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Interactions of Flavonoids with Iron and Copper Ions: A Mechanism for their Antioxidant Activity

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The metal chelating properties of flavonoids suggest that they may play a role in metal-overload diseases and in all oxidative stress conditions involving a transition metal ion. A detailed study has been made of the ability of flavonoids to chelate iron (including Fe^{3+}) and copper ions and its dependence of structure and pH. The acid medium may be important in some pathological conditions. In addition, the ability of flavonoids to reduce iron and copper ions and their activity–structure relationships were also investigated. To fulfil these objectives, flavones (apigenin, luteolin, kaempferol, quercetin, myricetin and rutin), isoflavones (daidzein and genistein), flavanones (taxifolin, naringenin and naringin) and a flavanol (catechin) were investigated. All flavonoids studied show higher reducing capacity for copper ions than for iron ions. The flavonoids with better Fe^{3+} reducing activity are those with a 2,3-double bond and possessing both the catechol group in the B-ring and the 3-hydroxyl group. The copper reducing activity seems to depend largely on the number of hydroxyl groups. The chelation studies were carried out by means of ultraviolet spectroscopy and electrospray ionisation mass spectrometry. Only flavones and the flavanol catechin interact with metal ions. At pH 7.4 and pH 5.5 all flavones studied appear to chelate Cu^{2+} at the same site, probably between the 5-hydroxyl and the 4-oxo groups. Myricetin and quercetin, however, at pH 7.4, appear to chelate Cu^{2+} additionally at the *ortho*-catechol group, the chelating site for catechin with Cu^{2+} at pH 7.4. Chelation studies of Fe^{3+} to flavonoids were investigated only at pH 5.5. Only myricetin and quercetin interact strongly with Fe^{3+}, complexation probably occurring again between the 5-hydroxyl and the 4-oxo groups. Their behaviour can be explained by their ability to reduce Fe^{3+} at pH 5.5, suggesting that flavonoids reduce Fe^{3+} to Fe^{2+} before association.

Keywords: Flavonoids; Metal chelation; Metal reduction; Antioxidant properties; Mass spectrometry

INTRODUCTION

Free radicals are continuously being formed in small amounts by normal processes of metabolism. Many of them serve useful physiological functions,[1,2] but they can damage the biomolecules when generated in excess, being implicated in the aetiology of several diseases and ageing.[3–5] In order to balance the physiological generation of free radicals, organisms have evolved a wide array of enzymatic and non-enzymatic endogenous antioxidant defenses.[6,7] Nevertheless, in situations of increased free radical generation the reinforcement of endogenous antioxidants with dietary antioxidants may be particularly important in diminishing the cumulative effects of oxidatively damaged molecules.

Recent work highlights the potential health-promoting properties of flavonoids:[8–10] these are phenolic compounds widely distributed in fruits, vegetables, plant extracts as well as in plant-derived beverages such as tea and red wine.[11,12] Flavonoids have generated interest because of their broad

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pharmacological effects such as vasoprotective, anti-inflammatory, antiviral and antifungal actions (reviewed in Ref. [13]). Many of these effects are related to their antioxidant properties, which may be due to their ability to scavenge free radicals[14–19] and to synergistic effects with other antioxidants.[20] Another antioxidant mechanism of flavonoids, not yet extensively studied, may result from the interactions between flavonoid and metal ions (especially iron and copper) leading to chelates formation that are only slightly active in the promotion of free-radical reactions.[21–27] Flavonoids have also been reported to show prooxidant effects, which have been related with their iron and copper reducing activities. These reduced metals can catalyse the production of hydroxyl radicals through Fenton reaction[28–31] and lipid radicals through the decomposition of preformed lipid hydroperoxides.[32,33]

In order to be an effective catalyst in those radical reactions, iron has to be present in an ionic or "free" form. Traces of iron salts are present in several body fluids, with the exception of blood plasma.[34] Usually iron is safely sequestered in proteins that normally bind iron hindering or preventing its action in catalysing radical reactions. Iron, however, can be released from those proteins at low pH[35] as a result of protein damage produced by peroxides[36,37] or by reductive mobilization by $O_2^{\cdot -}$.[38] Low pH values can be generated locally during phagocytosis and inflammation[39] and during the ischemia/reperfusion injury.[40] The nature of the small pool of iron, not bound to proteins, that exists in the cytosol of the cell for a variety of cellular functions and enzymes is unknown, but probably consists of both Fe^{2+} and Fe^{3+}.[41] The presence of both ferric and ferrous forms of iron in the cytosol is an important prerequisite for the initiation of lipid peroxidation.[42] In addition, desferrioxamine, a chelator of Fe^{3+}, has been shown to limit the amount of free radical production in several different cells and species.[43]

Most copper is "tightly bound" to the plasma protein caeruloplasmin, but some is attached to albumin and to aminoacids such as histidine that can catalyse free radical reactions.[42,44]

