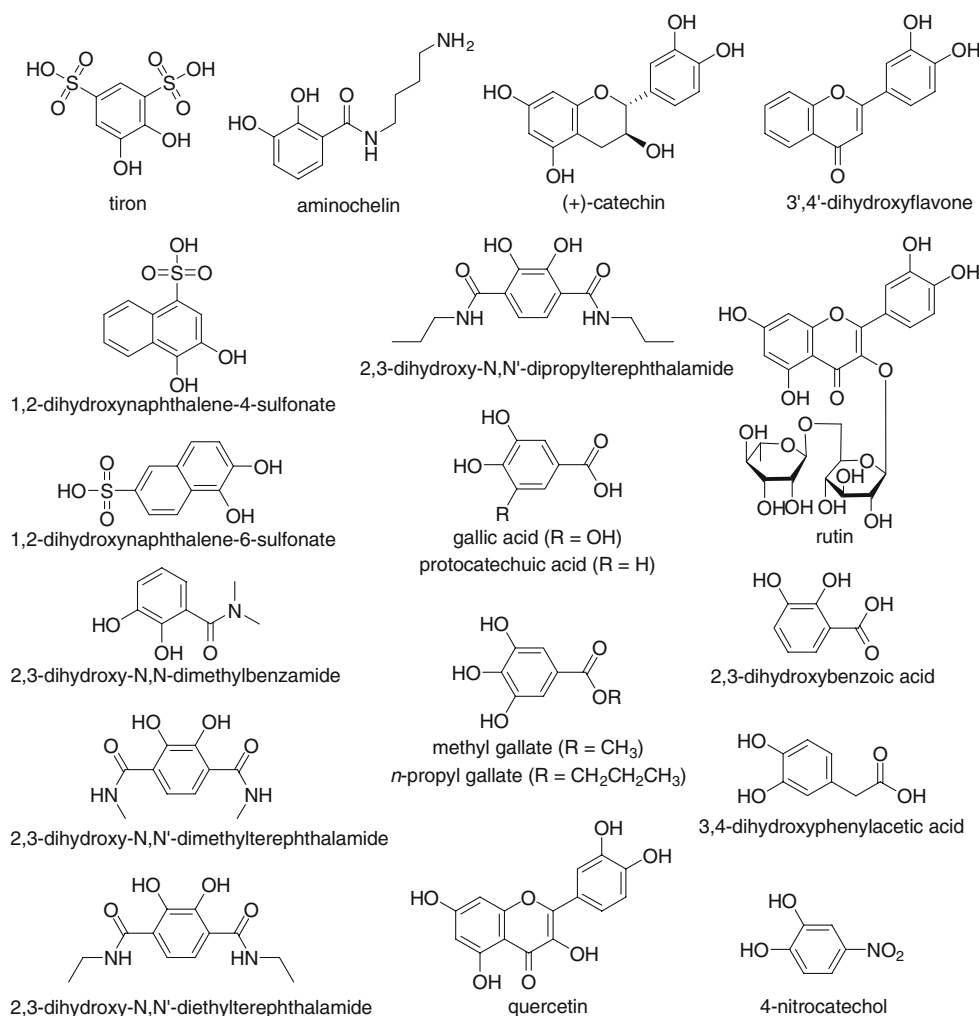
Table 1 Reported stability constants for catechol and gallol polyphenol ligands with both Fe^{2+} and Fe^{3+}

| Compound	Fe^{n+}, n =	Log K_1	Log K_2	Log K_3	Log β	Reference
1,2-Dihydroxynaphthalene-4-sulfonate	2	8.99	7.5		16.49	[139]
	3	19.84	15.11		34.95	[139]
1,2-Dihydroxynaphthalene-6-sulfonate	3	19.88	14.47	9.87	44.22	[297]
2,3-Dihydroxybenzoic acid	3	7.7[a]	4.0[a]	24.0[a]	35.7[a]	[135]
2,3-Dihydroxy-*N,N′*-diethylterephthalamide	3	16.22	14.32	11.48	42.02	[298]
2,3-Dihydroxy-*N,N*-dimethylbenzamide	3	17.8	13.9	8.5	40.2	[298]
	3				39.8	[299]
2,3-Dihydroxy-*N,N′*-dimethylterephthalamide	3	16.37	14.40	10.91	41.68	[298]
2,3-Dihydroxy-*N,N′*-dipropylterephthalamide	3	16.0	15.17	11.97	43.14	[298]
3,4-Dihydroxyphenylacetic acid	3	14.85[a]	8.9[a]		23.75[a]	[135]
	3				43.9	[299]
3′,4′-Dihydroxyflavone	3	20.87				[300]
4-Nitrocatechol	3	18.29[a]	14.17[a]	9.2[a]	41.66[a]	[135]
Aminochelin	3	19.06	11.71	10.54	41.31	[132]
(+)-Catechin	3	21.8	15.7	9.9	47.4	[301]
Catechol	2	7.95	5.55		13.5	[136]
	2	8.4				[137]
	3	20.01	14.69	9.06	43.76	[135]
3,3,4-CYCAM	3				40	[299]
Enterobactin	3				49	[134]
Gallic acid	2	7.0				[137]
	3	14	8.5	5	27.5	[180]
	3	11.15	8.53	4.42	24.09	[302]
LYSCAM	3				46	[303]
MECAM	3				46	[299]
Methyl gallate	3	12.4	11.7	9.93	34.03	[302]
n-Propyl gallate	2	9.1				[140]
Protocatechuic acid	3	19.50	14.32	8.70	42.52	[185]
Protochelin	3				44.6	[132]
Quercetin	2	9.44	3.86		13.3	[138]
	3				44.2	[187]
Rutin	3				44.1	[187]
Tiron	3	20.07[a]	15.01[a]	9.8[a]	44.88[a]	[135]
	3	20.7	15.2	11	46.9	[297]
	3	20.4	15	10.4	45.8	[136]
TRENCAM	3				43.6	[304]
TRENCAMS	3				43.6	[305]

[a] Listed values here are the averages of either two or three trials reported separately in the referenced publication

protonated and is therefore a neutral ligand [132]. According to Basolo and Pearson [159], Fe^{2+} is stabilized relative to Fe^{3+} by neutral unsaturated ligands due to the greater crystal field stabilization of a d^6 electronic configuration (Fe^{2+}) than that of a d^5 system (Fe^{3+}). These Fe^{2+}-semiquinone complexes are green in color, likely due to stabilization of the semiquinone radical by the aromatic ring, and may often be mistaken for mono(polyphenol) Fe^{3+} complexes at low pH. However, the presence of Fe^{2+} in these semiquinone complexes has been confirmed by both Mössbauer spectroscopy [160] and magnetic moment measurements [161].

