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On the ability of four flavonoids, baicilein, luteolin, naringenin, and quercetin, to suppress the fenton reaction of the iron-ATP complex

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Abstract

Four flavonoids, baicilein, luteolin, naringenin, and quercetin were investigated for their ability to suppress the Fenton reaction characteristic of the iron-ATP complex. Absorption spectroscopy indicates that under the conditions of 18.75% aqueous methanol, 0.0625 mM HEPES pH 7.4 buffer and 1.5:1 quercetin/iron-ATP ratio a mix ligand complex formed. All four flavonoids were found to interfere with the voltammetric catalytic wave associated with the iron-ATP complex in the presence of H_2O_2. Quercetin and luteolin were able to completely suppress the catalytic wave of the iron-ATP/H_2O_2 system when a minimum ratio of 1.5:1 of the flavonoid to iron-ATP was reached. At this ratio, the ability of the studied series of flavonoids to suppress the Fenton reaction characteristic of iron-ATP follows as quercetin $\approx$ luteolin $>$ naringenin $\approx$ baicilein. Both quercetin and luteolin contain catechol on the B ring, which may enhance the iron chelation of these species over baicilein and naringenin. The common structural feature of all of these flavonoids is the 4-keto, 5-hydroxy region, which may also contribute to the chelation of iron.

Introduction

Flavonoids are a group of polyphenols that are found widely in fruits and vegetables. Many of the flavonoids are widely described as antioxidants and have many types of pharmacological actions. Presently, a molecular-level basis for their antioxidant action or actions has not been fully described. Radical scavenging and iron chelation are the most frequently mentioned mechanisms in literature. Flavonoids are quite efficient with respect to oxygen radical scavenging (Husain *et al.* 1987; Rice-Evans & Miller, 1996). However, the aqueous solubility of these species, which is in the micromolar range, may be too low to protect effectively physiological species from free radical damage (de Groot & Rauen 1998; Deng *et al.* 1997; Sestili *et al.* 1998; Robak *et al.* 1998; Yasudisa *et al* 1998). Furthermore, the rates of radical scavenging by the flavonoids are very similar to all organic species. The metal binding properties of flavonoids offers another form of antioxidant action by encapsulation of pro-oxidant iron species, which generate hydroxyl radical species through the Fenton reaction (Ferrali *et al.* 1997; Morel *et al.* 1994; Moran *et al.* 1997; Afanas'ev *et al.* 1989; Yoshino *et al.* 1998; Gao *et al.* 1995). Although this has been hypothesized for some time, there are no molecular-level observations confirming this feature. The electrochemical behavior of iron-flavonoid species is therefore the subject of this study, since this characteristic would be able to characterize the Fenton reaction properties of the complex (Zhao *et al.* 1996; Cheng *et al.* 1996). Another aspect of this investigation is to discover which of the functional groups and regions of the flavonoids are important to the chelation of iron.

Previous electrochemical studies of the flavonoids primarily have focused on the voltammetric characteristics of this species and not on the metal complexes (Jørgensen *et al.* 1998; Vachàlkovà *et al.* 1997; Muralidharan *et al.* 1993; Chiavari *et al.* 1988; Lunte *et al.* 1988; Rapta *et al.* 1995; van Acker *et al.* 1996). Hendrickson and coworkers were able to identify three major set of voltammetric peaks on the flavonoid ring system (Hendrickson *et al.* 1994). Flavonoids with catechol 3′, 4′ hydroxy groups (Figure 1) have a set of cyclic voltammetric peaks appear within the potentials

