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Protection by Flavonoids Against the Peroxynitrite-mediated Oxidation of Dihydrorhodamine

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Peroxynitrite anion is a reactive and short-lived species and its formation *in vivo* has been implicated in several human diseases. In view of the potential usefulness of compounds that can protect against peroxynitrite or their reactive intermediates, a study focused on flavonoid compounds was carried out. Since the reactivity of peroxynitrite may be modified by CO_2/HCO_3^-, which is an important plasma buffer, the protection of flavonoids against peroxynitrite was evaluated by their ability to inhibit the peroxynitrite-mediated dihydrorhodamine (DHR123) oxidation with or without physiological concentrations of bicarbonate. Flavonoids from different classes were studied to elucidate which structural features are required for an effective protection. The most efficient flavonoids on protecting DHR123 against oxidation by peroxynitrite have their ability diminished in the presence of bicarbonate, but they maintain the hierarchy established in the absence of bicarbonate. The flavones are the most effective flavonoids and their effects depend mainly on the number of hydroxyl groups. These must include either a catechol group in the B-ring or a hydroxyl group at the 3-position. This work also included some isoflavones, flavanones and a flavanol, which enable us to conclude about the importance of another structural feature: the 2,3-double bond. These results indicate that the ability of flavonoids to protect against peroxynitrite depends on some structural features, also important to scavenge oxygen free radicals and to chelate metal ions. The most efficient flavonoids are effective at low concentrations with IC_{50} of the same magnitude as Ebselen, a selenocompound that has been reported to be excellent at protecting against peroxynitrite. Their effectiveness at low concentrations is an important aspect to take into account when characterizing a compound as an antioxidant with biological interest.

Keywords: Flavonoids; Peroxynitrite; Bicarbonate; Antioxidant properties

INTRODUCTION

Peroxynitrite anion ($ONOO^-$) is produced through the very fast, diffusion-controled reaction between nitric oxide radical ($^{\cdot}NO$) and superoxide anion radical anion ($O_2^{\cdot-}$) and its formation, *in vivo*, was proposed, in 1990, by Beckman *et al.*[1] Peroxynitrite anion exists in protonation equilibrium with peroxynitrous acid (ONOOH) with a $pK_a = 6.8$. Therefore, at pH 7.4, 80% of peroxynitrite will be in the anionic form.[2] $ONOO^-$ could react directly through one-electron oxidation with transition metal centers, two-electron oxidation with a target substrate RH and with CO_2.[3,4] This latter reaction is fast yielding a short lived intermediate, the nitrosoperoxocarboxylate ($ONO_2CO_2^-$), which rapidly decomposes to secondary oxidant radicals, $^{\cdot}NO_2$ and $CO_3^{\cdot-}$ (~35% yield), and isomerizes to nitrocarbonate (~65% yield). In addition, peroxynitrous acid can undergo homolysis to $^{\cdot}NO_2$ and $HO^{\cdot}$, in 30% yields at pH 7.4 and 37°C, while the remainder isomerizes directly to nitrate. [3,4]

A wide variety of biomolecules can be oxidized by $ONOO^-$ *in vitro* either by direct reactions or by the secondary radicals ($CO_3^{\cdot-}$, $^{\cdot}NO_2$, $HO^{\cdot}$). The reactions with sulfhydryls, transition metal centers and carbon dioxide represent the main biological targets for peroxynitrite.

Peroxynitrite also promotes nitration of aromatic and aliphatic aminoacid residues.[4] Most notably, protein tyrosine residues constitute key targets for

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peroxynitrite-mediated nitrations. The nitration process involves free radical mechanisms and requires the intermediate formation of secondary radicals from peroxynitrite.[5]

Peroxynitrite can directly initiate lipid peroxidation reactions, by abstracting a hydrogen atom from a polyunsaturated fatty acid, generating, in addition to peroxides and carbonyl compounds, some lipid nitrated derivatives.[6] Oxidised and/or nitrated/nitrosated products, leading to base modifications as well as single- and double-strand breaks, result from the reaction of peroxynitrite with DNA.[7]

Peroxynitrite formation and the consequent damages have been proposed to contribute to the pathogenesis of several diseases. The detection of 3-nitrotyrosine was considered a biomarker of peroxynitrite action *in vivo* and high levels of this molecule were detected in pathologies such as atherosclerosis, rheumatoid arthritis, neurodegenerative diseases and multiple sclerosis.[8–9]