Once the semiquinone form of the polyphenol is generated, it is capable of reducing another equivalent of Fe^{3+}, simultaneously oxidizing the semiquinone to the quinone (Fig. 7b) [132, 154–158]. The studies investigating this Fe^{3+} reduction behavior are performed at very low pH [1–3], which may be relevant for processes occurring during digestion in the stomach. At higher pH, the formation of bis- and tris-polyphenol complexes with iron
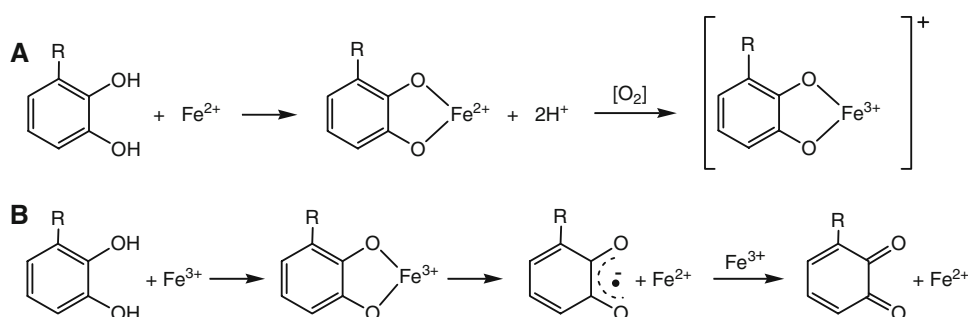
(two and three polyphenol ligands coordinated to a single iron, respectively) inhibit these Fe^{3+} reduction processes [155], so such reactions may occur much more slowly around pH 7. Nonetheless, this process of iron reduction is often attributed to both antioxidant and prooxidant activity of these compounds [154, 162]. Reduction of Fe^{3+} generates Fe^{2+} that can participate in the Fenton reaction and cause ROS generation [163–166]. This topic as it relates to DNA or cellular damage is discussed more in depth in the section on prooxidant activity of polyphenols.

Crystal Structures of Iron–Polyphenol Complexes

A number of X-ray structures of Fe^{3+}-polyphenol complexes have been published, with the majority of these synthesized as structural and functional models of catecholate dehydrogenase enzymes (summarized by Yamahara et al. in an excellent review) [167]. Because of their similarity to the enzyme active site, most of these synthetic structures contain catecholate ligands. Very few structures of gallate complexes with iron have been reported [168, 169], possibly due to their ability to form complexes with varying stoichiometry. Nuclearity of iron–polyphenol complexes with catecholate or gallate ligands ranges from mononuclear [170–174], to dinuclear [175] and supramolecular clusters [176], to extended polymeric structures [168, 169].

Stoichiometry and UV–Vis Spectroscopy of Iron–Polyphenol Complexes

Determining the binding interactions between polyphenol compounds and iron is vital to understanding their behavior. Generally, around physiological pH (7.2), a mixture of both the 2:1 and 3:1 ligand to metal species exist in solution, depending on the polyphenol compound and its stability constant with the metal [177]. It should be noted that changes in the ratio of metal to polyphenol as well as the pH can significantly change the coordinated species in solution. At slightly acidic pH (5–6.5), iron is bound by two catecholate or two–three gallate ligands per metal ion [138, 178–180], giving blue-purple Fe^{3+} complexes with

molar extinction coefficients on the order of 10^3 for their ligand-to-metal charge transfer (LMCT) bands [137, 179, 181, 182], and λ_{max} values ranging from 542 to 561 nm for gallates, or 561 to 586 nm for catecholates (Fig. 8) [181]. At more acidic pH (<4), polyphenols bind iron in a 1:1 ratio [140, 183, 184]. Fe^{3+} monocatecholate complexes are often blue-green, with λ_{max} values ~ 670 nm [147, 185]. At alkaline pH, the octahedral tris(polyphenol) complexes of Fe^{3+} predominate (Fig. 5), which are red in color [147]. Fe^{2+} complexes of polyphenol ligands are colorless in the absence of oxygen [137, 177, 181].

Flavonols, such as quercetin (Q) and myricetin (Myr), display unique absorbances in the UV–vis spectra because these compounds are colored due to their extended conjugation. In addition, flavonols possess a second iron-binding site between the carbonyl oxygen at the 4-position and either the 3-OH or 5-OH groups as well as the catechol or gallol moiety. At pH 6.0, iron complexes of Q have absorbance maxima at 407 and 548 nm, and iron complexes of Myr have absorbances at 443 and 589 nm [181].

Stoichiometry of polyphenol ligands to iron often varies in solution, as measured by Job's method. For example, quercetin (Q) has been shown by de Souza et al. [186] to
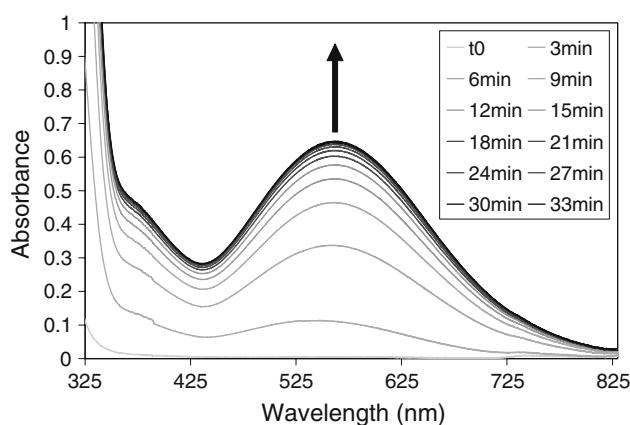

Fig. 8 Autooxidation of Fe^{2+} (145 μM) by O_2 upon binding of methyl-3,4-dihydroxybenzoate (MEPCA, 435 μM) as measured over time by UV–vis spectroscopy. Spectra were taken every 3 min after addition of Fe^{2+}. Sample was prepared in MES buffer (50 mM, pH 6.0)

bind iron in a 1:2 fashion in methanol, with a formula of $[Fe_2(Q)(H_2O)_8]Cl_2$ when synthesized from Fe^{2+} chloride tetrahydrate. However, rutin (Rut; quercetin-3-rutinoside) binds iron in a 2:3 ratio in methanol, with the formula $[Fe_3(Rut)_2(H_2O)_{12}]Cl_2$. Stoichiometry of products were confirmed by elemental analysis and ^{1}H NMR spectroscopy, with the Q complex showing iron bound not only at the catechol oxygens in the B ring, but also between the 3-OH and the 4-carbonyl oxygen of the C ring. ^{1}H NMR spectra of the rutin complex indicated iron ions bound to the two catechol groups of the B rings and an additional iron, likely bound at the 7-OH groups of ring A between two Rut molecules (Fig. 9) [186].

In contrast to the results reported by de Souza et al., Escandar and Sala reported only 1:1 and 2:1 metal-to-ligand complexes between rutin and iron (depending on pH), with no experimental evidence for the existence of the bridging iron at the 7-OH groups. Instead, Escandar and Sala proposed coordination of iron between the 4-carbonyl oxygen and the 5-OH group on the rutin molecule. However, they reached similar conclusions for the iron coordination of quercetin, stating that coordination occurred at both the catecholate group and between the 3-OH and 4-carbonyl oxygen in a 2:1 metal to ligand ratio, based on potentiometric and spectrophotometric results [187]. The conflicting results obtained for iron–rutin complexes illustrate the difficulty of determining iron coordination for polyphenol compounds with multiple iron binding sites.

Mechanisms of Polyphenol Antioxidant Activity

Cytoprotective Effects of Polyphenols Related to Iron-Binding

Iron is implicated in many oxidative-stress-related pathways and conditions, and is the primary generator of H_2O_2 and $\bullet OH$ (reactions 1 and 3) that damages DNA and other biomolecules [48, 109, 110]. Therefore, understanding the biochemistry of iron has been the focus of many experiments aimed at preventing, inhibiting, or intercepting the harmful products of oxidative stress. Despite the strong iron-binding properties of polyphenols, whether the iron-chelating ability of catechol or gallol containing polyphenols actually plays a key role in their antioxidant activity is a matter of some debate. Kawabata et al. and Yoshino and Murakami have shown that iron chelation by several polyphenols protects rat microsomes from lipid peroxidation by blocking the Fenton reaction [142, 143]. Yet van Acker et al. have reported that iron chelation by several different polyphenols does not play a significant antioxidative role in microsomal lipid peroxidation using UV–vis spectroscopy, and they have noted that "there are, however, several contradictions in the literature, and the outcome largely depends on the experimental conditions and the type of assay used" [188]. Sugihara et al. [189] showed concentration-dependent antioxidant and prooxidant activity for several flavonoid compounds in an iron-induced lipid peroxidation system with cultured hepatocytes. Under the same conditions, however, they also found that catechins were antioxidants at all concentrations, and attributed the antioxidant behavior to iron chelation [190].