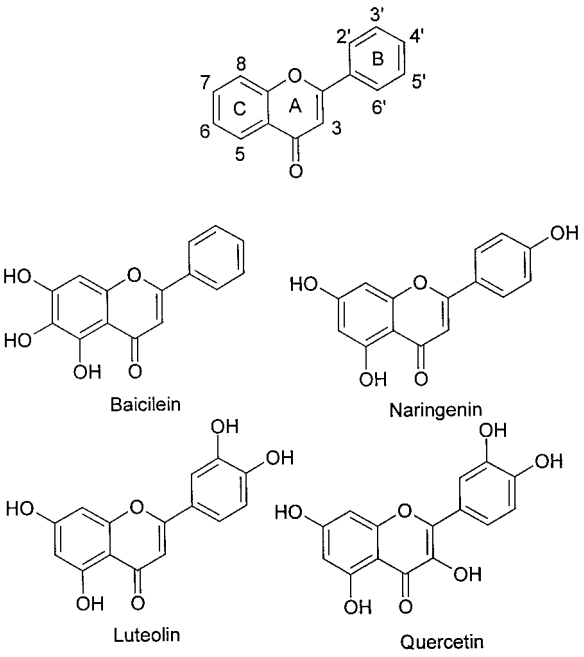

Figure 1. Flavonoids used in this study.

of 100 to 350 mV versus Ag/AgCl due to the oxidation of these species. These peaks are coupled to a chemical reaction of the oxidized catechol group. A second irreversible oxidative peak is attributable to the oxidation of the 3-hydroxy flavonoids at about 500 mV. A third wave is assigned to an irreversible oxidation of the 5, 7 dihydroxy groups. Electrochemical investigations of the antioxidant action of the flavonoids have found a possible correlation between the cyclic voltammetric oxidation peak potential of the 3′, 4′ or 4′ hydroxy groups of individual flavonoids with antioxidant action (Moran *et al.* 1997; van Acker *et al.* 1996; Hendrickson *et al* 1994).

Literature regarding the quantitative aspects of iron chelation is sparse. A study by van Acker et al, classified flavonoids on their ability to displace EDTA from Fe^{2+} in an aqueous 5% DMSO, pH 7.4, 50 mM phosphate buffer solution (van Acker *et al.* 1996). There are no reports of metal-flavonoid binding constants from 1967 to the present. An electrochemical study of metal-flavonoid complexes isolated from food reports small anodic shifts of the flavonoid voltammetric waves (Weber 1988). To our knowledge there are no other studies regarding the electrochemical properties of metal-flavonoid complexes.

Cyclic voltammetry is an ideal method for the study of the Fenton reaction activity of metal-flavonoid complexes (Zhao *et al.* 1994; Cheng *et al.* 1996). This is made possible by a catalytic reduction wave attributable to the following set of electrochemical-chemical (EC′) mechanism, which occur in the presence of a suitable iron complex (Bret & Bret 1993).

$$Fe^{III} + e = Fe^{II}, \quad (1)$$

$$H_2O_2 + Fe^{II} * = Fe^{III} + HO^- + HO^{\cdot}. \quad (2)$$

Due to the slow kinetics of the electro-reduction of hydrogen peroxide and the combination of the steps above, an amplified current due to the reduction of Fe(III) to Fe(II) results. The Fe(III)-ATP complex is a good example of a species that participates in this reaction sequence. Thus The Fe^{III}ATP-H_2O_2 system has a well characterized EC′ electrode mechanism (Zhao *et al.* 1994). Furthermore this complex is of interest since it is mentioned quite often as a physiological low-molecular iron species (Weaver *et al.* 1993, 1989; Lovstad 1992; Zhelyaskov 1992; Rao & Cederbaum 1997; Gurgueira 1996; Anghileri & Thouvenot 1997; Anghileri *et al.* 1997). It is important to note that at neutral pH another potent oxidizing agent, the ferryl species, is produced in competition with Reaction 2 (Pierre & Fontecave 1999).

In this investigation we studied the inhibition of the voltammetric catalytic wave of Reactions 1 and 2 by various flavonoids. Quercetin, luteolin, baicilein, and naringenin (4′,5,7-trihydroxyflavanone) were selected in order to gain structure-activity relationships (Figure 1). All four flavonoids have a common feature of 4-keto, 5 and 7 hydroxy groups. The 4-keto, 5 hydroxy region is a possible metal chelation site. Quercetin and luteolin have catechol groups on the B-ring, which are also logical metal binding sites. Baicilein contains 5, 6 and 6, 7 catechol-like groups that may aid in metal binding. Barring a chemical reaction to another form, naringenin contains only the 4-keto, 5 hydroxy binding site.