Few detailed studies have been made of the ability of flavonoids to chelate metal ions (Fe^{3+} in particular) or of the effect of varying their structure and the pH of the medium. The acid medium may be important in some pathological conditions. We have therefore used ultraviolet spectroscopy and electrospray ionisation mass spectrometry (ESI-MS) to investigate a large group of flavonoids (see Fig. 1) in order to study the influence of structure on their ability to chelate iron and copper at pH 7.4 and 5.5. In addition, the ability of flavonoids to reduce iron and copper ions and their activity–structure relationships were also investigated. The flavonoids studied were flavones (myricetin, quercetin, luteolin, rutin, kaempferol, and apigenin), isoflavones (genistein and daidzein), flavanones (taxifolin, naringenin and naringin) and a flavanol (catechin). Ultraviolet-visible absorption spectroscopy is a very useful technique for flavonoid structure analysis. For this reason, the interactions of flavonoids with metal ions can be demonstrated spectrophotometrically by changes in the absorption spectra. ESI-MS enables one, in principle, to study systems at biologically realistic concentrations and, among mass spectrometric techniques, it is the most closely related to solution.[45] ESI-MS studies were carried out in an attempt to obtain direct evidence for flavonoids/transition metal complexation, chelation sites and oxidation/reduction reactions.

MATERIALS AND METHODS

Chemicals

All the reagents were of the highest quality available and were used as supplied. Flavonoids were obtained from Sigma Chemical Company (St. Louis, MO, USA), cupric chloride from Baker Chemicals (Phillipsburg, NJ, USA), ferric chloride from Fluka (Buchs, Switzerland) and bathocuproinedisulfonic acid (BCDS) from Aldrich Chemical Company (Milwaukee, WI). All the other reagents were obtained from Merck (Darmstadt, Germany).

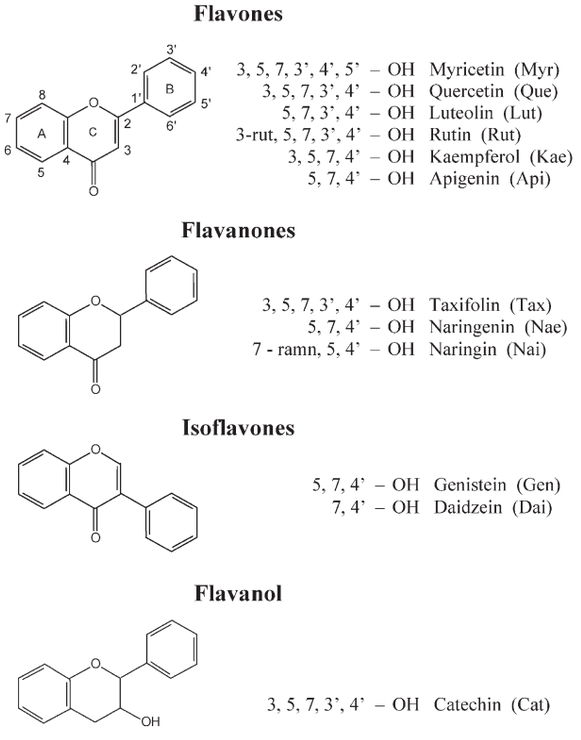

FIGURE 1 Structures of the flavonoids used in this study.

Metal Reduction Studies

Fe^{3+} Reducing Activity

The ability of flavonoids to reduce Fe^{3+} was assayed by a modified ferrozine method.[31] Ferrozine is a chromophoric chelator that strongly binds Fe^{2+} forming a stable complex with a high extinction coefficient at 562 nm.[46] The reaction mixture contained 50 mM Na-acetate buffer (pH 5.5), 1 mM ferrozine, 25 μM flavonoid and 100 μM $FeCl_3$. The reaction was started by the addition of $FeCl_3$ and the increase of absorbance at 562 nm after 3 min was recorded using a control lacking ferrozine. The Fe^{2+} concentration was determined by using an extinction coefficient for the $Fe(ferrozine)_3^{2+}$ complex of 27,900 $M^{-1}\,cm^{-1}$.[46]

Cu^{2+} Reducing Activity

The ability of flavonoids to reduce Cu^{2+} was evaluated by measuring the formation of the complex between Cu^{+} and bathocuproinedisulfonic acid (BCDS).[47] The reaction mixture contained 20 mM KH_2PO_4/KOH buffer (pH 7.4), 200 μM $CuCl_2$, 600 μM BCDS and either 10 μM ascorbate or 10 μM flavonoid. The mixtures were incubated at 37°C for 120 min and then the absorbances were read at 483 nm. The copper concentration was determined by using an extinction coefficient for the $(BCDS)_2Cu(I)$ complex of 12,900 $M^{-1}\,cm^{-1}$, which was evaluated by a calibration curve (ranging 5.0–125 μM) obtained by standard $CuCl_2$ solutions and by using 200 μM ascorbate as reducing agent.

Chelation Studies

Spectrophotometric Analysis

The modifications of the absorption spectra of 100 μM flavonoid solutions when combined with equimolar concentrations of either Fe^{3+} or Cu^{2+}, in 50 mM acetate buffer (pH 5.5) and 50 mM MOPS buffer (pH 7.4) at room temperature, were spectrophotometrically analysed with a Pye Unicam UV2-100 spectrophotometer. All the spectra were run against blanks containing the buffer and the metal ion.