Generally, iron chelation by polyphenols is attributed solely to antioxidant rather than prooxidant effects. Morel et al. [191, 192] have shown that the ability of polyphenols to chelate and remove iron from iron-loaded hepatocytes correlates with cytoprotective effects of these compounds, and Ferrali et al. [193] have shown that quercetin (Q, Fig. 6) protects mouse erythrocytes from iron-mediated lipid peroxidation by binding iron. Similarly, Anghileri and Thouvenot [194] have also shown that polyphenols from mate tea, green tea, and red wine extracts protect against iron-induced lipid peroxidation of mouse liver tissue suspensions.

Sestili et al. [195] observed a dose-dependent cytoprotective effect for Q on human leukemic cells (U937) exposed to t-butyl hydroperoxide (t-BuOOH). They also determined an IC_{50} for Q of 12.67 ± 0.86 μM, for inhibition of H_2O_2-mediated cytotoxicity. Cells incubated with desferrioxamine (DFO, a known cell-permeable iron-
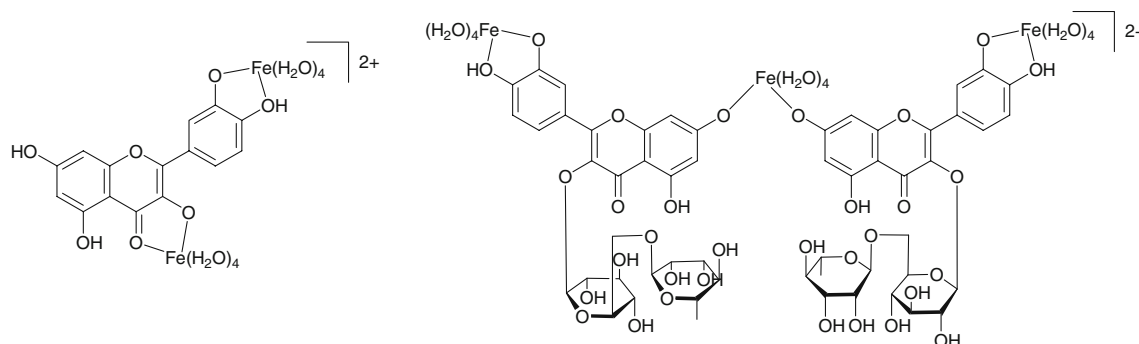

Fig. 9 Structures of the iron–quercetin (left) and iron–rutin (right) complexes proposed by de Souza et al.

chelator) showed the same inhibitory effect on DNA strand scission as Q, so Sestili et al. [195] inferred that iron-chelation was responsible for prevention of nuclear DNA damage by Q. Using similar methods, Sestili et al. [196] tested a variety of polyphenols and reported that only those compounds with catechol groups displayed both cytoprotective effects and DNA damage inhibition. Once again, these results suggested that iron binding contributes to the antioxidant activity of these compounds. Antioxidant activity of these compounds also correlated to their lipophilicity (ClogP), suggesting that a combination of iron-binding ability and the ability to cross cell membranes contribute to the antioxidant activity of polyphenol compounds [196]. Similar correlations between polyphenol iron-chelating ability and lipophilicity on prevention of DNA damage in H_2O_2-treated Jurkat cells were also noted by Melidou et al. [197].

Recently, Garcia-Alonso et al. [10] also studied the ability of a polyphenol-rich fruit juice blend to protect U937 cells from cell death by t-BuOOH. The blend of grape, cherry, and berry juices inhibited DNA damage similarly to the iron chelator *ortho*-phenanthroline so iron-chelation by the juice polyphenols was inferred to be the cause of antioxidant activity [10]. Boato et al. [198] observed that fruit juices high in polyphenols (>1 mg/ml), such as red grape and prune juices, limited bioavailability of iron for human colon cancer cells (Caco-2) in iron-enriched medium. Because juices low in polyphenols (<1 mg/ml) increased iron bioavailability, this reduced bioavailability was attributed to iron–polyphenol coordination preventing absorption by the cells. The increase in iron bioavailability was correlated to high ascorbate/polyphenol ratio, since ascorbate is known to reduce Fe^{3+} to the more water-soluble Fe^{2+}. Thus, Boato et al. [198] suggested a dietary balance of juices containing high ascorbate concentrations (for proper iron uptake) and high polyphenol content (for their cancer preventative properties). Several other groups have shown that polyphenols have a significant inhibitory effect on the bioavailability of iron not only in vitro [199–201], but also in rats [202], and in humans [203, 204]. This effect is usually assumed to occur either pre-ingestion (i.e., due iron-binding in the food or beverage) or in the gut, not after iron and polyphenols are separately absorbed or metabolized. The general bioavailability of polyphenol compounds, not related to iron, is an active research area [205, 206], but is beyond the scope of this review.

Kuo et al. [207] also examined the effects of polyphenols on Caco-2 cells but from a different perspective: metallothionein (MT) expression. MT is a sulfur-rich protein, which helps to control oxidative stress and heavy metal toxicity in vivo by chelating metal ions, including lead, mercury, cadmium, copper, and zinc. Kuo et al.

proposed that polyphenols might upregulate or downregulate MT expression, since polyphenol–metal chelation might prevent metal-mediated damage to biomolecules, resulting in lower MT levels. If the polyphenols themselves upregulate MT expression, increased MT levels could bind potentially damaging metal ions before they disrupt cellular function [207]. Using a ^{109}Cd-binding assay to determine MT expression, Kuo and coworkers observed that upon addition of the flavonoid quercetin, expression of MT was decreased in a dose- and time-dependent manner. On the other hand, kaempferol, genistein and biochanin-A-increased expression of MT, and flavone, catechin, and rutin had no effect on MT levels (Fig. 10). Although these results were primarily attributed to the ability of these compounds to bind either Cu^{2+} or Zn^{2+} (two metals that increase MT expression) [208–210], UV–vis spectroscopy also indicated interactions with Mn^{2+}, Fe^{2+}, and Fe^{3+} for some of these polyphenols. Interestingly, none of the polyphenol compounds that increased MT expression were shown to bind to metal ions under their experimental conditions [207].

In addition, Anghileri and Thouvenot reported that mate tea and green tea extracts and, to a lesser extent, red wine polyphenols, prevented iron-dependent calcium uptake in mouse liver tissue suspensions. They reasoned that iron binding by polyphenols limited the bioavailability of iron, thus inhibiting iron-dependent calcium uptake [194].

It is clear that polyphenols have varied cellular effects due to their metal-binding properties. A substantial body of work suggests that iron-binding by polyphenol compounds results in cytoprotective effects, and that both iron binding and lipophilicity are important factors contributing to overall antioxidant activity. However, definitive correlations between results from cell studies and prevention of oxidative stress-related diseases in animal or human clinical trials have not been established. Further investigation of indirect modes of polyphenol antioxidant activity, such as limitation of iron bioavailability and gene regulation, in animals or humans is also warranted.