Once known that peroxynitrite is a powerful oxidant, it is expected that it must affect the plasma antioxidant levels. Indeed, addition of peroxynitrite to plasma leads to rapid oxidation of the water-soluble antioxidants ascorbic acid, uric acid and plasma SH groups.[10]

In view of the potential usefulness of preventing peroxynitrite damage on target biomolecules, the search of compounds that can react with peroxynitrite or with their reactive intermediates is of fundamental importance. Selenium-containing amino acids and the organoselenium compound Ebselen were found to protect against oxidation and nitration reactions caused by peroxynitrite.[11] Flavonoids are phenolic compounds that possess chemical features that may also confer an efficient protection against peroxynitrite. These compounds are widely distributed in fruits and vegetables [12–13] and several epidemiological studies support the idea that a high intake of flavonoids in diet decreases the occurrence of cardiovascular and cerebrovascular diseases and of some forms of cancer.[14–16] Many of their pharmacological effects are linked to their antioxidant properties, which can be due to their ability to scavenge reactive oxygen species,[17–20] to chelate metal ions [21–22] and to synergistic effects with other antioxidants.[23]

In the last few years, several works have characterized the antioxidant properties of flavonoids, especially in relation to their capacity to scavenge reactive oxygen species. There are, however, few studies [24–28] about the interaction of flavonoids with reactive nitrogen species, namely with peroxynitrite. To further evaluate the protection of flavonoids against damage by peroxynitrite and to elucidate what structural features are required for an effective protection, the ability of a large group of flavonoids to inhibit the peroxynitrite-mediated dihydrorhodamine oxidation was investigated. In addition, taking into account that CO_2/HCO_3^- is an important plasma buffer system and reactivity of peroxynitrite may be significantly modified by bicarbonate,[29,30] the effect of flavonoids on peroxynitrite-mediated dihydrorhodamine oxidation was also investigated in the presence of physiological concentrations of bicarbonate. This purpose was accomplished by the study of four classes of flavonoids, which include the most common flavonoids in our diet: flavones (myricetin, quercetin, luteolin, kaempferol, rutin and apigenin), isoflavones (trihydroxisoflavone, daidzein and genistein), flavanones (taxifolin, naringenin and naringin) and a flavanol (catechin). Ebselen was also used as reference for comparative studies (Fig. 1). In a previous work a detailed study was already carried out to evaluate the ability of those flavonoids to scavenge reactive oxygen species and to chelate iron and copper ions in order to establish their structure-activity relationships.[20,22]

MATERIALS AND METHODS

Chemicals

All the reagents were of the highest quality available and were used as supplied. Flavonoids

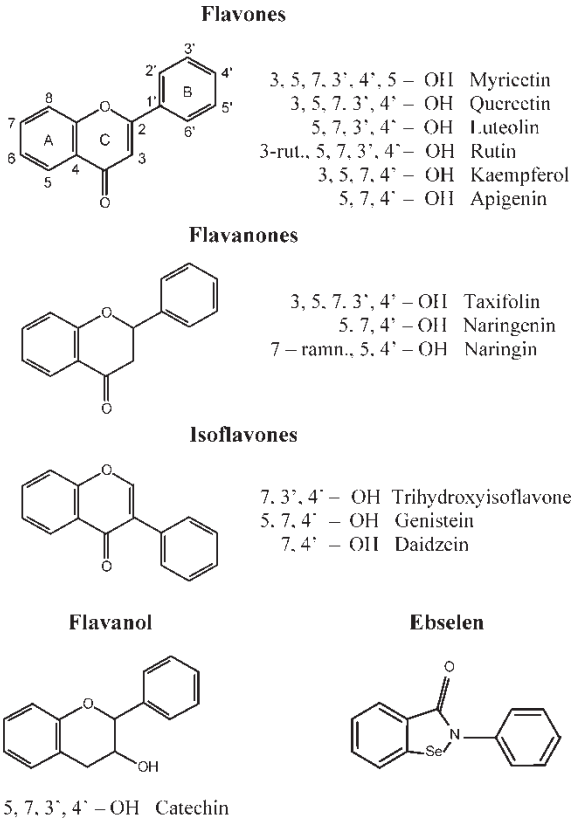

FIGURE 1 Structures of the flavonoids used in this study and Ebselen.

