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# Structure-antioxidant Activity Relationships of Flavonoids: A Re-examination

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The antioxidant and prooxidant activities of flavonoids belonging to several classes were studied to establish their structure–activity relationships against different oxidants. Special attention was paid to the flavonoids quercetin (flavone), taxifolin (flavanone) and catechin (flavanol), which possess different basic structures but the same hydroxylation pattern (3,5,7,3′,4′–OH). It was found that these three flavonoids exhibited comparable antioxidant activities against different oxidants leading to the conclusion that the presence of *ortho*-catechol group (3′,4′–OH) in the B-ring is determinant for a high antioxidant capacity. The flavone kaempferol (3,5,7,4′–OH), however, in spite of bearing no catechol group, also presents a high antioxidant activity against some oxidants. This fact can be attributed to the presence of both 2,3-double bond and the 3-hydroxyl group, meaning that the basic structure of flavonoids becomes important when the antioxidant activity of B-ring is small.

*Keywords*: Flavonoids; Antioxidant; Free radicals; Structure–activity relationships

## INTRODUCTION

There is overwhelming evidence indicating that natural and synthetic antioxidants play a role in wellness, health maintenance, and the prevention of chronic and degenerative diseases. Consequently, there has been a growing interest in the potential health-promoting properties of phytochemicals. Special attention has been given to flavonoids due to the many studies that suggest that these compounds have beneficial effects in age-associated diseases such as cardiovascular and cerebrovascular diseases, some forms of cancer and Parkinson's and Alzheimer's diseases.[1–5]

Flavonoids are polyphenolic compounds widely distributed in fruits, vegetables, plant extracts as well as in plant-derived beverages such as tea and red wine.[6–8] These compounds have long been recognized to possess antihepatotoxic, antiinflammatory, antiatherogenic, antiallergic, antiosteoporotic and anticancer activities (reviewed in Ref. [9]). Many of the pharmacological effects of flavonoids are related to their interaction with several enzymes[9–11] and to their antioxidant activity, which can be due to their ability to scavenge free radicals,[12–17] to chelate metal ions[18–22] and to synergistic effects with other antioxidants.[23]

The main objective of this work is to compare the antioxidant behaviour of some classes of flavonoids using different tests in order to elucidate their structure–activity relationships and to explain why some of these structural features are important. To accomplish this objective flavones (apigenin, luteolin, kaempferol, quercetin, myricetin and rutin), isoflavones (daidzein and genistein), flavanones (taxifolin, naringenin and naringin) and a flavanol (catechin) (Fig. 1) were subject to different tests, since most antioxidants can behave in different ways *in vitro*, depending on the oxidant.

The antioxidant properties of flavonoids were evaluated by studying the capacity of flavonoids to

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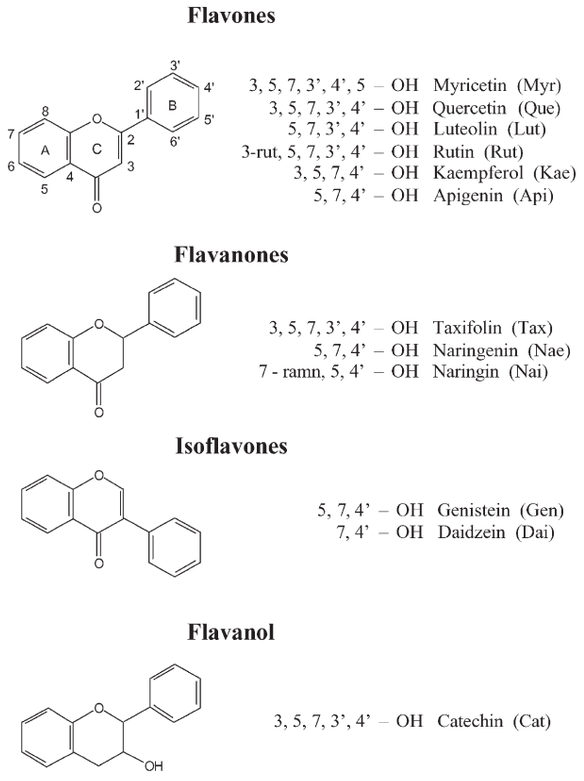


FIGURE 1 Structures of the flavonoids used in this study.

reduce ferrylmyoglobin, to inhibit lipid peroxidation and to scavenge hydrophilic peroxyl radicals, generated from 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH), and the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$).

Ferrylmyoglobin radical ($^{\bullet}X-Fe^{IV}=O$) is a two-electron oxidation product formed upon the reaction between metmyoglobin ($X-Fe^{III}$) and $H_2O_2$.[24] Ferrylmyoglobin is a radical species of biological significance as it may occur after reperfusion of the ischemic heart contributing to free radical damage of myocites.[25] In addition, as a result of violent exercise, muscle can be injured releasing myoglobin into the circulation,[26] where it could interact with any $H_2O_2$ available to cause oxidative damage to proteins[27] and lipids.[27,28]

Due to the relevance of lipid oxidation to biological systems is fundamental to evaluate the protection conferred by flavonoids against lipid peroxidation. In this work, the oxidation of liposomes was used as a model for studying the efficacy of flavonoids to protect polyunsaturated fatty acids against oxidation. The lipid peroxidation was induced either by the $Fe^{3+}$-EDTA-ascorbate system or by the azo compound AAPH.[29] The antioxidant potential of flavonoids to scavenge the hydrophilic peroxyl radicals generated from AAPH was also measured by the oxygen radical absorbance capacity (ORAC) assay as described by Cao *et al.*[14]

The study of the scavenging effects of flavonoids on DPPH$^{\bullet}$, a stable nitrogen-centered radical,[30,31] is another approach to establish the antioxidant hierarchy of flavonoids.