Protective Effects of Polyphenols in Blood and Plasma

Although iron homeostasis in the blood is tightly regulated, red blood cells may become a target of oxidative damage in diseases disrupting normal hemoglobin function, such as β-thalassemia, sickle cell anemia, and other hemoglobinopathies, due in large part to their high non-protein-bound iron content [211–214]. Since plasma concentrations of polyphenols in humans commonly reach 1–10 μM [25–29], these compounds might be expected to have a protective effect on red blood cells. Grinberg, et al. have reported such protective effects for polyphenols in green and black tea against red blood cell lipid peroxidation, noting a

Fig. 10 Structures, names, and abbreviations of selected polyphenol compounds mentioned in this review that have been tested for antioxidant activity
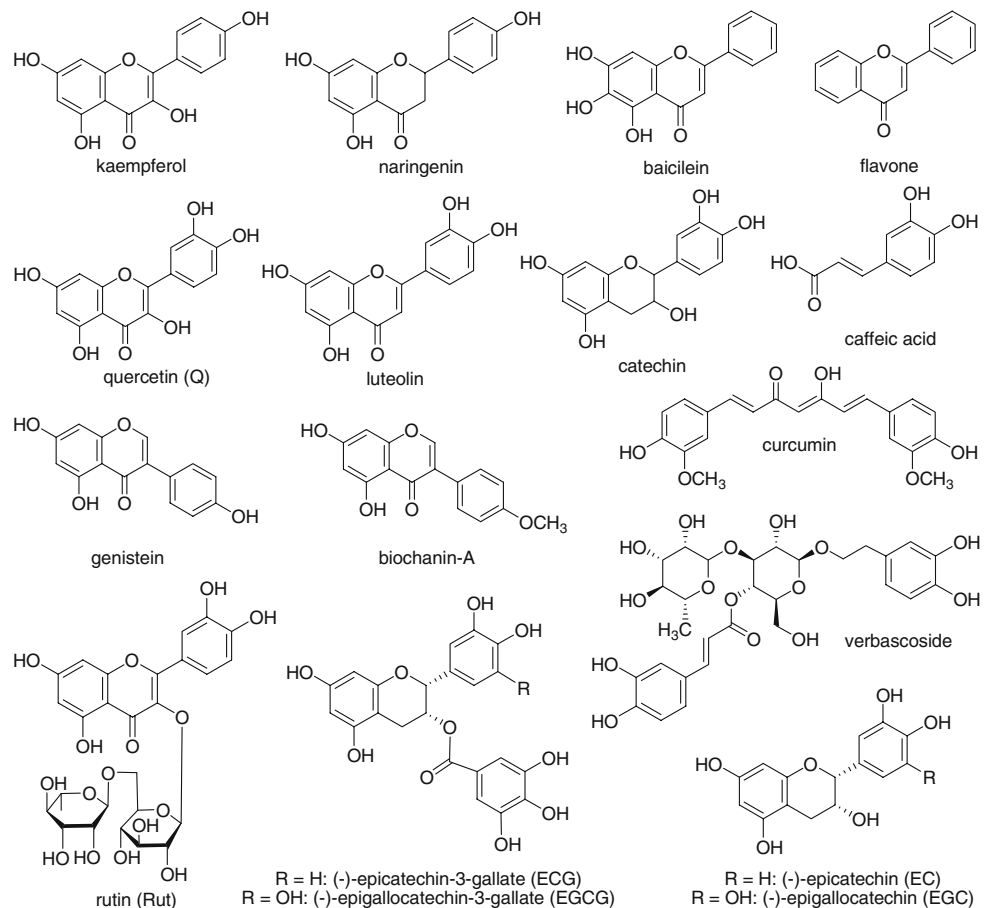
concentration-dependent inhibitory effect on $^{\bullet}OH$ production, and cytoprotection of red blood cells in vitro from primaquine-induced oxidative stress due to $O_2^{\bullet-}$ and H_2O_2 generation. These effects were attributed to iron-chelation by the tea polyphenols [215].

Similarly, Srichairatanakool et al. [216] found protective effects for green tea polyphenols against iron-overload symptoms in β-thalassemia patients and correlated these effects with both non-protein-bound iron chelation in thalassemic plasma, as well as radical scavenging, as measured by the TEAC assay. They also noted that polyphenols do not result in the adverse affects of typical iron-overload treatments, as DFO or deferiprone do. The individual green tea polyphenols (-)-epicatechin-3-gallate (ECG) and (-)-epigallocatechin-3-gallate (EGCG) (Fig. 10) also prevented oxidative stress in iron-treated erythrocytes similarly to the results observed for green tea [217].

Polyphenols Protect Against Neurodegenerative Diseases

A number of studies have linked iron homeostasis disruption to neurodegenerative diseases. Iron has been shown to accumulate in degenerating neurons [218], and also

induces aggregation and deposition of peptides such as amyloid beta (Aβ) [219] and α-synuclein [220, 221] in the brain, linking this metal to both Alzheimer's and Parkinson's diseases. Some comprehensive reviews of the protective effects and challenges of using green tea polyphenols for prevention or treatment of neurodegenerative diseases have been published by Singh et al. and Pan et al. [222, 223].

Green tea catechins are able to cross the highly selective blood-brain barrier (BBB) and protect against iron-induced neurodegeneration in mice [40, 224], as measured by their down-regulation of the amyloid precursor protein (APP) in the hippocampus region of the brain. APP can be converted to Aβ in the presence of divalent metal ions (such as Fe^{2+} and Cu^{2+}), generating neurotoxic amyloid fibrils [225]. Mandel et al. [226] have written an excellent review summarizing the neuroprotective effects of green tea polyphenols related to iron chelation.

Guo et al. [227] reported that green tea polyphenols protect brain synaptosomes against iron-induced lipid peroxidation. Both green tea and wine polyphenols were shown to inhibit aggregation and accumulation of Aβ fibrils [228], and to protect against Aβ neurotoxicity [229], perhaps as an indirect result of their iron-chelation ability.

Curcumin (Fig. 10), a polyphenol from turmeric, inhibits neurodegeneration in a mouse model, likely through a similar mechanism to green tea catechins [230, 231].

Additional work in the area of iron-mediated neurodegeneration prevention comes from Oboh and Rocha [13], who have used the thiobarbituric acid reactive species (TBARS) assay on homogenates of brain and liver cells of rats. TBARS is a common antioxidant assay that uses UV–vis spectroscopy to monitor the products of lipid peroxidation as they react with thiobarbituric acid and generate a colored species [232]. Oboh and Rocha's results show that polyphenols isolated from red pepper inhibit Fe^{2+}-mediated lipid peroxidation in both brain and liver cells by an iron-chelating mechanism, although iron chelation by the polyphenols was measured separately by UV–vis spectroscopy. In addition to iron binding, $^{\bullet}OH$ and $NO^{\bullet}$ scavenging were implicated as additional protective mechanisms for pepper polyphenols [233].

The interest in using antioxidant properties of polyphenols to prevent and treat neurodegenerative diseases is relatively new, and only a small number of compounds have been tested for this activity. Additional polyphenols should be tested for their effects on prevention of oxidative stress-induced neurodegeneration by chelating iron and copper ions as well as their ability to cross the BBB. These compounds should also be examined for their ability to inhibit APP aggregation. Identifying specific polyphenol compounds that have BBB permeability and metal chelating activity may lead to new treatments for neurodegenerative diseases.