(except 7,3′,4′-trihydroxisoflavone), Ebselen and diethylenetriaminepentaacetic acid (DTPA) were from Sigma Chemical Co. (St. Louis, MO, USA). 7,3′,4′-trihydroxisoflavone was purchased from Fluka (Buchs, Switzerland). Dihydrorhodamine 123 (DHR123) and rhodamine 123 (ROD123) were purchased from Molecular Probes (Leiden, The Netherlands). Dimethylformamide was obtained from May and Baker (Dagenham, UK). All the other chemicals were from Merck (Darmstadt, Germany). All the reagents were prepared using 18 MΩ cm deionised water (*Millipore*, MilliQ^{50} system).

Peroxynitrite Synthesis

Peroxynitrite was synthetized by mixing cold acidified hydrogen peroxide with nitrite, in a simple flow system, and quenching ONOOH with alkaline solution as peroxynitrite, mainly as described by Beckman *et al.*[31] and Saha *et al.*[32] Briefly cold solutions of 0.6 M $NaNO_2$ and 0.66 M H_2O_2 (in 0.7 M HCl) were mixed and peroxynitrite collected in 3 M NaOH. The yield of peroxynitrite was determined by measuring the absorbance at 302 nm using $\varepsilon_{302\,nm} = 1670\,M^{-1}\,cm^{-1}$. Typical yields of freshly prepared stock solution were 120–140 mM. Only fresh peroxynitrite solutions were used in this study. The nitrite and nitrate contaminations in fresh preparations of peroxynitrite were determined as described by Bolzan *et al.*[33] The following typical molar fractions were obtained: $n_{ONOO^-} = 0.61 \pm 0.10$, $n_{NO_2^-} = 0.25 \pm 0.12$ and $n_{NO_3^-} = 0.11 \pm 0.06$ ($n = 16$).

Assay of Peroxynitrite-mediated Oxidation of Dihydrorhodamine 123

The protection against peroxynitrite by either flavonoids or Ebselen was measured by the inhibition of peroxynitrite-mediated DHR123 oxidation to ROD123, as described by Kooy *et al.*,[34] with minor modifications. Flavonoids and Ebselen were dissolved in dimethylformamide (2 mM stock solutions) and stored at −20°C. DHR123 (28.9 mM) and ROD123 (0.5 mM) stock solutions were also prepared in dimethylformamide, purged with N_2 and stored at −20°C. The working solutions were prepared daily in saline phosphate buffer pH 7.4 (50 mM K_2HPO_4/KH_2PO_4, 90 mM NaCl, 90 mM KCl), supplemented with 100 μM DTPA and the $NaHCO_3$ solution (2.0 M) was prepared immediately before the assays. Briefly, 25 μM DHR123 and 25 mM $NaHCO_3$ were incubated with either flavonoids or Ebselen (0–10 μM) in saline phosphate buffer pH 7.4 with 100 μM DTPA, at 37°C. After addition of 1.2 μM peroxynitrite (diluted in 0.1 M NaOH from the stock solution), through a Hamilton syringe, fluorescence was measured immediately on a spectrofluorometer SLM-Aminco 8100, with excitation and emission wavelenghts 493 nm and 524 nm, respectively. Assays were performed at 37°C in a thermostatized cuvette holder and with magnetic stirring. All fluorescence intensities measured were corrected for background fluorescence and referred to that of the fluorescence standard of the instrument. The control assay (reaction in the absence of either flavonoid or Ebselen) corresponds to maximal oxidation of DHR123 and, therefore, to 100% of fluorescence. In this assay approximately 400 nM of ROD123 were produced. In addition the relative fluorescence values observed for the control assays in the presence of added bicarbonate (0.343 ± 0.063, $n = 55$) were not significantly different from those in the absence of bicarbonate (0.329 ± 0.077, $n = 37$). In the presence of either flavonoids or Ebselen, a decrease in the fluorescence intensity may occur. The fluorescence measurements were expressed in percentage relative to the fluorescence of the control assay (%control). Decomposed peroxynitrite in buffer, hydrogen peroxide, nitrite, and nitrate did not interfere with oxidation of DHR123 by $ONOO^-$ (data not shown).