Electrospray Ionisation Mass Spectrometry (ESI-MS) Analysis

The flavonoids studied by this technique were the flavones myricetin, quercetin, luteolin and kaempferol and the flavanol catechin. All experiments were performed in a Quattro II QhQ tandem quadrupole instrument (Micromass Manchester) fitted with an electrospray ionisation source operated in positive ion mode. The tip of the capillary was maintained at the potential of 3 kV and the sampling cone was maintained at potentials within the range −30 to −120 V relative to ground, in order to optimise the spectra. The mobile phase consisted of methanol/water 1:1 and 0.1% of acetic acid and the optimum flow rate of the mobile phase was found to be 5 μl/min. Solutions of cupric chloride or ferric chloride and a ligand in a ratio 1:1 were prepared in concentrations in the range 35–65 μM in each component. The pH of these solutions was ≤5.5. Mass spectra were acquired by scanning the first quadrupole mass analyser from m/z (mass to charge ratio) 2000 to 50–100 and ions were detected by means of a scintillator detector positioned after the first quadrupole. Collision induced dissociation (CID)[48] spectra were obtained by selecting a precursor ion in the first quadrupole mass analyser and transferring it into the collision cell containing argon. Product ion spectra were obtained by scanning the second quadrupole mass analyser. The pressures and the voltages applied to the collision cell were in the ranges $(1–4) \times 10^{-3}$ mbar and (20–60) eV, respectively, in order to optimise the conditions. All data were processed by means of the MassLynx data system (Micromass).

RESULTS

Reduction Studies

Fe^{3+} Reduction Study

The reduction of Fe^{3+} ions by flavonoids was studied at pH 5.5, due to the low solubility of iron at physiological pH. In aqueous solution Fe^{3+} exists as the hydrated ion $Fe(H_2O)_6^{3+}$ which, except at low pH, hydrolyses and polymerises, precipitating hydrated ferric oxides.[41] The reduction of iron was monitored using the chromophoric chelator ferrozine which strongly binds Fe^{2+}, forming a stable complex with a high extinction coefficient. Our results (Table I) show that only flavones myricetin and quercetin reduced Fe^{3+} effectively. Rutin, catechin and taxifolin were moderately active and kaempferol and luteolin were relatively poor reductants. All other flavonoids studied showed no activity.

Cu^{2+} Reduction Study

The reduction of Cu^{2+} by flavonoids was measured using the bathocuproinedisulfonic acid (BCDS), which forms a complex with Cu^{+}. As can be inferred from the values represented in Table II, flavonoids reduce copper ions with a high stoichiometry. Myricetin, with six hydroxyl groups, was the better reductant followed by the flavonoids with five hydroxyl groups quercetin (flavone), taxifolin (flavanone) and catechin (flavanol). The flavones

TABLE I Fe^{3+} reducing activity of flavonoids

Classes	Compounds	Substituents	μmoles Fe^{2+}/μmole flavonoid
Flavones	Myricetin	3, 5, 7, 3′, 4′, 5′–OH	1.2 ± 0.0
	Quercetin	3, 5, 7, 3′, 4′–OH	1.0 ± 0.0
	Kaempferol	3, 5, 7, 4′–OH	0.1 ± 0.0
	Luteolin	5, 7, 3′, 4′–OH	0.1 ± 0.0
	Rutin	3-rut, 5, 7, 3′, 4′–OH	0.2 ± 0.0
	Apigenin	5, 7, 4′–OH	0
Flavanol	Catechin	3, 5, 7, 3′, 4′–OH	0.2 ± 0.0
Isoflavones	Genistein	5, 7, 4′–OH	0
	Daidzein	7, 4′–OH	0
Flavanones	Taxifolin	3, 5, 7, 3′, 4′–OH	0.3 ± 0.0
	Naringenin	5, 7, 4′–OH	0
	Naringin	7-ramn, 5, 4′–OH	0

Values represent means ± standard deviation (SD) of at least four independent experiments.

luteolin (5,7,3′,4′–OH) and kaempferol (3,5,7,4′–OH) show the same Cu^{2+} reducing activity and apigenin (5,7,4′–OH) has about half of their activities. The flavone apigenin and the isoflavone genistein possessing the same hydroxyl groups (5,7,4′–OH) were less active than the flavonoids with four hydroxyl groups; the flavanone naringenin (5,7,4′–OH), however, was twice more effective on reducing the copper ions.

Chelation Studies

Spectrophotometric Study

The flavonoid spectra typically consist of two absorption maxima in the ranges 240–285 nm (band II) and 300–550 nm (band I) depending on hydroxyl substitution. In our study, we examined a large group of flavonoids from different structural classes, flavones, flavanones, isoflavones and a flavanol (Fig. 1), in order to test their ability to chelate Cu^{2+} and Fe^{3+} at both pH 7.4 and pH 5.5. Our results showed that spectral changes were observed for flavones with Cu^{2+} ions at both pH values and with Fe^{3+} at pH 5.5 only, due to the solubility problems of iron.[41] Small modifications in the absorption spectrum of the flavanol catechin were also observed upon addition of Cu^{2+} at pH 7.4. The spectra of isoflavones (daidzein and genistein) and flavanones (taxifolin, naringenin and naringin) were unchanged.