Assays Quantifying the Inhibition of Iron-Mediated DNA Damage by Polyphenols

Whole-cell assays involve many variables and can make it difficult to definitively attribute polyphenol antioxidant activity to metal binding. For this reason, many in vitro methods have been used to examine the iron-binding antioxidant mechanism of polyphenols in order to correlate the results to those observed in biological systems. Two common methods for assessing inhibition of iron-mediated DNA damage by polyphenols are DNA gel electrophoresis and the deoxyribose assay, an assay that uses UV–vis spectroscopy to quantify malonaldehyde (a product formed from $^{\bullet}OH$ degradation of 2-deoxyribose) by its condensation reaction with thiobarbituric acid [182, 234, 235]. The major benefit of the deoxyribose assay is that it allows for faster screening of compounds, but its conditions do not closely resemble those of biological systems: the substrate is not truly DNA, but only 2-deoxyribose. In addition, many Fe^{3+}-polyphenol complexes absorb at or near the wavelength of the deoxyribose degradation product (532 nm), making this technique inherently problematic for

measurements of polyphenol inhibition of iron-mediated damage. Therefore, gel electrophoresis is perhaps the best technique because it both directly determines DNA damage and closely simulates biologically relevant conditions (such as pH, buffering capacity, and ionic strength).

Romanová et al. [236] have shown prevention of iron-mediated DNA damage for three flavonoid compounds (including quercetin), citing their iron-chelation ability, measured by UV–vis spectroscopy, as the most likely cause for DNA protection. In addition, Lopes et al. [182] used the deoxyribose assay to show that tannic acid prevents the Fenton reaction and protects 2-deoxyribose from oxidation by chelating Fe^{2+}. In contrast, Moran et al. observed prooxidant activity for polyphenol compounds, including gallic acid and methyl gallate (Fig. 6), using a modification of the deoxyribose assay. However, they used Fe^{3+}-EDTA in place of aqueous Fe^{2+}, and cited both the ability of polyphenols to reduce Fe^{3+} and EDTA blocking polyphenol chelation as likely causes of this prooxidant activity [237]. Prooxidant activity was also observed for polyphenols in a DNA damage assay using the Fe^{3+}-bleomycin complex as the iron source. Again, reduction of Fe^{3+}-bleomycin by polyphenols was described as the mechanism for prooxidant activity [237]. Laughtin et al. [165] also reported prooxidant activity for polyphenols in the presence of Fe^{3+}-EDTA/H_2O_2 and with the Fe^{3+}-bleomycin complex.

Using gel electrophoresis, both Zhao et al. and Perron et al. [181, 238] have studied the effects of polyphenols on plasmid DNA damage resulting from Fe^{2+} and H_2O_2. Zhao et al. [238] observed that verbascoside (Fig. 10) inhibited DNA damage from the Fenton reaction in a dose-dependent manner, and attributed this antioxidant activity to iron binding by the polyphenol. In an extension of this work, Perron et al. [181] assayed 12 different phenolic compounds, showing that all of the compounds with catechol or gallol groups inhibited 50% of the DNA damage from Fe^{2+} and H_2O_2 (IC_{50}) at concentrations between 1–59 μM. Notably, the IC_{50} value for quercetin (10.8 ± 1 μM) was identical within error to the IC_{50} reported by Sestili et al. for inhibition of H_2O_2-mediated cytotoxicity in U937 cells. Perron et al. [181] also directly confirmed the necessity of iron binding for DNA damage prevention by showing that EGCG (Fig. 10) did not inhibit DNA damage when Fe^{2+} was coordinated with EDTA prior to addition of the polyphenol, although the Fe^{2+} EDTA complex generated hydroxyl radical in the presence of H_2O_2.

Generally, polyphenols protect DNA from damage in systems involving Fe^{2+} at biologically relevant, low micromolar concentrations. While some experiments with Fe^{3+} have shown prooxidant activity of polyphenol compounds, it is expected that most non-protein-bound iron in cells is in the Fe^{2+} oxidation state [113]. Because research

in vitro is extremely promising, it will be important to confirm iron binding as a primary mechanism of antioxidant activity in animal models. The next logical step would be testing of polyphenol compounds in clinical trials for treatments or prevention of diseases attributed to iron-mediated DNA damage and other oxidative damage, such as cancer, cardiovascular diseases, and neurodegenerative diseases. One such clinical trial by the Mayo Clinic, in collaboration with the National Cancer Institute, is currently recruiting participants; their goal is to test green tea extract with high EGCG content in patients with chronic lymphocytic leukemia to observe the effects on this form of cancer [239].

In Vitro Iron-Binding Structure–Activity Relationships of Polyphenols

Because of the immense variety and many different classes of polyphenol compounds, determining structure–activity relationships (SARs) for antioxidant properties of polyphenols is a challenging undertaking. For identification of effective polyphenol antioxidants, determining SARs for these compounds is required to realize their potential to treat and prevent diseases caused by oxidative damage.

Cheng and Breen have used cyclic voltammetry to show that four polyphenol compounds, baicilein, naringenin, luteolin, and quercetin (Fig. 10) effectively suppress reduction of H_2O_2 by the Fe^{2+}-ATP complex. The two compounds with catechol moieties on the B ring (luteolin and quercetin) are more potent inhibitors of the Fenton reaction than the two compounds without catechols (baicilein and naringenin) [240]. Based on the large stability constants for iron–catecholate complexes, it would seem, therefore, that iron binding at the catecholate group may be responsible for the greater antioxidant activity observed for luteolin and quercetin. However, baicilein has a gallol group on the A ring rather than the B ring, which could also bind iron. Since baicilein was a weaker antioxidant in this system, Cheng and Breen concluded that substituents on the B ring more significantly affect antioxidant activity.

The higher antioxidant activity of phenol substituents on the B ring as compared to the A ring was confirmed by Jovanovich et al. [241], as well as Arora et al. [242], who showed that a catechol group on the B ring gives rise to iron binding and antioxidant activity. In addition to reporting that compounds with no phenol groups had negligible antioxidant activity in a lipid peroxidation model evaluating antioxidant activities of polyphenols, Arora et al. stated that phenol substituents on the A ring contribute little to the antioxidant activity of polyphenols. However, in this study they also tested one compound (7,8-dihydroxyflavone) with a catechol substituent on the A ring that had similar antioxidant activity to compounds with B

ring catechol substituents. Thus, the lower antioxidant activity of A ring polyphenols compared to B ring polyphenols may not apply universally.

Khokar and Apenten [243] compared polyphenol compounds with catechol and gallol moieties on the B ring (as well as tannic acid), and concluded that these structural motifs are optimal for iron binding and antioxidant activity in vitro. They also proposed that the presence of a hydroxy-keto group (a 3-OH or 5-OH plus a 4-C=O), as well as a large number of catechol/gallol groups (as in the case of tannic acid, Fig. 11), also contributes to iron-binding and antioxidant activity [243].

By quantifying a representative selection of polyphenol compounds for prevention of iron-mediated DNA damage, Perron et al. [181] determined that compounds with gallol groups were more potent antioxidants than those with only catechol groups. Furthermore, the IC_{50} values for twelve polyphenols reported by Perron and coworkers correlated to the pK_a value of the most acidic phenolic hydrogen of the polyphenol compounds (Table 2), representing the first predictive model of antioxidant potency as a function of iron-binding ability (Fig. 12). This correlation, along with additional experiments, directly established iron binding as the mechanism of the observed antioxidant activity [181, 244]. This predictive model allows the library of polyphenols to be efficiently screened for those with the highest iron-binding antioxidant activity.