## MATERIALS AND METHODS

### Chemicals

All the reagents were of the highest quality available and were used as supplied. 2,2′-Azobis(2-amidino-propane)dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) were purchased from Aldrich Chemical Company (Milwaukee, WI). Potassium ferricyanide was obtained from May and Baker (Dagenham, UK). All flavonoids, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT) and β-phycoerythrin (β-PE) from *Porphyridium cruentum* were obtained from Sigma Chemical Company (St Louis, MO, USA). The β-PE used in these experiments had the same lot number and usually lost more than 95% of its fluorescence within 30 min in the presence of 4 mM AAPH. All the other reagents were obtained from Merck (Darmstadt, Germany).

### Kinetic Analysis of Ferrylmyoglobin Reduction

Metmyoglobin was obtained by oxidizing commercial horse heart myoglobin with equimolar potassium ferricyanide. After mixing, the solution was allowed to stand at room temperature for 15 min. Metmyoglobin was further purified by gel filtration on a Sephadex G-25 column pre-equilibrated with phosphate buffered saline (20 mM $Na_2HPO_4/NaH_2PO_4$, 110 mM NaCl, pH 7.4), and collecting the brown fraction eluted with buffer. The total haem and metmyoglobin concentrations were calculated from their molar absorptivity coefficients at 525 and 630 nm, respectively.[32]

Ferrylmyoglobin was obtained by reaction of metmyoglobin with $H_2O_2$ in the following conditions: a solution of phosphate buffered saline (pH 7.4) containing 25 μM metmyoglobin was supplemented with 37.5 μM $H_2O_2$ to form ferrylmyoglobin. After 10 min of reaction, 10 μM of flavonoid was added to the reaction mixture. The reactions were performed at 37°C. Kinetic analysis of spectral modifications of ferrylmyoglobin (450–700 nm region) in the presence of flavonoids was carried out with a Pye Unicam UV2-100 spectrophotometer (connected to a computer with Vision Software operating under Microsoft Windows environment). To calculate the initial rates of ferrylmyoglobin disappearance, absorbances at 550 and 630 nm were recorded immediately after the addition of

flavonoid during a 15 s interval. The ferrylmyoglobin concentration was calculated according to the following formula: [ferrylmyoglobin]/μM = 249 × $A_{550\,nm}$ −367 × $A_{630\,nm}$.[33] Flavonoids were dissolved in ethanol, except for catechin which is soluble in water.

### Effects of Flavonoids on Oxidation of Liposomes

Liposomes were prepared from rat hepatic microsomes isolated from male Sprague–Dawley rats by differential centrifugation. Total microsomal lipid was extracted from freshly prepared microsomes by the method of Folch *et al.*[34] Lipid content was measured as the amount of total lipid phosphorus by the method of McClare *et al.*[35] All solvents were purged with nitrogen and all operations were performed under nitrogen at 4°C. The extracted lipid was stored at −20°C under nitrogen in chloroform. Aqueous suspensions of microsomal lipid were prepared by transferring an aliquot of the stock lipid solution to a round bottom flask, removing the chloroform under a stream of nitrogen, adding nitrogen-saturated 50 mM Tris–HCl pH 7.4 buffer to the thin lipid film and then capping the flask under nitrogen. The sealed flask was shaken vigorously for 2 min. The final concentration of lipid in the suspension was 5 μmol of lipid phosphorus per ml. The oxidation of liposomes was induced either by the $Fe^{3+}$-EDTA-ascorbate system or by peroxyl radicals generated at a constant rate in the aqueous phase by thermal decomposition of the azo compound AAPH. The assays for the peroxidation of liposomes promoted by $Fe^{3+}$-EDTA-ascorbate were performed as follows: reaction mixtures were made to a final volume of 1.0 ml by mixing 50 mM Tris–HCl pH 7.4 buffer with 100 mM NaCl, 500 μM liposomes, flavonoid (three concentrations were tested for each flavonoid in order to determine the concentration corresponding to 50% inhibition-$IC_{50}$) and 50 μM $Fe^{3+}$-50 μM EDTA. After mixing and following a 5 min incubation period, peroxidation was initiated by the addition of 150 μM ascorbate. The assay conditions for the peroxidation of liposomes promoted by AAPH were the same omitting $Fe^{3+}$-EDTA-ascorbate and initiating the reaction by the addition of 20 mM AAPH. The reaction mixtures were incubated at 37°C for 30 min in the assays with $Fe^{3+}$-EDTA-ascorbate and for 3 h in the assays with AAPH. A control assay was done in the absence of flavonoid. The extent of lipid peroxidation was determined through the thiobarbituric acid (TBA) method. After the incubation period we added to all the assays 90 μl of 2% (w/v) butylated hydroxytoluene in ethanol, 1 ml of 20% (w/v) trichloroacetic acid and 1 ml of 0.67% (w/v) TBA. The mixtures were heated in a boiling water bath for 10 min. After cooling, 1 ml of each mixture was taken, then 2 ml of 1-butanol was added and the solution was mixed for 1 min to extract the chromophore. Phases were separated by centrifugation for 5 min at 1000*g*. The clear upper butanol phase was read at 532 nm against appropriate blanks. Results obtained in the presence of flavonoids were expressed in terms of percentage of lipoperoxidation inhibition in comparison with a control assay.