Materials and methods

Chemicals

Baicalein, 4′,5,7-trihydroxyflavanone, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Aldrich Chemical Co., Milwaukee, WI, USA), luteolin, adenine triphosphate (ATP), catechol (Sigma Chemical Co., St. Louis, MO, USA), 30% hydrogen peroxide, ferric nitrate (Fisher Scientific, Pittsburgh, PA,

USA) and quercetin (Acros, USA), were as received. Methanol (Fisher Scientific) was of HPLC grade and used without further purification. All solutions in this study were 18.75% (v/v) methanol and made using 18 MΩ-cm quality water obtained from a Millipore-Q system. The buffer system was 0.0625 M HEPES adjusted to pH 7.4 with concentrated HCl. Ferric-ATP complex was formed by slowly adding pH 7.4 HEPES buffered aqueous solution to dry ATP and ferric nitrate (2:1) and vigorous agitation. Insoluble ferric hydroxides were not observed using this method. The solution was then refrigerated and used during the course of several weeks. The efficacy of the ferric-ATP solution was tested by cyclic voltammetry in the presence and in the absence of hydrogen peroxide. No degradation of the buffered ferric-ATP solution was noticed during the course of this investigation.

Solutions

In this study all solutions had a composition of 18.75% methanol in water which was buffered at pH 7.4 by 0.0625 M HEPES. Depending on the study solutions may have had the concentrations of the following species, 0.25 mM Fe^{3+}, 0.50 mM ATP, 0.375 mM flavonoid, and 8.6 mM H_2O_2.

Electrodes

The carbon disk working electrode (3 mm diameter) and Ag/AgCl reference electrode were purchased from Bioanalytical Systems (West Lafayette, IN, USA). The carbon disk was polished with an aqueous slurry of 1.0 micron alumina between voltammetric runs unless specified in the Results section.

Equipment

All voltammetric experiments were conducted on a Bioanalytical Systems Model CV-50W potentiostat controlled by a Pentium class personal computer. Cyclic voltammetric sweep rates were kept at 20 mV/second and the laboratory temperature was 22 ± 2 °C. Optical absorbance experiments were carried out on a Hewlett-Packard HP 8453 UV-vis photodiode array.

Results

Iron-ATP complex is a well-known redox cycling agent for the Fenton reaction and thus this complex

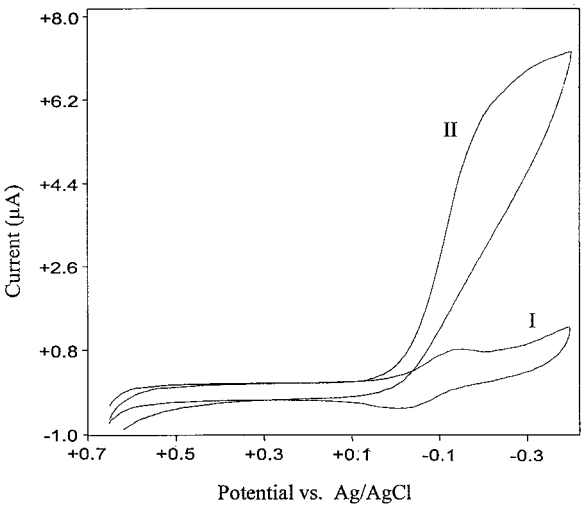

Figure 2. Cyclic voltammograms of 0.25 mM Fe^{III}ATP at a carbon disk electrode in 18.75% aqueous methanol, 0.0625 mM HEPES pH 7.4 buffer at 20 mV/s. Bottom) 0 mM H_2O_2, Top) 8.6 mM H_2O_2.