The interactions of the flavones myricetin, quercetin, luteolin, kaempferol, and apigenin with equimolar concentrations of Cu^{2+} at pH 7.4 produced large bathochromic shifts in band I maxima which were associated with decreases in absorbance, very significant for kaempferol and apigenin. In addition, it was observed that for myricetin and quercetin (Fig. 2a) the bathochromic shifts led the band I maxima to shift to about the same wavelength (436 nm), for luteolin and kaempferol to 408 nm, whereas for apigenin the band I becomes a shoulder (408 nm) (Fig. 2b). The bathochromic shifts and decreases in absorbance of band II maxima recorded were smaller. Under similar conditions, band I of the rutin spectrum also demonstrated a bathochromic shift to about 408 nm but with an increase in absorbance. Band II demonstrated a very small hypsochromic shift and a small increase in absorbance too.

At a lower pH (pH 5.5) the band I of flavones myricetin, quercetin, kaempferol, luteolin, and apigenin undergoes a large bathochromic shift to about the same wavelength (412 ± 4 nm), with

TABLE II Cu^{2+} reducing activity of flavonoids

Classes	Compounds	Substituents	μmoles Cu^{+}/μmole flavonoid
Flavones	Myricetin	3, 5, 7, 3′, 4′, 5′–OH	12.5 ± 0.4
	Quercetin	3, 5, 7, 3′, 4′–OH	9.2 ± 0.2
	Luteolin	5, 7, 3′, 4′–OH	6.7 ± 0.5
	Kaempferol	3, 5, 7, 4′–OH	6.6 ± 0.6
	Rutin	3-rut, 5, 7, 3′, 4′–OH	8.6 ± 0.3
	Apigenin	5, 7, 4′–OH	3.3 ± 0.9
Flavanol	Catechin	3, 5, 7, 3′, 4′–OH	9.4 ± 1.0
Isoflavones	Genistein	5, 7, 4′–OH	4.0 ± 1.0
	Daidzein	7, 4′–OH	2.7 ± 0.5
Flavanones	Taxifolin	3, 5, 7, 3′, 4′–OH	9.4 ± 0.4
	Naringenin	5, 7, 4′–OH	7.1 ± 1.1
	Naringin	7-ramn, 5, 4′–OH	2.4 ± 0.4

Values represent means ± standard deviation (SD) of at least four independent experiments.

significant decreases in absorbance. These bands became shoulders for luteolin and apigenin (Fig. 3). Band II demonstrated a small bathochromic shift and a small decrease in absorbance. Band I and band II of the rutin spectrum showed small decreases in absorbance.

The interaction of the flavanol catechin with an equimolar concentration of Cu^{2+} at pH 7.4 produced a bathochromic shift of 16 nm that led the small band I maxima to 296 nm with a small increase in absorbance.

Upon the addition to flavones, at pH 5.5, of an equimolar concentration of Fe^{3+}, significant spectral changes occurred in the case of myricetin and quercetin only. Band I maxima of myricetin and quercetin showed large bathochromic shifts (56 nm) to about the same wavelength (426 nm) that were associated with large decreases in absorbance. Band II presented smaller bathochromic shifts (14 nm) with decreases in absorbance (Fig. 4a). Luteolin, rutin and kaempferol band I maxima showed small bathochromic shifts (14, 10 and 6 nm, respectively) with large decreases in absorbance (Fig. 4b). The band I of kaempferol, however, presented a shoulder between 410–430 nm. On doubling the Fe^{3+} concentration, the band I maxima of luteolin and rutin are shifted 45 and 20 nm, respectively. In these conditions, the shoulder of kaempferol becomes a peak at 410 nm, i.e. the band I maximum of the kaempferol spectrum was shifted 45 nm after the addition of iron. For apigenin the band I does not undergo a bathochromic shift but shows a shoulder between 390–430 nm.

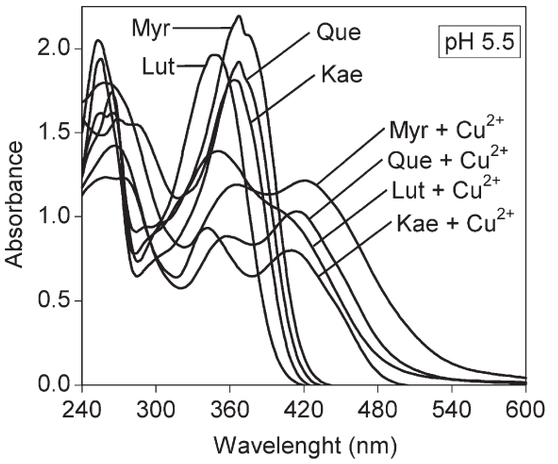

FIGURE 3 Absorption spectra of flavonoids myricetin, quercetin, luteolin, and kaempferol and Cu-flavonoid complexes at pH 5.5. The absorption spectra of flavonoids (100 μM) in 50 mM acetate buffer (pH 5.5) were recorded against the buffer and the differential spectra of Cu-flavonoid complexes against the buffer solution with Cu^{2+} (100 μM).