The SARs of polyphenols related to iron binding have generally been established for catechol and gallol containing compounds. In addition to these iron-binding groups, hydroxy-keto moieties may also contribute to antioxidant activity by binding iron. Although the
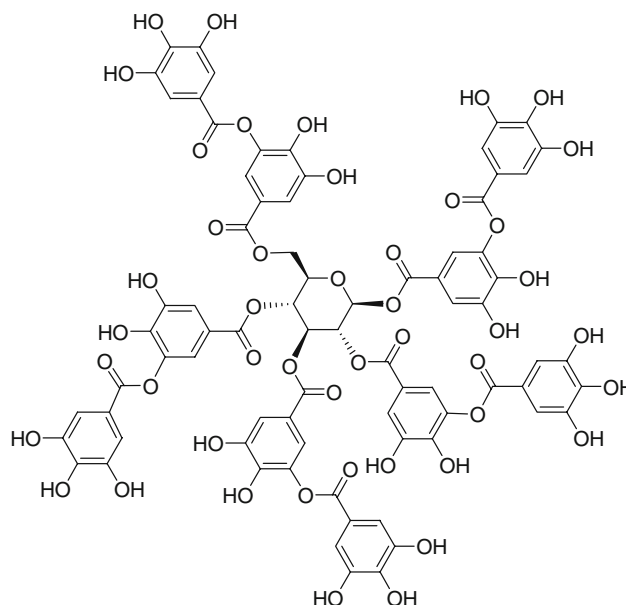

Fig. 11 Structure of tannic acid

important iron-binding functional groups are known, better predictive models must now be developed to screen the enormous number of polyphenol compounds based on their physical and chemical properties (lipophilicity/bioavailability, molecular weight, shape, π-acidity, etc.) for those with optimal antioxidant activity in vitro. After selecting the best candidates, the in vitro experiments should be followed by cell and animal studies, and eventually clinical trials for the most promising polyphenol antioxidants.

Polyphenols as Preservatives

Iron-mediated oxidative damage is not limited to living organisms. Due to the presence of iron in the environment, iron generated $^\bullet$OH is also responsible for food spoilage and wood rotting [245, 246], and conflicting reports exist about whether polyphenols are suitable for use as preservatives of food and wood. Contreras et al. [247] have reported increased degradation of wood after treatment with several catechol polyphenols, including catechol, protocatechuic acid, and 2,3-dihydroxybenzoic acid (Fig. 6), and similar to compounds that have been isolated from brown-rot fungi [248]. Under mildly acidic conditions, these compounds were able to reduce Fe^{3+} to Fe^{2+} in their mono (catecholate) complex forms [247]. Tiron (Fig. 6), however, was not shown to participate in the redox-cycling mechanism, as this catechol compound

cannot reduce Fe^{3+} [249]. In contrast, Binbuga et al. [140] have shown preservation of wood using n-propyl gallate (Fig. 6), citing iron chelation as a likely mechanism even under the mildly acidic conditions present in wood. This preservative effect of n-propyl gallate was attributed primarily to its antifungal properties, since it may interfere with the Fenton reaction and redox cycles that wood-decaying brown rot fungi use to decompose wood [248, 250, 251].

In addition to wood preservation, polyphenol compounds have been widely studied for use as preservatives for food, cosmetics, and pharmaceuticals, with many patents for these applications both nationally and internationally related to their antioxidant properties [252–258]. The FDA has also approved n-propyl gallate for use as a food preservative [259]. However, most patents discussing polyphenol preservative mechanisms do not cite metal chelation as a cause of their antioxidant activity, instead claiming the radical scavenging ability often attributed to these compounds.

The formation of iron–polyphenol complexes has been attributed to negative aspects in foods as well, such as off-color development or browning of bruised or sliced fruits [260]. Similar polyphenol oxidation pathways occur in polyphenol oxidase and tyrosinase, two enzymes that utilize both a metal ion and O_2 to oxidize phenolic compounds [261, 262]. While these enzymes typically

Table 2 pK_a values for phenolic compounds and IC$_{50}$ values for iron(II)/H$_2$O$_2$-mediated DNA damage inhibition by phenolic compounds[a]

Compound	IC$_{50}$ (μM)	pK_{a1}[b]	pK_a reference
(−)-Epigallocatechin-3-gallate (EGCG)	1.1	7.55 ± 0.03	[306]
(−)-Epicatechin-3-gallate (ECG)	2.3	7.6	[306]
		7.74	[307]
Methyl 3,4,5-trihydroxybenzoate (MEGA)	4.0	7.92	[62]
		8.03	[308]
Propyl gallate (PrEGA)	5.1	7.77 ± 0.04	[140]
(−)-Epigallocatechin (EGC)	9.83	8.51 ± 0.04	[306]
		7.87	[307]
Gallic acid (GA)	14.0	8.80 ± 0.02	[309]
		8.70 ± 0.02	[310]
		8.45 ± 0.06	[310]
Methyl 3,4-dihydroxybenzoate (MEPCA)	15.6	8.12 ± 0.18	[311]
Protocatechuic acid	34.4	8.90 ± 0.03	[309]
		8.79 ± 0.05	[136]
		8.64 ± 0.05	[310]
		8.81 ± 0.01	[312]
(−)-Epicatechin (EC)	59.1	8.76 ± 0.02	[306]
		8.72 ± 0.01	[312]
		8.91 ± 0.23	[313]
Vanillic acid (VA)	140.0	9.39	[314]
		9.391	[315]
		9.17	[316]

[a] IC$_{50}$ values are taken from Perron et al. (reference [181]) and are all ± 1 μM

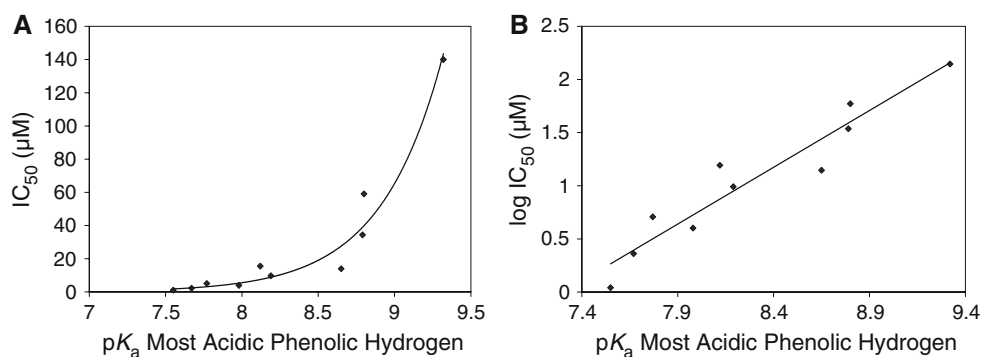[b] pK_a values are for the most acidic phenolic hydrogen

Fig. 12 a Graph of IC_{50} versus pK_a for the most acidic phenolic hydrogen showing the best-fit exponential correlation to the data for polyphenols (*solid line*, $R^2 = 0.91$). The data points for myricetin and quercetin were omitted from the data set because of their non-gallol and non-catechol binding sites, respectively. Error bars for IC_{50} values are within the size of the data points. **b** Linear representation of the correlation between IC_{50} and polyphenol pK_a, shown by plotting log IC_{50} versus pK_a (*solid line*, $R^2 = 0.91$). pK_a values used in this figure are the averages of the literature values listed in Table 2

have copper-containing active sites, an iron-containing tyrosinase has been isolated from tea leaves [263].