was used throughout this study as a model for low molecular weight iron species (Zhao et al. 1994). Cyclic voltammetry of (0.25 mM Fe^{3+}, 0.51 mM ATP) this complex in an aqueous, buffered at pH 7.4, 18.75% methanol (v/v) solution results in a quasi-reversible set of waves and is very similar to reported results in pure aqueous solutions (Figure 2) (Zhao *et al.* 1994). In a large excess of H_2O_2 (8.6 mM) a large reduction wave is observed with an absence of any wave due to the oxidation of Fe^{II}ATP (Figure 2). This is consummate with the EC′ mechanism outlined in the Introduction and in the pure aqueous system of a previous study (Zhao *et al.* 1994). No voltammetric wave due to the reduction of H_2O_2 was observed in the absence of Fe^{III}ATP. The height of the catalytic voltammetric wave was averaged over the course of six runs and found to be 7.22 μA at −0.400 V.

Quercetin (37.9 μM) exhibits a slight yellow color with a UV absorption peak of 375 nm in pH 7.4 HEPES buffer (Figure 3). The mixture of 25.3 μM Fe^{III} with 37.9 μM quercetin results in a brown complex with an absorption peak shifted to 389 nm and an increased broadband absorption extending to 875 nm. The results are similar to those reported in another study using an aqueous 5% dimethylsulfoxide pH 7.4 phosphate buffer solution (van Acker *et al.* 1996). A combination of 37.9 μM quercetin to pH 7.4 HEPES buffered 25.3 μM Fe^{III}ATP (50.0 μM ATP) solution results in a brown complex similar to the color observed in the Fe^{III}-quercetin system. However, the

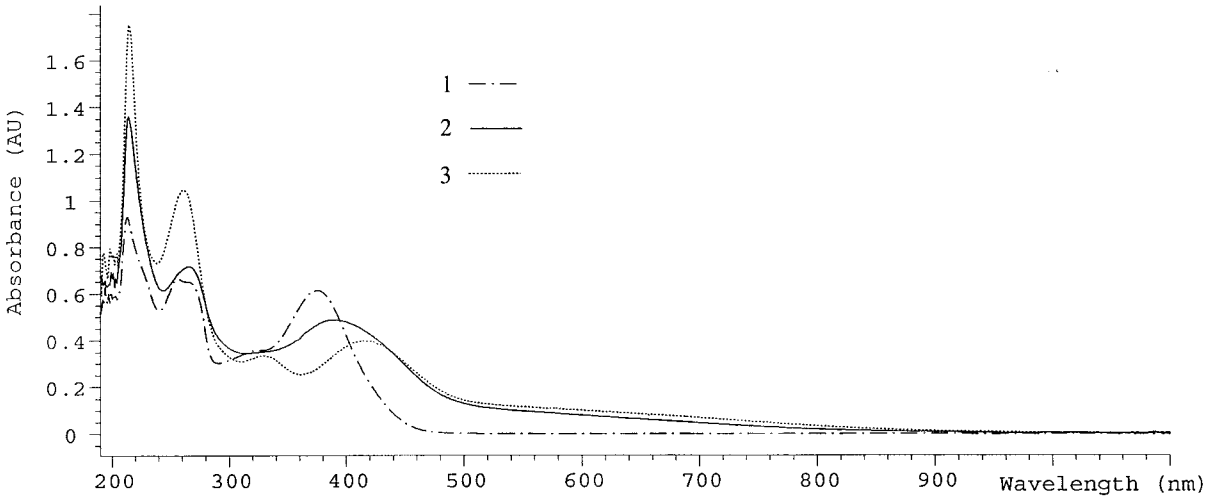

Figure 3. UV-visible absorption characteristics of (1) 37.9 μM quercetin, (2) 25.3 μM Fe^{III}, 37.9 μM quercetin (3) 25.3 μM Fe^{III}, 37.9 μM quercetin, 50 μM ATP (bottom). Other conditions 1.9% methanol with pH 7.4 6.25 mM HEPES. 50 μM ATP and 25.3 μM Fe^{III}, 50.0 μM ATP HEPES were found to be optically transparent between 300 to 800 nm.

mixed ligand system has two peaks at 325 and 415 nm. Neither Fe^{III}ATP nor ATP were found to absorb at wavelengths longer than 300 nm. The results indicate that quercetin may form mixed ligand complexes of Fe^{III} in the presence of ATP.