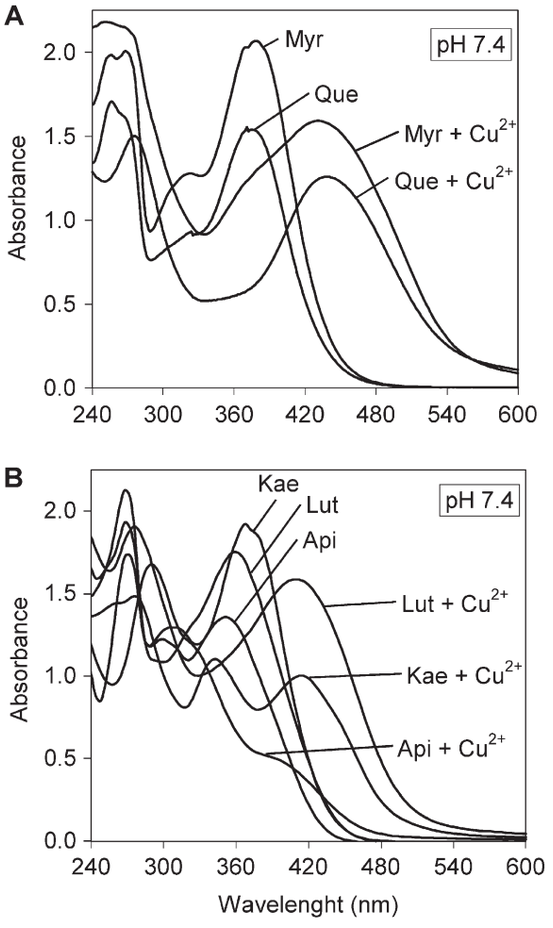

FIGURE 2 Absorption spectra of flavonoids and Cu-flavonoid complexes at pH 7.4. (A) myricetin and quercetin; (B) luteolin, kaempferol, and apigenin. The absorption spectra of flavonoids (100 μM) in 50 mM MOPS buffer (pH 7.4) were recorded against the buffer and the differential spectra of Cu-flavonoid complexes against the buffer solution with Cu^{2+} (100 μM).

Electrospray Ionisation Mass Spectrometry (ESI-MS) Study

Under ESI-MS conditions, at pH ≤5.5, all the flavonoids studied by this technique form complexes with copper and iron with the stoichiometries 1:1 and 1:2 (metal ion/flavonoid). With copper, these complexes correspond to the ions $[Cu(II) + (M - H)]^+$ and $[Cu(II) + M + (M - H)]^+$. The flavonoid molecule and its deprotonated form are symbolised by M and (M − H), respectively. In Fig. 5, the more abundant complex of kaempferol with copper, whose stoichiometry is 1:2 $[Cu(II) + M + (M - H)]^+$, is shown. In all cases, the relative intensities of the isotopic peaks of the copper-containing species agree within experimental error with the naturally occurring abundances, providing further support for the above assignments.

In the case of iron complexes, the metal presents mainly two oxidation states, Fe^{2+} being preferred with myricetin, Fe^{2+}/Fe^{3+} with quercetin, and Fe^{3+}

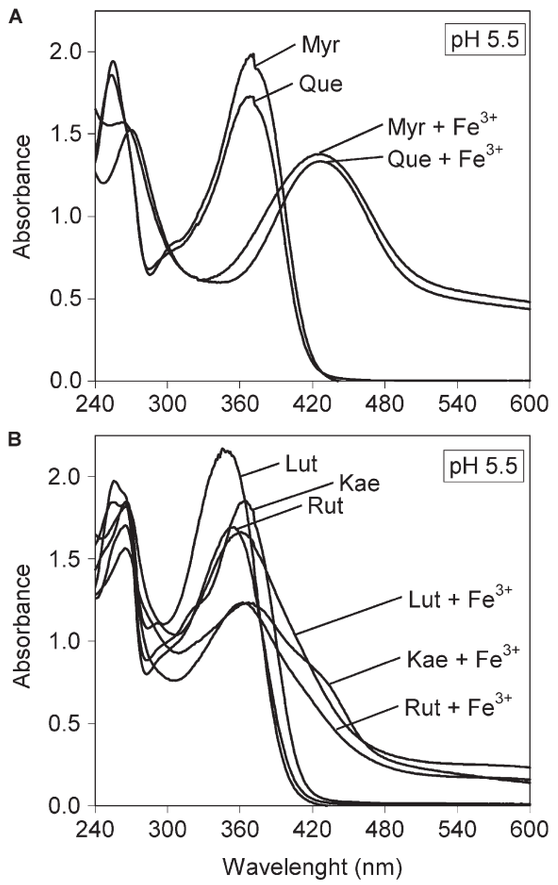

FIGURE 4 Absorption spectra of flavonoids and Fe-flavonoid complexes at pH 5.5. (A) myricetin and quercetin; (B) luteolin, rutin, and kaempferol. The absorption spectra of flavonoids (100 μM) in 50 mM acetate buffer (pH 5.5) were recorded against the buffer and the differential spectra of Fe-flavonoid complexes against the buffer solution with Fe^{3+} (100 μM).