### Assay for the Peroxyl Radicals from AAPH (ORAC Assay)

The ORAC assay was used essentially as described by Cao *et al.*[14] in order to estimate peroxyl radical absorbing activities ($ORAC_{ROO\bullet}$) of some flavonoids. β-PE, a fluorescent protein, was used as a target of peroxyl radicals generated from the azo compound AAPH. The fluorescence decay, at an excitation wavelenght of 545 nm and an emission wavelenght of 572 nm, was followed using a Shimadzu RF-5000 recording spectrofluorophotometer. The flavonoids studied were apigenin, quercetin, rutin and catechin. All the solutions were prepared in 75 mM $NaH_2PO_4$–$K_2HPO_4$ buffer (pH 7.0), except for the flavonoids apigenin, quercetin and rutin which were dissolved first in acetone (5 mM) and then diluted with buffer (20 and 100 μM). When acetone was used in a sample, it was also used in the blank and standard, although acetone itself has a very small effect on the ORAC assay. Briefly, the final assay mixture contained 16.5 nM β-PE and either 1 μM Trolox (control standard) or flavonoid samples (0.125–2 μM) and 4 mM AAPH in 2.0 ml of phosphate buffer, in 10 mm quartz fluorometer cells at 37°C and with a magnetic stirrer. The concentration of β-PE was determined spectroscopically through its molar absorptivity coefficient of $2.41 \times 10^6\,M^{-1}\,cm^{-1}$ at 545 nm.[36] β-PE was routinely preincubated with phosphate buffer in a fluorometer cell at 37°C for 15 min before the beginning of each experiment. The reactions were started by the addition of AAPH freshly prepared and stored on ice. Fluorescence readings were taken every 40 s for as long as 2 h. All fluorescence measurements were expressed relative to the initial reading. $ORAC_{ROO\bullet}$ activity was calculated using the differences of areas under the β-PE decay curves between the blank and a sample and expressed as μmol Trolox equivalents $[(Area_{Sample} - Area_{Blank})/(Area_{Standard} - Area_{Blank})]$. The slope of the plots of $ORAC_{ROO\bullet}$ activity against flavonoid concentration gives $ORAC_{ROO\bullet}$ activity for each flavonoid, expressed as μmol Trolox equivalents per μmol flavonoid.

### $DPPH^\bullet$ Assay

$DPPH^\bullet$ is a stable radical that has a high absorption at 517 nm. When the odd electrons of $DPPH^\bullet$ are

paired off with electrons taken up from phenolic compounds, the absorption at 517 nm decreases.[31] The degree of decolourisation is a measure of the reducing capacity of flavonoids and whereby it enables to evaluate their antioxidant activity. DPPH$^{\bullet}$ scavenging activity was measured essentially as described by Cotelle *et al.*[31] and Schlesier *et al.*[37] The final assay mixture contained 100 μM DPPH$^{\bullet}$ and flavonoids (0–20 μM) both dissolved in ethanol. The decrease in absorbance at 517 nm caused by the addition of the flavonoid was followed during 30 min. After this period the variation of absorbance per min ($\Delta A\,min^{-1}$) for all flavonoid concentrations studied was approximately equal to the small ΔA per min displayed by the control (assay containing only DPPH$^{\bullet}$). The DPPH$^{\bullet}$ scavenged after 30 min for each flavonoid concentration was determined by using an extinction coefficient of $8317\,M^{-1}\,cm^{-1}$, which was evaluated by a calibration curve (ranging 0–100 μM) obtained by standard DPPH$^{\bullet}$ solutions. The slope of the plots of DPPH$^{\bullet}$ scavenged against flavonoid concentrations gives the moles of DPPH$^{\bullet}$ scavenged per mol of flavonoid.