Analysis of the Data

Values represent mean ± standard deviation (s.d.) of at least four independent experiments, unless otherwise stated. The significance of differences between the fluorescence mean values obtained in the absence or in the presence of added bicarbonate was evaluated by two sample unpaired two-tailled Student's t test using Microsoft Excel (Microsoft, USA). In order to test whether the presence of a flavonoid structural feature influences the protection against peroxynitrite by flavonoids, the significance of differences between the fluorescence mean values obtained for the flavonoids, with and without that structural feature, was evaluated by the same significance test. In addition, the correlation between the fluorescence values and the number of total hydroxyl groups in each flavonoid was assessed using the Pearson coefficient (r), which was evaluated using Microcal Origin 3.5 (Microcal Software Inc., USA). Curve fitting and half-maximal inhibitory concentrations (IC_{50}), for the inhibition of DHR123 oxidation, were performed by non-linear regression analysis, using Microcal Origin 3.5 software.

RESULTS AND DISCUSSION

Protection by Flavonoids against Peroxynitrite-mediated Oxidation of DHR123

In the presence of effective flavonoids, less DHR123 is oxidized by peroxynitrite to the fluorescent

TABLE I Protection conferred by 1 μM flavonoids against DHR 123 oxidation by peroxynitrite.

Class	Flavonoid	Substituents	DHR oxidation (% control ± s.d. (*n*)) Buffer	Buffer + 25 mM $NaHCO_3$
Flavones	Myricetin	3,5,7,3′,4′,5′ - OH	39 ± 2 (5)	51 ± 1** (6)
	Quercetin	3,5,7,3′,4′ - OH	47 ± 1 (6)	58 ± 2** (6)
	Kaempferol	3,5,7,4′ - OH	68 ± 3 (9)	84 ± 1** (8)
	Luteolin	5,7,3′,4′ - OH	65 ± 3 (6)	71 ± 3* (6)
	Rutin	3-rut.,5,7,3′,4′ - OH	69 ± 1 (6)	74 ± 2** (6)
	Apigenin	5,7,4′ - OH	102 ± 3 (4)	104 ± 1 (3)
Flavanol	Catechin	3,5,7,3′,4′ - OH	64 ± 3 (6)	72 ± 2* (5)
Flavanones	Taxifolin	3,5,7,3′,4′ - OH	75 ± 2 (7)	75 ± 1 (6)
	Naringenin	5,7,4′ - OH	101 ± 3 (5)	99 ± 2 (3)
	Naringin	5,7-ramno,4′ -OH	103 ± 1 (3)	97 ± 2 (3)
Isoflavones	Trihydroxisof.	7,3′,4′ - OH	60 ± 2 (7)	64 ± 2* (4)
	Genistein	5,7,4′ - OH	96 ± 1 (3)	94 ± 3 (7)
	Daidzein	7,4′ - OH	97 ± 1 (6)	93 ± 3 (5)
Selenocompound	Ebselen		55 ± 2 (7)	71 ± 2** (7)

The results obtained with Ebselen are shown for comparison. *$p < 0,05$, **$p < 0,001$ comparing no HCO_3^- addition with HCO_3^- addition.

ROD123 and, therefore, smaller values of fluorescence, relative to the control assay, are obtained. Table I lists the protection of all tested flavonoids (1 μM) against the oxidation of DHR123 after the addition of a peroxynitrite bolus in the absence or in the presence of added bicarbonate. For comparison, the results obtained with Ebselen are also shown.

The inhibition of peroxynitrite-mediated oxidation of DHR123 by flavonoids, with or without bicarbonate in the reaction media, was concentration dependent, as illustrated in Fig. 2 for some representative flavonoids, and the respective half-maximal inhibitory concentrations (IC_{50}) are presented in Table II. The protection against peroxynitrite by quercetin displays concentration-dependent curves similar to those present by Ebselen. Quercetin, however, has smaller IC_{50} values than Ebselen. It was also observed that those flavonoids that at 1 μM concentration were ineffective in preventing the DHR123 oxidation keep their ineffectiveness against peroxynitrite, even at higher concentrations.

The effect of some flavonoids on peroxynitrite-mediated DHR123 oxidation was modified in the presence of 25 mM bicarbonate. The ability of effective flavones and of the flavonol catechin, tested at 1 μM concentration, is significantly decreased. (Table I). Consequently IC_{50} values calculated for some of those flavonoids increased (Table II). In contrast, the ability of less effective flavonoids, the flavone apigenin and all the flavavones and isoflavones studied (except trihydroxisoflavone), was not significantly different in the presence of added bicarbonate.