It was assumed that the other flavonoids used in this investigation have the ability to form mixed ligand systems with Fe^{III}ATP based on color changes and on the voltammetric results below. Further evidence of quercetin complexation of Fe^{III}ATP is offered by the cyclic voltammetry of 0.25 mM Fe^{III}ATP in the presence of 0.375 mM quercetin. At this molar ratio of 1.5:1 of quercetin to FeATP the cyclic voltammetric wave associated with the latter disappears, which is an indication of the formation of a new complex. Comparison of Figures 2 and 4 demonstrate the loss of $Fe^{II/III}$ATP voltammetry in the presence of excess quercetin. Voltammetry of the other studied flavonoids, baicilein, luteolin, and naringenin all followed this trend of forming flavonoid complexes with the Fe^{III}ATP complex at the flavonoid: metal ratio of 1.5:1.

It is interesting to note that there was no identifiable metal centered voltammetric waves solely associated with Fe^{III}quercetin/ATP complex between the voltage limits of 650 to -350 mV versus Ag/AgCl. However, the quercetin voltammetric waves were influenced by the presence of Fe^{III} (Figure 4). The quercetin cyclic voltammetric peak waves were shifted to positive potentials. Both of these observations are consistent with literature (Weber 1988). Luteolin had a larger change in voltammetric characteristics when Fe^{III}ATP is added (Figure 4). The cyclic waves associated with the oxidation-reduction of the catechol groups on the B ring of 0.375 mM luteolin indicate a chemical reaction of the oxidized form. The ratio of the cyclic voltammetric peak currents, $i_{p,a}/i_{p,c}$ is 0.45. In the presence of 0.25 mM Fe^{III}ATP the set of waves are shifted more positively by 120 mV and the cyclic voltammetric $i_{p,a}/i_{p,c}$ ratio becomes a more reversible 0.69. It is plausible that the observed shift in the cyclic voltammetric waves of luteolin and quercetin may be attributable to complexation of iron by this functional group. However, the cyclic voltammetry of the $Fe(catechol)_3^{3-}$ complex indicated no shift in potential and little perturbation of the catechol reduction-oxidation waves.

Naringenin lacks the 6,7 and the 3′,4′ catechol groups of the previous set. This species has a 4′ hydroxy group. It was generally observed that flavonoid species with less than 3 hydroxy groups were not soluble in the solution used in this study (see Experimental). Naringenin lacks any cyclic voltammetric waves between the potentials of 450 to -400 mV. Oxidation of the 5,7 hydroxy groups of the C ring is apparent at an onset potential of 450 mV for the highly irreversible wave. The electrochemical oxidation of naringenin was anodically shifted by about 200 mV in the presence of 1.5:1 molar ratio of this species relative to Fe^{III}ATP.

Baicilein lacks catechol hydroxy groups on the B ring but has a set of nearly reversible waves within

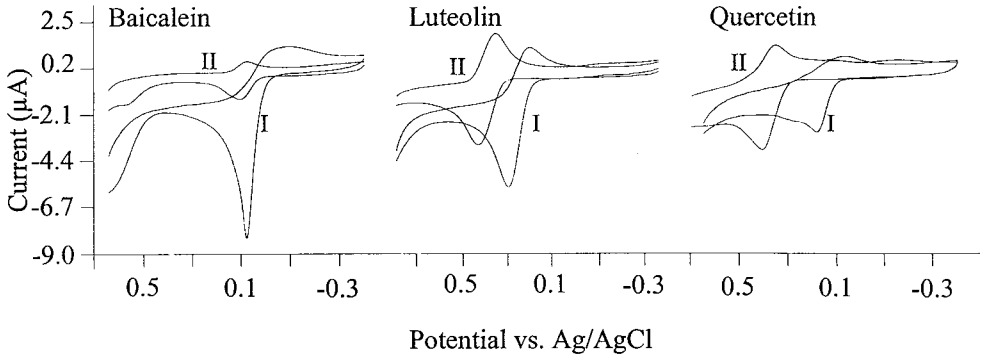

Figure 4. Cyclic voltammetric results of 0.375 mM flavonoid in the absence (I) and in the presence of 0.25 mM $Fe^{III}ATP$ (II). Other conditions were 18.75% aqueous methanol, 0.0625 mM HEPES pH 7.4 buffer at 20 mV/s.