### Analysis of the Data

Values represent means ± standard deviation (SD) of at least four independent experiments unless otherwise stated. Area-under-curve was quantified using Integration in Microcal Origin 3.5 for Windows (Microcal Software Incorporation, 1991–1994). Linear regression analyses of $ORAC_{ROO\cdot}$ activities vs. flavonoid concentrations were computed using Microsoft Excel 97 for Windows (Microsoft, 1995–1997). Using the regression lines percentage of inhibition/flavonoid concentration, the half maximal inhibitory concentration ($IC_{50}$) values and standard errors of estimates were determined by inverse prediction, based on the criterion of least squares.[38]

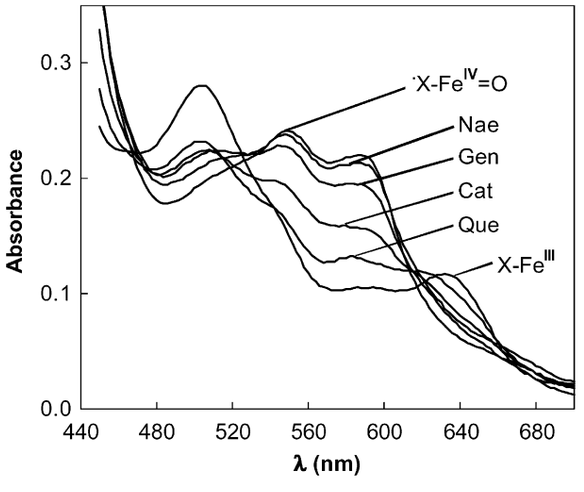


FIGURE 2 Spectral changes of ferrylmyoglobin upon its reaction with some of the flavonoids studied. A solution of phosphate buffer (20 mM $Na_2HPO_4/NaH_2PO_4$, 110 mM NaCl, pH 7.4) containing 25 μM metmyoglobin was supplemented with 37.5 μM $H_2O_2$ to form ferrylmyoglobin. After 10 min of reaction, 10 μM of each flavonoid was added to the reaction mixture and the spectra were scanned 2 min later. Representative spectra with the flavonoids quercetin, catechin, genistein and naringenin are shown.

## RESULTS AND DISCUSSION

The antioxidant activities of 12 flavonoids having different basic chemical structures (Fig. 1), were studied to establish their structure–activity relationships with different oxidants. Special attention was paid to the flavonoids quercetin (flavone), taxifolin (flavanone) and catechin (flavanol) that have the same pattern of OH substitutions.

### Optical Analysis of the Reaction of Ferrylmyoglobin with Flavonoids

The reaction of flavonoids with ferrylmyoglobin was assessed through kinetic analysis of spectral modifications of ferrylmyoglobin. Figure 2 shows the spectra of ferrylmyoglobin produced after the addition of $H_2O_2$ to metmyoglobin and the ferrylmyogobin spectral changes produced within 2 min after the addition of some of the flavonoids studied. As seen in the spectra taken at different times (data not shown) the changes induced by each flavonoid are practically identical. The characteristic peaks of ferrylmyoglobin at around 550 and 580 nm progressively disappear and a spectrum resembling that of the native metmyoglobin (peaks at 503 and 630 nm) begins to appear. However, the initial rates of the spectral changes induced by flavonoids are different (Table I). The loss of ferrylmyoglobin spectrum indicates that flavonoids are effective "quenchers" of ferryl species. The flavones myricetin (3,5,7,3′,4′,5′–OH), quercetin (3,5,7,3′,4′–OH), kaempferol (3,5,7,4′–OH) and luteolin (5,7,3′,4′–OH) are the flavonoids with higher reducing activity. Rutin (3-rut,5,7,3′,4′–OH) with the same hydroxyl groups as luteolin but with a bulky rutinoside group at the 3-position has a smaller activity. The flavanol catechin and the flavanone taxifolin, which have the same hydroxyl groups as quercetin, have about half of its activity. This observation provides evidence for the importance of the 2,3-double bond in the C-ring towards the reducing activity of flavonoids. The remaining flavonoids possessing a smaller number of hydroxyl groups show a lower activity.

### Effect of Flavonoids on $Fe^{3+}$-EDTA-ascorbate-dependent Oxidation of Liposomes

The structural features of flavonoids required for the inhibition of lipid peroxidation correspond to the sum

TABLE I Rates of ferrylmyoglobin reduction upon its reaction with flavonoids

| Classes | Compounds | Substituents | $-d[Mb^{IV}=O]/dt$ ($\mu M\,min^{-1}$) |
|---|---|---|---|
| Flavones | Myricetin | 3,5,7,3′,4′,5′–OH | 52.8 ± 0.9 |
| | Quercetin | 3,5,7,3′,4′–OH | 40.8 ± 2.2 |
| | Kaempferol | 3,5,7,4′–OH | 47.2 ± 1.2 |
| | Luteolin | 5,7,3′,4′–OH | 39.0 ± 1.0 |
| | Rutin | 3-rut,5,7,3′,4′–OH | 26.7 ± 0.3 |
| | Apigenin | 5,7,4′–OH | 10.1 ± 1.4 |
| Flavanol | Catechin | 3,5,7,3′,4′–OH | 21.9 ± 0.5 |
| Isoflavones | Genistein | 5,7,4′–OH | 5.6 ± 1.7 |
| | Daidzein | 7,4′–OH | 5.1 ± 2.0 |
| Flavanones | Taxifolin | 3,5,7,3′,4′–OH | 23.9 ± 3.3 |
| | Naringenin | 5,7,4′–OH | 2.9 ± 2.0 |
| | Naringin | 7-ramn,5,4′–OH | 1.7 ± 0.3 |