There is some controversy in the literature concerning the mechanism of peroxynitrite-mediated oxidation of DHR123. Kooy *et al.*[34] suggested that ROD123 formation results from a direct reaction of $ONOO^-$ with DHR123. In contrast, a mechanism by which DHR123 oxidation occurs via the free radical intermediates, formed during the spontaneous decomposition of $ONOO^-$, was proposed.[35] In a recent work, the DHR123 oxidation caused by reaction of intermediates formed during the decay of peroxynitrous acid was also excluded.[36] In the presence of bicarbonate, however, nitrogen dioxide ($^{\cdot}NO_2$) and trioxocarbonate ($CO_3^{\cdot -}$) are formed and these reactive species should be responsible for DHR123 oxidation.[36] Carbonate radical is a relatively strong one-electron oxidant and $^{\cdot}NO_2$ is a more moderate oxidant and also a nitrating agent.[4] Kooy *et al.* [34] suggested a mechanism for DHR123 oxidation based on one electron oxidation promoted by either $ONOO^-$ or $CO_3^{\cdot -}$ to form DHR123 radical, which can then dismutate to form ROD123 and DHR123. Through an alternative mechanism, as proposed by Glebska and Koppenol,[36] $ONOO^-$ may form an adduct with DHR123, followed by protonation and oxidation of DHR123.

Analysis of Structure-activity Relationships

The most effective flavonoid at inhibiting the oxidation of DHR123 by peroxynitrite, with or without bicarbonate in the assay, is the flavone myricetin, which possesses 6 hydroxyl groups, followed by quercetin with 5 hydroxyl groups. In spite of having different hydroxylation patterns, the flavones with 4 hydroxyl groups (kaempferol, luteolin and rutin), were equally efficient inhibitors of DHR123 oxidation, with or without bicarbonate in the assay. This fact suggests that the protecting effects of flavones depend largely on the number of hydroxyl groups and on the presence of either a catechol group in the B-ring or a hydroxyl group at the 3-position. These facts may be sustained by the comparison of luteolin (5, 7, 3′, 4′ —OH) and rutin (3-rut, 5, 7, 3′, 4′ —OH) with apigenin (5, 7, 4′ —OH)