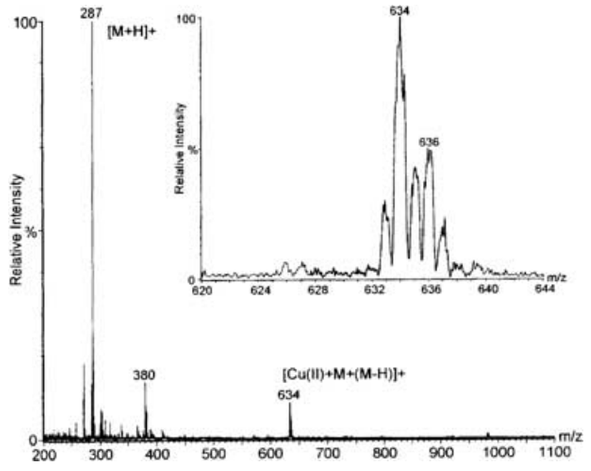

FIGURE 5 Electrospray mass spectrum of a solution of cupric chloride and kaempferol. Peak of m/z 634 corresponds to $[Cu(II) + M + (M - H)]^+$ ion M represents the mass of the flavonoid molecule and (M − H) its deprotonated form. The inset presents the isotopic copper pattern for $m/z = 634$.

with the other flavonoids. The oxidation states are inferred from the combination of the charges of the species involved in the complexes and considering the observed, mass to charge ratio, m/z, of the complex ion. The complex ions formed were of the type $[Fe(II) + (M - H)]^+$ and $[Fe(III) + (M - 2H)]^+$, for the stoichiometry 1:1 (M − 2H), standing for the loss of two protons from the flavonoid molecule. For stoichiometry 1:2 the ions were $[Fe(II) + M + (M - H)]^+$ and $[Fe(III) + 2(M - 2H)]^+$. Figure 6 illustrates mainly complex ions of stoichiometry 1:2 for quercetin with Fe^{2+}/Fe^{3+} (Fig. 6a) and for kaempferol with Fe^{3+} (Fig. 6b). This latter spectrum also contains a much less intense peak at $m/z = 966$ which corresponds to the stoichiometry 2:3. Some other stoichiometries, corresponding to less intense peaks such as 2:2 (myricetin and kaempferol), 2:3 (luteolin) and 1:3 (quercetin) with copper and 2:3

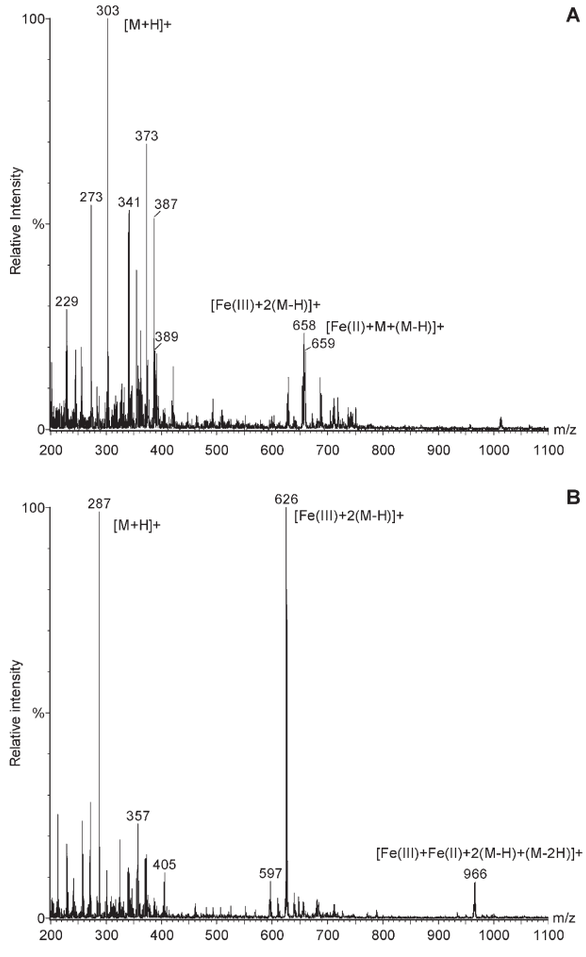

FIGURE 6 Electrospray mass spectrum of a solution of ferric chloride and quercetin (A). The peak at $m/z = 659$ corresponds to the ion $[Fe(II) + M + (M - H)]^+$. Electrospray mass spectrum of a solution of ferric chloride and kaempferol (B). The main complex peak at $m/z = 626$ corresponds to the ion $[Fe(III) + 2(M - H)]^+$ and a less intense peak at $m/z = 966$ corresponds to the ion $[Fe(III) + Fe(II) + (M - 2H) + 2(M - H)]^+$.

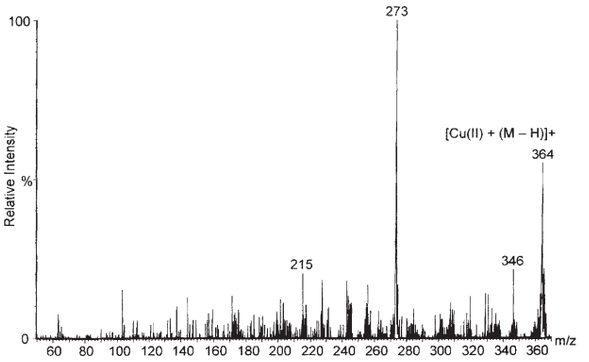

FIGURE 7 CID mass spectrum of m/z 364 corresponding to $[Cu(II) + (M - H)]^+$ ion from a solution of cupric chloride and quercetin. The ion at $m/z = 273$ results from the loss of CuCO from the ion $[Cu(II) + (M - H)]^+$ at $m/z = 364$.

(myricetin and luteolin) with iron, were also observed in other spectra not shown.

In general, collision induced decomposition (CID) spectra showed losses of H_2O, CO, and CuCO, from 1:1 (metal/flavonoid) complexes. The loss of CuCO giving the ion at $m/z = 273$ is prominent in Fig. 7, for quercetin complexed with Cu^{2+} ($m/z = 364$).