Based on the limited research in this area, the use of polyphenol compounds as preservatives for food and wood seems promising, although selection of specific polyphenol compounds with antioxidant properties will be important, since some appear to actually accelerate rotting processes under certain conditions. Therefore, as with polyphenol antioxidants for use in vivo, further testing is needed to determine both the conditions under which polyphenols have preservative effects, and the SARs of polyphenol compounds as related to antioxidant, preservative, and antibiotic properties.

Prooxidant Activity of Polyphenols Related to Iron Binding

Hydroxyl Radical Production by Iron–Polyphenol Complexes

Although the focus of this review is on the antioxidant activity of polyphenol compounds, several reports have described prooxidant behavior for polyphenols, and this activity must not be overlooked. Prooxidant activity may arise from the ability of polyphenol compounds to bind and reduce Fe^{3+} to the hydroxyl radical generating Fe^{2+}. EGCG and ECG, with two gallol groups or one gallol and one catechol group, respectively, have been reported to reduce up to four equivalents of Fe^{3+} in this manner [154].

In addition to the prooxidant activity observed for polyphenol reduction of Fe^{3+} (discussed in Reduction of Fe^{3+} by Polyphenol Ligands and Assays Quantifying the Inhibition of Iron-Mediated DNA Damage by Polyphenols sections), Hiramoto et al. [264], Moran et al. [237], and Ohashi et al. [144] have observed DNA strand scission by

Fe^{3+}-polyphenol complexes in vitro, for gallic acid and the green tea compounds EGC and EGCG, among others. Puppo [164] also observed an increase in $^\bullet OH$ production from the Fenton-type reaction of the Fe^{3+}-EDTA complex in the presence of polyphenols myricetin, quercetin, and catechin, among others. A review of the prooxidant and toxic effects of polyphenols both in vitro and in vivo was published by Schweigert et al. [166] primarily addressing copper-mediated prooxidant activity but also prooxidant activity with iron as well.

Prooxidant activity of polyphenol compounds is believed to arise from the ability of polyphenols to reduce Fe^{3+} or Cu^{2+} and the prevention of polyphenol binding by EDTA or other chelating ligand already present. Thus, experiments exploring the prooxidant properties of polyphenol compounds are typically performed either in the presence of metal chelators (such as EDTA, bleomycin, or ADP) [164, 165, 237, 265], that block chelation of iron by the polyphenol ligand, and/or often use oxidized metal ions, such as Fe^{3+} or Cu^{2+}. In these cases, the polyphenol compound can reduce the metal ion via an outer-sphere electron transfer, making it available for reaction with H_2O_2 or other sources of radicals.

In contrast to the prooxidant effects of polyphenols with Fe^{3+}-EDTA or Fe^{3+}-bleomycin complexes, Inoue et al. [266] have shown by gel electrophoresis that the polyphenol caffeic acid (Fig. 10) does not damage DNA in the presence of aqueous Fe^{3+} and H_2O_2. Once again, this implies that iron binding is required for polyphenol antioxidant activity, or at the very least, prevention of prooxidant activity.

It must be noted that the intracellular cytoplasmic environment is known to be quite reducing, due to the many reductants present inside the cell, such as NADH, glutathione, thioredoxin, ascorbic acid, and citric acid [114, 115, 267]. Thus, any non-protein-bound metal ions would

most likely be present in their reduced forms in vivo [113]. Due to the deleterious effects of prooxidant activity, however, it is essential to understand both the antioxidant and prooxidant behavior of polyphenol compounds.

It is interesting that polyphenol compounds can display both antioxidant and prooxidant activity under very similar conditions. Often these conditions are quite similar to those in biological systems, however, there are not usually ligands as strongly chelating as EDTA other than proteins in cells, and these are highly specialized and prevent iron-mediated prooxidant activity. Plus, iron is not usually found as Fe^{3+} in any appreciable intracellular concentration. Therefore, the conditions for polyphenol prooxidant activity are actually quite limited, often not biologically relevant, and therefore may be of less concern to humans who ingest these compounds in food sources. However, this body of work showing prooxidant activity for polyphenols when they cannot directly chelate iron does stress the essentiality of iron-binding as a viable antioxidant mechanism for these compounds.

Superoxide Dismutase-Like Activity of Polyphenols and Iron-Polyphenol Complexes

Polyphenol compounds and their complexes with Fe^{2+} are reported to react with superoxide ($O_2^{\bullet-}$) to form H_2O_2 and a semiquinone radical, resulting in superoxide dismutase (SOD)-like behavior [66, 186, 259, 268, 269]. The reactivity is similar to SOD, an enzyme present in all aerobic organisms, which catalyzes the dismutation of two $O_2^{\bullet-}$ anions under physiological pH into H_2O_2 and O_2 (reaction 7) [270].

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (7)$$
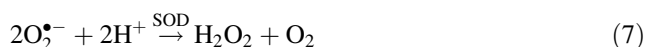
Since these reactions form H_2O_2, polyphenol compounds may contribute to cellular oxidative stress. An example of this proposed SOD-like activity was reported by de Souza et al. for the Fe^{2+}-quercetin complex. Upon deprotonation and iron binding, the oxidation potential of the flavonoid is decreased so that it is oxidized in the presence of $O_2^{\bullet-}$ to yield a Fe^{2+}-quercetin semiquinone radical complex and H_2O_2 [186] (Fig. 13). This mechanism was proposed from cyclic voltammetry measurements based on the absence of normally observed oxidation waves for the Fe^{2+}-quercetin complex in the presence of $O_2^{\bullet-}$, suggesting that the Fe^{2+}-quercetin complex scavenges $O_2^{\bullet-}$ radicals. However, the products of this reaction were not isolated or characterized; therefore, such reaction products must be further characterized to confirm the SOD-like mechanism for iron-polyphenol complexes.

Since Fe^{3+} from enzymes such as ferritin [88], hydrolyases [87], or dehydratases [87, 89], can be reduced by

$O_2^{\bullet-}$ to generate Fe^{2+} (reactions 3–5), it has been hypothesized that H_2O_2 generated from SOD-like reactions could react with Fe^{2+} to form the more reactive hydroxyl radical ($^\bullet OH$) via the Fenton reaction (reaction 1) [271, 272]. If polyphenol compounds decompose $O_2^{\bullet-}$, they may directly prevent $O_2^{\bullet-}$ from reducing iron and subsequent iron release from proteins. Polyphenol compounds can also chelate Fe^{2+}, so the iron released from proteins may be bound by polyphenol compounds, resulting in an additional antioxidant mechanism as proposed by Reddan et al. [259]. This polyphenol-bound iron would not be available to react with H_2O_2, and thus the H_2O_2 would be decomposed by catalase or peroxidase enzymes, such as glutathione peroxidase [273–275].

The interactions between iron, polyphenols, and ROS and RNS are extremely complex, so it is necessary to explore these systems carefully, using diverse and complementary experimental techniques (studying the structure of iron–polyphenol complexes, for example, combined with their ROS reactivity as examined using EPR spectroscopy), to positively identify the mechanisms and products of these reactions.