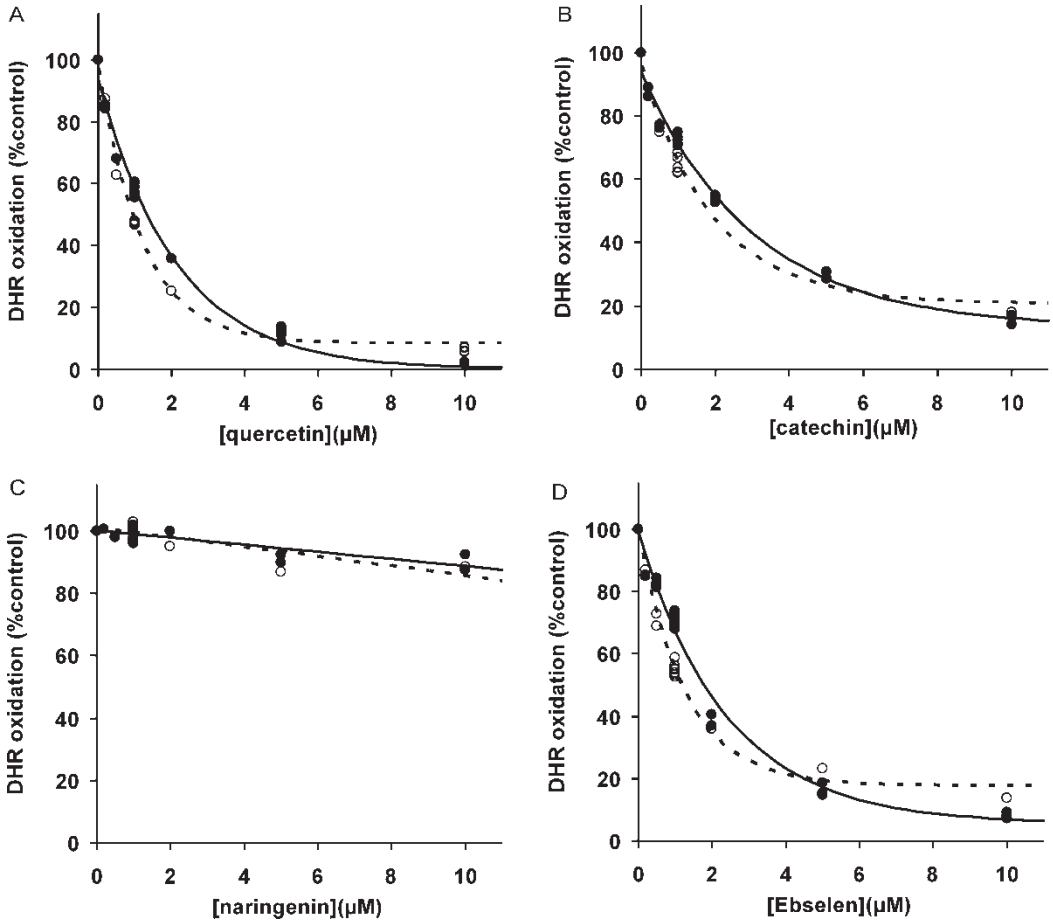

FIGURE 2 Effect of some flavonoids against dihydrorhodamine 123 oxidation by peroxynitrite. A—quercetin; B—catechin and C—naringenin. The results obtained with the standard drug Ebselen are shown for comparison—D. %control in the absence of 25 mM HCO_3^- (O,- - -); %control in the presence of 25 mM HCO_3^- (•,—). In each assay, 25 μM DHR 123 was incubated with either flavonoids or ebselen (0–10 μM) and with or without 25 mM HCO_3^- at 37°C and, after addition of 1.2 μM peroxynitrite, the fluorescence was immediately measured. The fluorescence measurements were expressed in percentage relative to the fluorescence of the control assay (reaction in the absence of either flavonoid or ebselen), which corresponds to 100% of fluorescence. Values represented are from independent experiments ($n = 2–9$).

and kaempferol (3, 5, 7, 4′ —OH) with apigenin (5, 7, 4′ —OH), respectively. In addition, the protective effects of flavones myricetin (6 hydroxyl groups) and quercetin (with 5 hydroxyl groups) are more effective than the one presented by Ebselen, which is considered to be particularly efficient at protecting against peroxynitrite ($k = 2 \times 10^6\ M^{-1}\ s^{-1}$).[11]

TABLE II Half-maximal inhibitory concentrations (IC_{50}) of flavonoids in peroxynitrite mediated oxidation of DHR 123

		IC_{50} (μM)	
Compound	Substituents	Buffer	Buffer + 25 mM $NaHCO_3$
Quercetin	3,5,7,3′,4′ - OH	0.9	1.3
Kaempferol	3,5,7,4′ - OH	1.8	2.3
Luteolin	5,7,3′,4′ - OH	1.7	2.4
Catechin	3,5,7,3′,4′ - OH	1.8	2.4
Naringenin	5,7,4′ - OH	33	44
Genistein	5,7,4′ - OH	23	16
Ebselen	––	1.2	1.8

The results obtained with Ebselen are shown for comparison.

The structural difference between the flavones and the isoflavones is the location of the B-ring, which in the isoflavones is at the 3-position of the C-ring. The three isoflavones studied have a low number of −OH substituents and, therefore, it was expected that these compounds would be less efficient at protecting against peroxynitrite. In fact, this was observed for genistein and daidzein, but not for trihydroxyflavone, which exhibited a fluorescence value similar to the one of Ebselen. This isoflavone possesses a catechol group in the B-ring and most likely this structural feature is important for the protection against peroxynitrite.

The flavanones naringin and naringenin with, respectively, two and three hydroxyl groups showed no activity, as expected. Taxifolin, however, with the same five hydroxyl groups as quercetin (3, 5, 7, 3′, 4′ —OH) also presented a low activity. The flavanol catechin with the same hydroxylation pattern and lacking both the double bond and the 4-oxo group has also a moderate activity.

These results provide evidence for a significant contribution of the 2,3-double bond towards the protection by flavonoids against peroxynitrite.