The oxidation of myricetin and quercetin in the presence of iron is also inferred from the loss of H_2 from the flavonoids in association with the reduction of iron, detected through its oxidation state in the complex.

DISCUSSION

Our results clearly demonstrated that from the interaction of flavonoids with iron and copper ions can result the formation of chelates and the reduction of the metal ions, both depending on flavonoid structures.

All flavonoids studied show higher reducing capacity for copper ions than for iron ions. This can be rationalized in terms of the standard redox potentials of the metals. The standard reduction potential of the Cu^{2+}/Cu^{+} couple (+0.15 V) is much lower than that for Fe^{3+}/Fe^{2+} couple (+0.77 V).

Only the flavones myricetin (3,5,7,3′,4′,5′–OH) and quercetin (3,5,7,3′,4′–OH) presented a high Fe^{3+} reducing activity, providing evidence for the importance of the simultaneous presence of both the catechol group in the B-ring and the 3-hydroxyl group in C-ring. The presence of the 2,3-double bond in conjugation with the 4-oxo group in the C ring is also particularly important for Fe^{3+} reducing activity, as become evident by the comparison of quercetin with catechin and taxifolin structures.

The Cu^{2+} reduction studies show that a large number of copper ions per molecule of flavonoid were reduced. It seems that for some flavonoids the number of electrons involved in their oxidations is higher than those expected from the number of –OH groups. This unusually large number of electrons for the oxidation of flavonoids may be ascribed to a reproduction of –OH groups by oxidative polymerisation.[49] The copper reducing activity seems to depend largely on the number of hydroxyl groups present in the molecules, having their basic structures a small influence. In fact myricetin (3,5,7,3′,4′,5′–OH) was the better reductant followed by the flavonoids with five hydroxyl groups quercetin (flavone), taxifolin (flavanone) and catechin (flavanol). As the last three flavonoids belong to different classes, this suggests that the 2,3-double bond in the C-ring is not important for the reducing activity. The much higher Cu^{2+} reducing activity displayed by the flavones luteolin (5,7,3′,4′–OH) and kaempferol (3,5,7,4′–OH) compared with that shown by apigenin (5,7,4′–OH), again, emphasize the importance of the catechol structure in the B-ring and of the 3-hydroxyl group for a good reducing activity of flavonoids.

In relation to the formation of chelates, and according to the literature there are three possible metal-complexing sites within a flavonoid molecule containing hydroxyl groups at 3, 5, 3′, and 4′ positions. These sites are between the 3-hydroxyl group and the 4-oxo group, the 5-hydroxyl group and the 4-oxo group, and between the *ortho*-hydroxyl groups in the B-ring.[22] Our results showed that among the different classes of flavonoids studied the major spectral changes occurred on interaction of flavones with metal ions. This can be explained by the fact that in flavones the presence of the 2,3-double bond increases the planarity of the molecule. This double bond in the C-ring confers higher rigidity to the ring and holds the A and C rings in a more coplanar position allowing the 3-hydroxyl/4-oxo groups and 5-hydroxyl/4-oxo groups to be closer.

Based on spectrophotometric studies, the formation of metal–flavone chelates is indicated by the appearance of new peaks in the spectra. From the interaction of flavones kaempferol (3, 5, 7, 4′–OH), luteolin (5, 7, 3′, 4′–OH) and apigenin (5, 7, 4′–OH) with Cu^{2+} ions, peaks at about 408 nm appeared in their spectra at both pH 7.4 and pH 5.5. This observation suggests that at both pH values these three flavones probably chelate Cu^{2+} ions at the same site. The chelation site common to these flavones is between the 5-hydroxyl group and the 4-oxo group.

When Cu^{2+} ions are added to myricetin (3, 5, 7, 3′, 4′, 5′–OH) and quercetin (3, 5, 7, 3′, 4′–OH) at pH 7.4, the band I of these flavonoids is shifted to 436 nm. Since these flavonoids possess the three possible metal-complexing sites, the shift of band I to a longer wavelength may reflect the contribution of the three copper-complexing sites. At pH 5.5, however, after

the addition of Cu^{2+} ions, band I of these flavones undergoes a shift to about the same wavelength as the shifts observed for kaempferol, luteolin and apigenin. Therefore, it seems that at pH 5.5 all those flavones interact with Cu^{2+} ions through the same site, most probably between the 5-hydroxyl and the 4-oxo groups. These arguments lead to the conclusion that quercetin and myricetin should have more chelation sites at pH 7.4 than at pH 5.5. In fact, the complexing ability of a catechol-type B ring increases as the pH becomes more alkaline.[22] In summary, our experimental data in solution support the idea that for chelation of copper the site between the 5-hydroxyl group and the 4-oxo group is the most important chelating site. Our results are in agreement with those of Thompson and Williams[50] who found that the stability of the complex is higher in case of chelation via 5-OH, this probably being associated with the stability of the six-membered ring over that of the five-membered ring complexes.

The flavanol catechin spectrum also presented modifications after the addition of Cu^{2+} ions at pH 7.4. Since catechin lacks the 4-oxo group we can infer that catechin chelates Cu^{2+} through the *ortho*-catechol group. At pH 5.5, however, catechin does not chelate copper ions. This behaviour of catechin is in accordance with the fact that the complexing ability of the catechol group increases as the pH increases.