To calculate the initial rates of ferrylmyoglobin disappearance absorbances at 550 and 630 nm were taken immediately and the ferrylmyoglobin concentration was calculated according to the following formula: $[\text{ferrylmyoglobin}]/\mu M = 249 \times A_{550\,nm} - 367 \times A_{630\,nm}$. Values given represent the mean ± SD of 5 to 9 independent experiments.

of the structural features for the different processes involved. Besides the scavenging of lipid radicals, the inhibition of lipoperoxidation by flavonoids is also influenced by their lipophilicity, which affects their penetration into bilayers, and by their ability to scavenge the initiating radicals. In addition, for the lipoperoxidation induced by $Fe^{3+}$-EDTA-ascorbate, synergistic effects between ascorbate and flavonoid radicals may also occur. The lipoperoxidation induced by $Fe^{3+}$-EDTA-ascorbate was evaluated by measuring the TBA reactive substances and the oxygen consumption. Using the TBA method, lipoperoxidation inhibitions were not observed with the flavones naringenin (5, 7, 4′–OH) and naringin (7-ramn,5,4′–OH) and with the isoflavone daidzein (7,4′–OH) for concentrations up to 100 μM. All the other flavonoids inhibited the lipoperoxidation. This inhibition was concentration dependent with half inhibition concentration ($IC_{50}$) values indicated in Table II. In general, flavonoids with lower $IC_{50}$ values are the ones that possess a larger number of hydroxyl groups in their structures. Flavonoids of different classes but with the same number of hydroxyl groups have $IC_{50}$ values of the same magnitude. This can be observed for the flavonoids possessing five hydroxyl groups (3,5,7,3′,4′–OH) such as quercetin (flavone), catechin (flavanol) and taxifolin (flavanone), with $IC_{50}$ values ranging from 3–9 μM, as well as for the flavonoids possessing three hydroxyl groups (5,7,4′–OH) apigenin (flavone) and genistein (isoflavone) with 90 μM $IC_{50}$ values. In addition, the higher antioxidant activities of quercetin, catechin and taxifolin may be attributed to the highly significant contribution of the 3′,4′-dihydroxy (catechol) structure on the B-ring towards the antioxidant activity of these compounds. Kaempferol (3,5,7,4′–OH), however, in spite of bearing no catechol structure, also presents a high antioxidant activity in contrast with apigenin (5,7,4′–OH) that has a very low activity. This seems to enhance the importance of the 3-hydroxyl group for a high antioxidant activity of flavones. This difference in antioxidant activity based on the presence of the 3-hydroxyl group is probably a result of the enhanced planarity of the molecule. The 3-OH moiety interacts with the B-ring through a hydrogen bond with the 6′- or 2′-hydrogen and in this way "fixates" the position of ring B in the same plane as rings A and C.[16,39] Rutin (3-rut,5,7,3′,4′–OH) with the same hydroxyl groups as luteolin but with a bulky rutinoside group at the 3-position has a slight decrease in activity. This effect is probably due to a loss of coplanarity of ring B with the rest of the molecule.

The oxygen consumption resulting from lipid peroxidation in the presence of flavonoids is in agreement with the antioxidant hierarchy that was determined for these compounds through the TBA method. In addition, it was verified that in order to inhibit lipid peroxidation with the same magnitude as quercetin, luteolin and catechin, the flavonoids naringin, naringenin and daidzein must be in a concentration 12 times higher (data not shown).

### Effect of Flavonoids on AAPH-dependent Oxidation of Liposomes

The inhibitory effects of flavonoids on lipid peroxidation induced by the aqueous peroxyl radical generator AAPH and measured by the TBA method

TABLE II Half maximal inhibition concentrations ($IC_{50}$) of flavonoids on $Fe^{3+}$-EDTA-ascorbate-dependent oxidation of liposomes

| Classes | Compounds | Substituents | $IC_{50}$ ± SD (μM) |
|---|---|---|---|
| Flavones | Myricetin | 3,5,7,3′,4′,5′–OH | 3.4 ± 0.4 |
| | Quercetin | 3,5,7,3′,4′–OH | 3.0 ± 0.5 |
| | Luteolin | 5,7,3′,4′–OH | 3.5 ± 0.5 |
| | Kaempferol | 3,5,7,4′–OH | 7.4 ± 0.4 |
| | Rutin | 3-rut,5,7,3′,4′–OH | 15.7 ± 2.6 |
| | Apigenin | 5,7,4′–OH | 90.5 ± 10.7 |
| Flavanol | Catechin | 3,5,7,3′,4′–OH | 4.5 ± 0.5 |
| Isoflavone | Genistein | 5,7,4′–OH | 89.5 ± 16.2 |
| Flavanone | Taxifolin | 3,5,7,3′,4′–OH | 9.1 ± 1.7 |

Values given represent the mean ± SD of 4 to 10 independent experiments.