the potential range of 0 to 55 mV, which is normally associated with this group. This set may be due to the oxidation-reduction of 6,7 or 5,6 hydroxy groups on the C ring. This assumption is made based on the observed potential for the completely irreversible oxidation-reduction waves of the 5,7 dihydroxy groups of the other flavonoid species which normally fall positive of 500 mV and that this potential falls into the range normally associated with catechol. The presence of $Fe^{III}ATP$ increased the reversibility of the baicilein catechol groups. The ΔE_p for baicilein decreased from 174 mV to 25 mV in the presence of $Fe^{III}ATP$. The wave heights of baicilein were greatly affected by the presence of $Fe^{III}ATP$. The oxidative peak current for 0.375 mM baicilein decreased from 7.95 to 1.13 μA in the presence of 0.25 mM $Fe^{III}ATP$ and from 1.33 to 0.58 μA for the reductive process.

The Fenton reaction suppression characteristic of each flavonoid species was examined by the ability to moderate the catalytic current of the aforementioned $Fe^{III}ATP$-H_2O_2 system. A type of titration of the $Fe^{III}ATP$-H_2O_2 system was conducted in order to obtain the optimal quercetin/Fe^{3+} molar ratio required for complete Fenton reaction suppression. At a constant $Fe^{III}ATP$ concentration (0.25 mM Fe^{3+}, 0.51 mM ATP) the quercetin/Fe^{3+} molar ratio was varied from 0 to 1.5. The height of the catalytic wave at -0.400 V was measured. The resulting set of voltammograms and a plot of catalytic current as a function of quercetin/Fe^{3+} molar ratio are shown in Figure 5. It is apparent from the plot that complete suppression of the Fenton reaction occurs at a molar ratio of 1.5:1 quercetin:Fe^{3+} (Figure 5). Luteolin, naringenin, and, baicilein were also studied using the same protocol as above. Luteolin was able to completely suppress the Fenton reaction character-

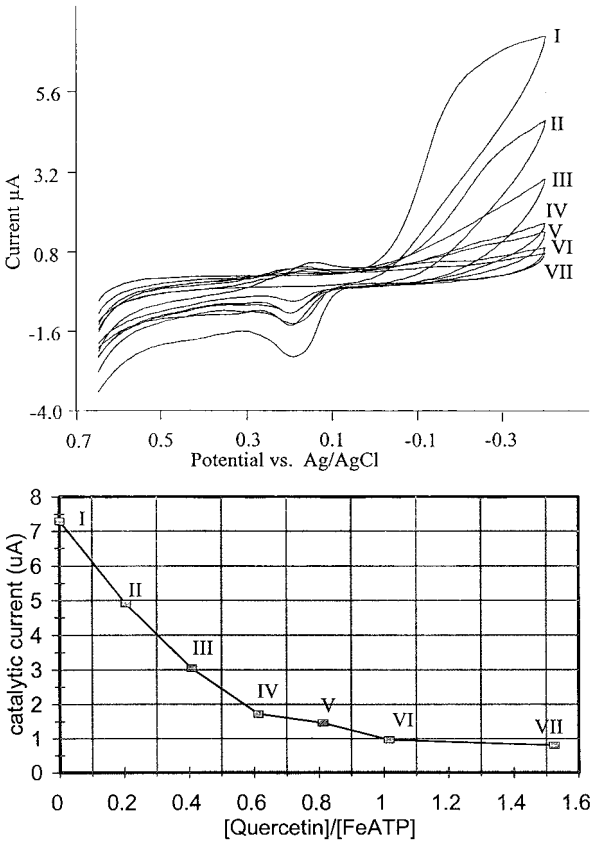

Figure 5. Top) Moderation of the Fenton reaction characteristic of 0.25 mM $Fe^{III}ATP$, 8.6 mM H_2O_2 in 0.0625 M HEPES buffer pH 7.4 by quercetin. The top graph is the cyclic voltammograms taken at 20 mV/s for various quercetin/iron ratios. The quercetin/$Fe^{III}ATP$ ratios (I–VII) are indicated on the *x*-axis of the bottom graph. The catalytic wave at -0.400 V are plotted versus the quercetin/iron ratio in the bottom graph. The current of point VII at -0.400 V is the same as the background current (0.00 mM H_2O_2).