The ESI-MS studies provided a direct evidence for the formation of complexes of various stoichiometries, among which the 1:1 and 1:2 (metal/flavonoid) had previously been considered as the more likely.[22] Moreover, with the exception of catechin, all the flavonoids studied by ESI-MS lose CuCO as indicated by CID experiments. These losses occur in flavonoids that have in common the 4-oxo group and either the 3 or 5-hydroxyl group as opposed to catechin which does not possess the 4-oxo group. This reinforces our prediction of chelation at the 4-oxo and 5-OH groups, based on spectrophotometric measurements, since luteolin, which does not have the 3-OH group, also shows chelation of Cu^{2+}. The behaviour of catechin confirms that the preferred chelation site must involve the 4-oxo group and either one of the 5-OH or 3-OH groups. When these pairs are not available, chelation will take place at the 3′ and 4′–OH groups. On the other hand, the CID mass spectrum of the complex catechin/copper with stoichiometry 1:1 contains an intense peak due to ion Cu^{+}. This result suggests that the complex catechin/copper might be very labile in acid medium[51] and might explain why this complex at pH 5.5 was not observed in our spectrophotometric studies.

In the chelation studies of iron to flavonoids at pH 5.5 we observed that only the flavones myricetin and quercetin at an equimolar concentration present a large bathochromic shift. At this acid medium, the complexation site is probably between the 5-hydroxyl group and the 4-oxo group. In fact, the chelating ability of the catechol group decreases as the pH decreases.[22] This is probably because the chelate formation requires both 3′, 4′–OH groups to be dissociated and the extent of dissociation may differ. In addition, the dissociation pattern of individual hydroxyl groups occurs in the sequence 7–OH > 4′–OH > 5–OH[52] predicting a more favoured dissociation for the 5-OH than for 3-OH group. All the other flavones present a moderate interaction with Fe^{3+}. This can be rationalized in terms of the number of hydroxyl groups. In fact, luteolin, rutin and kaempferol possess fewer –OH groups than quercetin resulting in a lower negative charge density at the chelation site. Furthermore, we observed that, at pH 5.5 and equimolar concentrations, all the flavones studied chelate Fe^{2+} (data not shown). This suggests that flavonoids chelate iron more effectively when the metal ion is in its bivalent form, which would mean that the flavonoid needs to reduce Fe^{3+} to Fe^{2+}, before association. The results of the Fe^{3+} reduction study by flavonoids, in fact, demonstrated that only the flavones myricetin and quercetin reduce Fe^{3+} at pH 5.5 and this does explain why they were the more effective flavones in iron chelation.

The complexation of iron and the various flavonoids at pH $\leq$ 5.5 was also observed by means of ESI-MS, the stoichiometries 1:1 and 1:2 (metal ion/flavonoid) being the most favoured, leading to complexes of higher abundance. In this case, experiments at high cone voltages were performed, rather than CID experiments, for kaempferol and myricetin, leading to the loss of FeCO. This observation provided extra confirmation of the chelation site being located between the 4-oxo and either the 3- or 5-hydroxyl groups.

Another important observation obtained by MS was that the most abundant complexes of quercetin and myricetin with iron involve Fe^{2+}, while kaempferol, luteolin and catechin complexes preferentially involve Fe^{3+}. These data confirm our assumption, based on the spectrophotometric studies, that quercetin and myricetin chelate Fe^{2+} since they are the only flavones able to reduce Fe^{3+} to Fe^{2+}.

When one compares the ability of flavones to chelate copper and iron, it is important to note that the degree of complexation follows the order: $Cu^{2+} > Fe^{2+} > Fe^{3+}$. This order is to be expected, based on the prediction of Crystal Field Theory of an "extra" stabilization energy, due to the splitting of the d orbitals on forming the complex.[53] This energy is dependent on Δ, the ligand field splitting. Since these ions form high-spin octahedral complexes having d^9 (Cu^{2+}), d^6 (Fe^{2+}), and d^5 (Fe^{3+}) configurations, the stabilization energies decrease,

respectively, in the following order $3/5\Delta_o$, $2/5\Delta_o$ and 0 (Δ_o values are typically about 10,000–2000 cm^{-1} for divalent and trivalent ions).

In conclusion, when flavonoids reduce transition metal ions, it is assumed that these flavonoids can exert pro-oxidant effects on promoting Fenton or Haber–Weiss reactions. We demonstrated, however, that those flavonoids also show capacity to chelate iron and copper. The chelation of metal ions by flavonoids may render those ions inactive in generating radicals or, alternatively, the generated radicals will be intercepted by the flavonoids themselves. Therefore, the metal chelating properties of flavonoids support the assumption that these compounds may play an important role in metal-overload diseases such as Wilson's disease (copper overload) and hemochromatosis (iron overload).[54] It is noteworthy that in the plasma of iron-overload patients non-transferrin-bound iron is present, apparently as complexes with citrate and acetate.[55] In addition, the chelating properties of flavonoids may also be important in all oxidative stress conditions where a transition metal ion is involved. One of such conditions is atherosclerosis where the oxidative modifications of LDL *in vivo* appear to involve reactive oxygen species and transition metal ions such as copper.[56]

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