TABLE III Half maximal inhibition concentrations ($IC_{50}$) of flavonoids on AAPH dependent oxidation of liposomes

| Classes | Compounds | Substituents | $IC_{50}$ ± SD (μM) |
|---|---|---|---|
| Flavones | Myricetin | 3,5,7,3′,4′,5′–OH | 18.0 ± 1.4 |
| | Quercetin | 3,5,7,3′,4′–OH | 11.0 ± 1.0 |
| | Luteolin | 5,7,3′,4′–OH | 15.3 ± 2.4 |
| | Kaempferol | 3,5,7,4′–OH | 74.0 ± 5.0 |
| | Rutin | 3-rut,5,7,3′,4′–OH | 17.0 ± 1.3 |
| | Apigenin | 5,7,4′–OH | >300 |
| Flavanol | Catechin | 3,5,7,3′,4′–OH | 16.0 ± 1.2 |
| Isoflavone | Genistein | 5,7,4′–OH | 192.0 ± 10.0 |
| Flavanone | Taxifolin | 3,5,7,3′,4′–OH | 22.0 ± 1.3 |

Values given represent the mean ± SD of 4 to 7 independent experiments.

are represented in Table III. In this system, flavonoids may interfere not only with the propagating lipid radicals, but also with the initiating radicals generated from AAPH. All the flavonoids were less effective at inhibiting the AAPH-dependent peroxidation than the $Fe^{3+}$-EDTA-ascorbate-dependent peroxidation. The flavones myricetin (3,5,7,3′,4′,5′–OH), quercetin (3,5,7,3′,4′–OH), luteolin (5,7,3′,4′–OH) and rutin (3-rut,5,7,3′,4′–OH), the flavanol catechin (3,5,7,3′,4′–OH) and the flavanone taxifolin (3,5,7,3′,4′–OH) have similar $IC_{50}$ values. These flavonoids have, in common, the presence of the catechol structure on the B-ring. The flavone kaempferol (3,5,7,4′–OH) possessing four hydroxyl groups as luteolin and rutin, but not the catechol structure on the B-ring, has a much higher $IC_{50}$ value. This observation provides evidence for the highly significant contribution of the catechol structure on the B-ring towards the antioxidant activity of those compounds.

## Scavenging Activity of Flavonoids on the Peroxyl Radicals Generated from AAPH

In this work, we determined by means of the ORAC assay the peroxyl radical scavenging activity of catechin, apigenin and rutin only, because the activities of the remaining flavonoids were already determined by Cao *et al.*.[14] We also determined the activity of quercetin to compare with the value obtained by Cao *et al.* in order to use the activities reported by these authors for the discussion about flavonoid structures-peroxyl radical scavenging

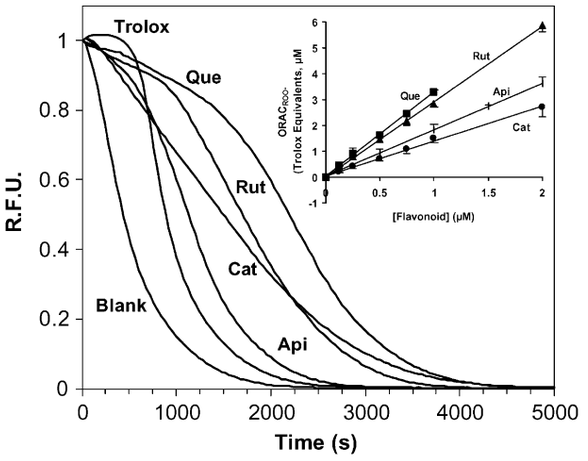


FIGURE 3 Effects of either Trolox or flavonoids on relative fluorescence decay of β-PE promoted by AAPH. The reactive mixtures contained 75 mM phosphate buffer, pH 7.0, 1.65 nM β-PE, either 1 μM Trolox (control standard) or 1 μM flavonoid and 4 mM AAPH. The β-PE fluorescence decay was followed and expressed as relative fluorescence units (RFU). The curves presented are means of at least four independent experiments. (Inset) $ORAC_{ROO}$ activity (Trolox equivalents, μM) as a function of flavonoid concentration (μM).

activity relationships. This assay is based on the susceptibility of the fluorescent protein β-PE to oxidative damage, induced by AAPH, with concomitant loss of its fluorescence character. To accomplish this, the kinetics of β-PE fluorescence decay in the presence of Trolox, catechin, apigenin, rutin and quercetin were followed (Fig. 3). The inset of this figure represents the concentration-dependent $ORAC_{ROO}$· activity obtained for each flavonoid. The slopes of the lines obtained by linear regression represent the $ORAC_{ROO}$· activities of the tested flavonoids equivalents to 1 μM Trolox. The $ORAC_{ROO}$· values obtained for quercetin, rutin, apigenin and catechin were 3.28, 2.90, 1.79 and 1.45, respectively (Table IV). These results show that flavonoids with several –OH substituents have higher antioxidant activity than the control standard Trolox. For flavonoids with the same basic chemical structure (quercetin, rutin and apigenin) the $ORAC_{ROO}$· activity increases with the number of –OH substituents. Catechin, a flavanol without the 2,3-double bond and without the 4-oxo group in the C ring, has a much smaller antioxidant activity