istics of FeATP at the molar ratio of 1.5:1. Baicilein and naringenin were less successful at this task. At the molar ratios of 1.5:1 flavonoid:Fe^{III}ATP, naringenin and baicilein both reduced the catalytic wave by 73% (2.0 μA). The observed reduction in the catalytic wave of the Fe^{III}ATP-H_2O_2 system was not attributable to the irreversible adsorption and passivation by flavonoids to the electrode surface. This is demonstrated by the conditioning of the carbon electrode surface in a solution of 0.5 mM quercetin by repeated scanning between the potentials of 800 to -400 mV at 20 mV/s. After 5-6 scans the conditioned electrode surface was re-immersed into the previously described Fe^{III}ATP-H_2O_2 solution and examined for a catalytic wave. The conditioned surfaces were found to have 80% of a freshly polished electrode's catalytic wave. It is clear that most of the decrease of the catalytic wave due to hydrogen peroxide reduction is due to the complexation of Fe^{III} by the flavonoids. The overall observed trend for the Fenton reaction suppression of the FeATP system is quercetin $\approx$ luteolin $>$ naringenin $\approx$ baicilein.

Discussion

The suppression of the catalytic EC′ voltammetric wave clearly illustrates that the mechanism of antioxidant action of the investigated flavonoids may occur by the suppression of the production of the hydoxyl radical and/or ferryl species. The flavonoid ring system has many possible metal binding sites. The catechol group on the B ring of quercetin and luteolin is a logical metal chelation site on these flavonoids. Many types of siderophores, low-molecular weight plant-borne iron transport and uptake species, are based on the chelation of iron through catechol functional groups (Theil *et al.* 1994). In this study, it was observed that the two flavonoids containing catechol functional groups on the B ring were more adept at controlling the redox cycling Fenton reaction characteristics of the $Fe^{II/III}$ATP couple. Luteolin lacks a 3-hydroxy group when compared with quercetin, it is on this basis that the 3-hydroxy, 4-ketone region is reasoned as not being an important metal chelation center in the deactivation of FeATP as a Fenton reaction center. Although naringenin and baicilein lacked the catechol moiety on the B ring both were able to effect partial deactivation of the Fenton reaction ability of FeATP. The structural feature shared by the flavonoids examined in this study was the 4-keto, 5-hydroxy region. It is for this reason that we theorize that this metal chelation region is important for the deactivation of the FeATP towards the Fenton reaction. The iron-baicilein complex lacks the anodic shift of its ligand voltammetry that the other three studied flavonoids had exhibited and was previously mentioned in literature (Weber 1996). We expect that this lack in redox potential shift is due to the competition for of the 5, 6 and 6, 7 hydroxy groups with the deactivating 4 keto, 5 hydroxy region for iron. The lack of enhanced deactivation of the FeATP Fenton reaction by baicilein over naringenin indicates that the 5,6 and 6,7 catechol groups are apparently not important FeATP deactivating centers on the baicilein ring system. The B-ring catechol containing species in this study, luteolin and quercetin experienced an anodic shift in their respective electrode reactions when complexed to iron along with naringenin. This may indicate that the 4-keto, 5-hydroxy region is important iron binding region of luteolin and quercetin despite the presence of the catechol region on the B-ring. However, the 3′, 4′ catechol group of luteolin and quercetin apparently enhances the deactivation of FeATP. Further studies will be aimed at the isolation, and structural determination of the metal-flavonoid complexes.

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