Interactions Between Polyphenols and Copper

Although the purpose of this review is to highlight the iron binding properties of polyphenol compounds, due to similarities in copper-mediated ROS generation, the interactions between polyphenols and copper should not be ignored. Copper also generates $^\bullet OH$ in a Fenton-like reaction with H_2O_2, and this copper-generated $^\bullet OH$ can damage DNA (Fig. 14) [86, 276]. Superoxide also reduces Cu^{2+} to form H_2O_2 and Cu^+ (reaction 8) [277].

$$Cu^{2+} + O_2^{\bullet-} + 2H_2O \rightarrow Cu^+ + H_2O_2 + O_2 \qquad (8)$$
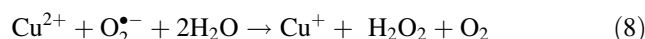
Polyphenols have fairly strong binding interactions with Cu^{2+}, a borderline-hard Lewis acid similar to Fe^{2+}; in fact, stability constants for Cu^{2+} catecholate complexes are larger than for Fe^{2+} [136, 278]. However, polyphenol ligands are widely reported to reduce Cu^{2+} to Cu^+, a soft Lewis acid for which polyphenols have little affinity [133]. For this reason, stability constants for Cu^+ polyphenol complexes have not been reported. This weak interaction between polyphenols and Cu^+ may also explain why so little has been published on the antioxidant effects of polyphenols related to copper chelation. In contrast with other metal ions found in biological systems, Cu^{2+} has a positive reduction potential in aqueous solution, facilitating reduction of Cu^{2+} to Cu^+ and promoting Cu^{2+} binding to electron-rich ligands such as oxygen atoms [279]. This tendency toward copper reduction, coupled with the tendency of polyphenol compounds to oxidize results in

Fig. 13 SOD-like reactions of the Fe^{2+}-quercetin complex, as proposed by de Souza et al.
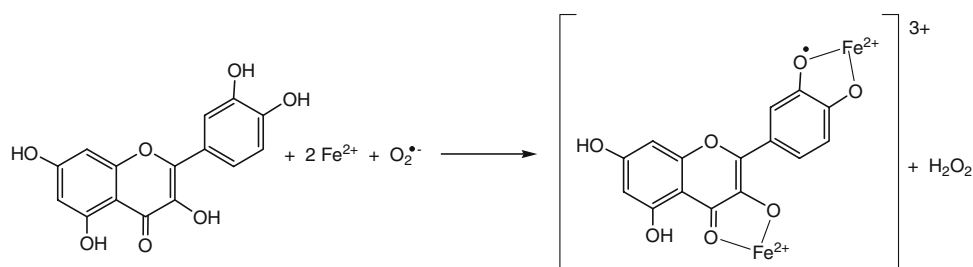
complex copper–polyphenol interactions, especially in the presence of ROS [280–282].

To the best of our knowledge, only Andrade et al. and Perron, et al. [283, 284] have reported antioxidant effects for protection of copper-induced DNA damage by polyphenols. Andrade et al. examined the antioxidant activity of tannic acid (Fig. 10) using the deoxyribose assay with Cu^+/H_2O_2 (see the Assays Quantifying the Inhibition of Iron-Mediated DNA Damage by Polyphenols section). From their results, they calculated an IC_{50} value of 5.3 ± 0.8 μM for tannic acid [283].

Using gel electrophoresis, Perron et al. tested 12 phenolic compounds for their effects on Cu^+/H_2O_2-mediated DNA damage and observed both antioxidant and prooxidant behaviors. They showed inhibition of copper-mediated DNA damage for EGCG (Fig. 10) resulting in an IC_{50} of 249 ± 1 μM. In contrast, EC and EGC displayed prooxidant activity under the same conditions, whereas ECG displayed both prooxidant activity and antioxidant activity at low (0.1–4 μM) and high (10–1,000 μM) concentrations, respectively. Very similar experiments with Fe^{2+} instead of Cu^+ resulted in an IC_{50} of 1.1 ± 1 μM for inhibition of iron-mediated DNA damage: nearly 250 times more potent. The greatly diminished antioxidant potency for polyphenols in the copper system was attributed to the weak interactions between polyphenols and Cu^+. Based on these experiments, a copper redox-cycling mechanism was proposed for the prooxidant activity observed for some polyphenols under these conditions [132, 276, 285, 286].

The prooxidant activity for polyphenol compounds under certain conditions has prompted some cautionary advice on consuming large amounts of these compounds [287, 288], but copper homeostasis is even more tightly regulated than iron; it has been estimated that the intracellular concentration of non-protein-bound Cu^+ is less than 10^{-18} M in unstressed yeast cells, corresponding to
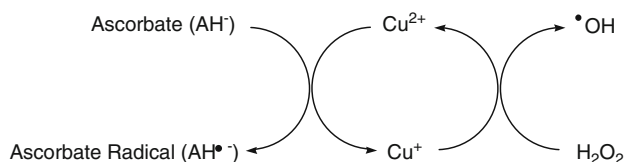
less than one non-protein-bound Cu^+ ion per cell [289]. Furthermore, compounds such as glutathione (1–15 mM) [122, 290] are present at much higher concentrations in vivo than those attainable by polyphenols ($\sim$1–10 μM), and have much higher affinity for Cu^+ than polyphenols.

Recently, however, non-protein-bound copper pools have been found in the mitochondria and neuronal cells of higher organisms [291, 292], and mis-regulation of copper homeostasis results in higher cellular concentrations and increased oxidative stress [277, 293–296]. Therefore, elucidating the antioxidant and prooxidant mechanisms of polyphenol–copper interactions will be important for a complete understanding of polyphenol activity in vivo.

Conclusions and Future Directions

Although both Fe^{2+} and Cu^+ perform Fenton-like reactions with H_2O_2, polyphenol compounds containing metal binding catechol and gallol groups have very different activities, depending on the metal ion. Antioxidant activity is commonly observed for polyphenols in Fe^{2+}/H_2O_2 systems using a number of different assays, including cell studies and in vitro DNA damage inhibition experiments. In contrast, when testing polyphenols in Cu^+/H_2O_2 systems, greatly diminished antioxidant potency or even prooxidant activity is often observed from the interactions between polyphenols and copper. These findings stress the need for caution in experimental design: in addition to the polyphenol compounds, the metal ion must be specifically chosen to examine the metal–antioxidant interactions. Since results are highly dependent on experimental conditions, it should be the goal of researchers to design the most biologically relevant experiments possible.

Because iron-mediated damage to biomolecules such as lipids and DNA is implicated in disease development, the iron-chelating mechanism of polyphenol antioxidant activity must be fully explored in addition to radical scavenging to understand polyphenol antioxidant behavior. Polyphenols with gallol or catechol groups are generally the most potent antioxidants, primarily because of the large iron-binding stability constants for these groups. Also, compounds with the hydroxy-keto moiety can chelate iron, giving rise to antioxidant activity. As compared to radical

Fig. 14 The Fenton-like reaction with copper

scavenging, however, the iron-binding mechanism for polyphenol antioxidant activity is relatively underdeveloped. Therefore, additional research is needed in several areas, including stability constant measurements for iron binding, pK_a measurements of polyphenols, and additional cell studies and DNA damage prevention experiments to correlate in vitro and in vivo antioxidant activity of these compounds. The goal of this work should be to develop biologically relevant predictive models and SARs for polyphenol compounds, as well as high-throughput screening methods for determining antioxidant (or prooxidant) behavior. These further studies will enable identification of highly effective polyphenol antioxidants for clinical trials to prevent or treat diseases caused by oxidative stress.

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