TABLE IV Peroxyl radical scavenging activity of flavonoids evaluated by the $ORAC_{ROO}$· assay

| Classes | Compounds | Substituents | $ORAC_{ROO}$ ± SD (μM Trolox equivalents/μM flavonoid) |
|---|---|---|---|
| Flavones | Quercetin | 3,5,7,3′,4′–OH | 3.3 ± 0.1 |
| | Rutin | 3-rut,5,7,3′,4′–OH | 2.9 ± 0.2 |
| | Apigenin | 5,7,4′–OH | 1.8 ± 0.1 |
| Flavanol | Catechin | 3,5,7,3′,4′–OH | 1.4 ± 0.3 |

$ORAC_{ROO}$· activity for each flavonoid, expressed as μmol Trolox equivalents per μmol flavonoid, was obtained from the slope of the plots of $ORAC_{ROO}$· activity against flavonoid concentration (inset of Fig. 3). Values given represent the mean ± SD of 4 to 7 independent experiments.

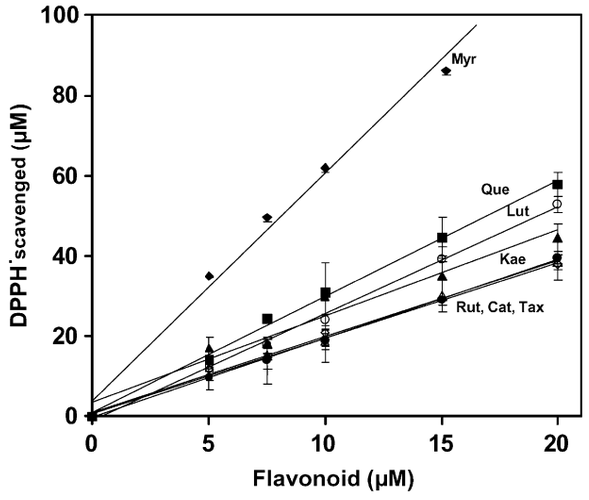


FIGURE 4 DPPH• scavenging activity of flavonoids. The concentration-dependent DPPH• scavenging activity was evaluated, after a period of 30 min, through the decrease in absorbance at 517 nm caused by the addition of flavonoid (0–20 μM) to 100 μM DPPH• 30 min later.

than flavone quercetin that possesses the same hydroxyl groups. Cao *et al.*,[14] however, showed that taxifolin, a flavanone with the same hydroxyl groups as quercetin, presents a similar $ORAC_{ROO\bullet}$ activity. The difference between these two flavonoids is the absence of the 2,3-double bond in the C ring of taxifolin. This suggests that the conjugation between rings A and B through the 2,3-double bond in the C ring is not particularly important for the peroxyl radical absorbing activity ($ORAC_{ROO\bullet}$ activity) of a flavonoid.

### Scavenging Effects of Flavonoids on DPPH•

The scavenging activity of several flavonoid concentrations (0–20 μM) on the stable radical DPPH• was evaluated through the decrease of absorbance at 517 nm. The concentration-dependent DPPH• scavenging activities obtained for the most effective flavonoids (the flavones myricetin, quercetin, kaempferol, luteolin and rutin, the flavonol catechin and the flavanone taxifolin) are represented in Fig. 4. Reaction stoichiometries (Table V) were obtained from the line slopes. All the other flavonoids did not present DPPH• scavenging activity, for that concentration range. The flavone myricetin (3,5,7,3′,4′,5′–OH) presents a very high stoichiometry. This flavone scavenges six DPPH• molecules per flavonoid molecule and, therefore, the number of electrons involved in its oxidation corresponds to the number of –OH groups. This unusually large numbers of electrons for the oxidation of flavonoids may be ascribed to a reproduction of –OH groups by oxidative polymerisation.[40] The other flavonoids with DPPH• scavenging activity present stoichiometries between 2 and 3. These flavonoids are the flavones quercetin(3,5,7,3′,4′–OH), kaempferol (3,5,7,4′–OH), luteolin (5,7,3′,4′–OH) and rutin (3-rut,5,7,3′,4′–OH), the flavonol catechin (3,5,7,3′,4′–OH) and the flavanone taxifolin (3,5,7,3′,4′–OH). All these flavonoids, except kaempferol, possess the *o*-catechol group in the B-ring. The presence of a catechol group in the B-ring thus appears essential for a good scavenging activity. In addition, for the flavonoids possessing the *o*-catechol group the presence of the 2,3-double bond in conjugation with the 4-oxo group in the C ring is not a determinant structural feature for the scavenging of DPPH•. This may be sustained by the flavonoids quercetin, catechin and taxifolin that possess the same hydroxyl groups (3,5,7,3′,4′–OH) but belong to different classes. The presence of a 3–OH group in the flavones also seems to confer radical scavenging activity and it may explain the DPPH• scavenging activity of kaempferol (3,5,7,4′–OH) in contrast with apigenin (5,7,4′–OH) that has no activity.

## CONCLUSIONS

Bors *et al.*[41] have proposed that three structural determinants should be responsible for effective radical scavenging by flavonoids: (1) the

TABLE V DPPH• scavenging activity of flavonoids

| Classes | Compounds | Substituents | μmol DPPH• scavenged per μmol flavonoid |
|---|---|---|---|
| Flavones | Myricetin | 3,5,7,3′,4′,5′–OH | 5.7 ± 0.3 |
| | Quercetin | 3,5,7,3′,4′–OH | 2.9 ± 0.1 |
| | Kaempferol | 3,5,7,4′–OH | 2.2 ± 0.2 |
| | Luteolin | 5,7,3′,4′–OH | 2.7 ± 0.1 |
| | Rutin | 3-rut,5,7,3′,4′–OH | 2.0 ± 0.0 |
| | Apigenin | 5,7,4′–OH | 0 |
| Flavanol | Catechin | 3,5,7,3′,4′–OH | 1.9 ± 0.1 |
| Isoflavones | Genistein | 5,7,4′–OH | 0 |
| | Daidzein | 7,4′–OH | 0 |
| Flavanones | Taxifolin | 3,5,7,3′,4′–OH | 1.9 ± 0.1 |
| | Naringenin | 5,7,4′–OH | 0 |
| | Naringin | 7-ramn,5,4′–OH | 0 |

The stoichiometries were obtained from the slopes of the plots of DPPH• scavenged against flavonoid concentrations (Fig. 4). Values given represent the mean ± SD of 3 independent experiments.

*ortho*-dihydroxy or catechol group in the B-ring, which confers a high stability to the radical formed; (2) the conjugation of the B-ring to the 4-oxo group via the 2,3-double bond, which ensures the electron delocalisation from the B-ring and (3) the 3- and 5-OH groups with the 4-oxo group, which allows electron delocalisation from the 4-oxo group to both substituents. The combination of all of these structural features enables a higher electron delocalisation conferring, therefore, a higher stability to aroxyl radicals. The *ortho*-catechol group confers a high stability to the resulting radical since when the OH bond is broken a strong H-bond is formed between the radical and the other OH group, which stabilizes the radical and decreases the O–H bond dissociation enthalpy.[42,43] In addition, when the B-ring possesses a pyrogallol (3′,4′,5′–OH) group the central O–H bond is the weakest due to the presence of two ortho groups which can form two hydrogen bonds with the radical.[42]

In this context the flavone myricetin is expected to be the most efficient flavonoid antioxidant followed by quercetin. In fact this was observed in the present study, but the flavanol catechin and the flavanone taxifolin exhibited antioxidant capacities comparable to that of quercetin against different oxidants. These flavonoids have the same hydroxylation pattern but catechin lacks the determinants (2) and (3), and taxifolin the determinant (2). This behaviour is in agreement with van Acker *et al*. hypothesis,[39] according to which the B-ring determines the antioxidant activity whereas the basic structure has only a small influence. Indeed for most flavonoids, the part of the polyphenol molecule with better electron-donating properties is the B-ring.[44] The influence of the basic structure increases when the antioxidant activity of B-ring decreases. Moreover, Bors and Saran[45] also concluded that substances with a saturated heterocyclic ring are predominantly attacked at the *ortho*-dihydroxy site in the B-ring and the semiquinones formed are quite stable.

As a matter of fact, the flavonoids quercetin, catechin and taxifolin displayed similar and high antioxidant potencies against lipoperoxidation induced by $Fe^{3+}$-EDTA-ascorbate providing evidence for the significant contribution of the *ortho*-catechol structure towards the antioxidant activity. However, kaempferol (3,5,7,4′–OH), in spite of bearing no catechol structure also presents a high antioxidant activity. This fact can be attributed to the presence of both the 2,3-double bond and the 3-OH group. In the latter case, the basic structure can compensate the low antioxidant activity of B-ring. On lipoperoxidation induced by AAPH, however, kaempferol did not show such a good antioxidant capacity. In this system, flavonoids may interfere not only with the propagating lipid radicals, but also with the initiating radicals $ROO^{\bullet}$ generated from AAPH. As was shown by Cao *et al*.,[14] kaempferol has a smaller $ROO^{\bullet}$ scavenging activity than both quercetin and taxifolin.

The scavenging effects of flavonoids on $DPPH^{\bullet}$ provide additional support for the conclusions mentioned before: the antioxidant activity of flavonoids is determined by B-ring, the presence of *ortho*-catechol group being the most important for a high antioxidant capacity; for flavonoids lacking a catechol group the basic structure becomes important, i.e., the presence of both 2,3-double bond and 3-OH group is of fundamental importance for a high antioxidant activity. This difference in antioxidant activity based on the presence of both 2,3-double bond and the 3-OH group is probably a result of the enhanced planarity of the molecule. The 2,3-double bond in the C-ring confers higher rigidity to the ring and holds the A and C rings in a more coplanar position. In addition, the 3-OH moiety interacts with B-ring through an intramolecular hydrogen bond with the 2′- or the 6′-hydrogen and this conformational arrangement places the ring B more or less in the same plane as rings A and C.[16,39] The planarity of the flavonoid molecules also seems to be essential for these compounds to achieve efficiently the two electron reduction of ferrylmyoglobin ($^{\bullet}X–Fe^{IV}=O$) to metmyoglobin.

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