To sum up, these results suggest that the most efficient flavonoids on protecting DHR123 against oxidation by peroxynitrite have their ability diminished in the presence of bicarbonate, but they keep the hierarchy established in the absence of bicarbonate. That is, myricetin is still the most effective flavone followed by the flavones possessing 5 and 4 hydroxyl groups. In addition, some structural features of flavonoids are essential to confer a good protection against DHR123 oxidation by peroxynitrite. The statistical analysis of the data provides further support for the above assignment. Strong correlations were observed between the number of hydroxyl groups present in each flavonoid and the DHR123 oxidation in the absence and in the presence of added bicarbonate ($r = -0.79$ and -0.80, respectively) (Fig. 3). This means that flavonoids with a large number of hydroxyl groups are more efficient at preventing the DHR123 oxidation by peroxynitrite. In addition, the flavonoids possessing the *o*-catechol group in B-ring are more efficient at protecting against peroxynitrite. The mean of %control of flavonoids possessing the *o*-catechol group is significantly lower (60.8 ± 11.6, $n = 42$; no HCO_3^-) and (66.6 ± 8.8, $n = 39$; with HCO_3^-) than that of flavonoids without this group (90.2 ± 15.1, $n = 30$; no HCO_3^-) and (93.4 ± 7.0, $n = 32$; with HCO_3^-) ($p < 0.001$). The flavonoids with the 3-OH substituent at C-ring are also more effective; they present a mean value of %control significantly lower (60.0 ± 13.2, $n = 33$; no HCO_3^-) and (68.9 ± 12.2, $n = 31$; with HCO_3^-) than those without that structural feature (83.6 ± 18.1, $n = 39$; no HCO_3^-) and (86.3 ± 13.7, $n = 40$; with HCO_3^-) ($p < 0.001$). The presence of the double bond between C2 and C3 is other important flavonoid structural feature for preventing the DHR123 oxidation by peroxynitrite. The mean of %control of flavonoids possessing the double bond is significantly lower (69.3 ± 19.6, $n = 51$; no HCO_3^-) and (76.3 ± 16.0, $n = 51$; with HCO_3^-) than that of flavonoids lacking this structural feature (82.1 ± 16.6, $n = 21$; no HCO_3^-) and (84.7 ± 12.7, $n = 20$; with HCO_3^-) ($p < 0.05$).

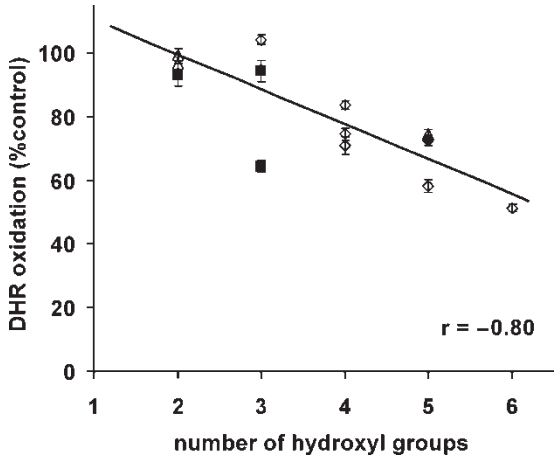

FIGURE 3 Influence of the number of hydroxyl groups on the flavonoid molecule on the dihydrorhodamine 123 oxidation by peroxynitrite, in the presence of 25 mM HCO_3^-. Flavones (◇), isoflavones (■), flavanones (△) and flavanol (◆). The values presented are those shown in Table I. *r*, correlation coefficient.

In summary, the flavones are the most effective flavonoids at protecting against peroxynitrite and their effects depend largely on the number of hydroxyl groups that must include either a catechol group in the B-ring or the 3-hydroxyl group. This conclusion is in agreement with the one presented by Haenen *et al.*,[24] but their study was focused on flavones only. Our work also included some isoflavones, flavanones and a flavanol, which enables us to conclude about the importance of another structural structure: the 2,3-double bond. The most powerful flavonoids are effective at low concentrations with IC_{50} of the same magnitude as Ebselen reported to be excellent at protecting against peroxynitrite. Their effectiveness at low concentrations is an important aspect to consider when characterizing a compound as antioxidant with biological interest.

The mechanism by which flavonoids protect against peroxynitrite-mediated oxidation is not yet established. Previous studies conducted in the absence of added bicarbonate have shown that polyphenolic compounds may be both oxidized and nitrated by peroxynitrite.[25,26] Catechol-containing compounds have the ability to reduce peroxynitrite to nitrite, in a two-electron pathway, resulting in their oxidation to the corresponding *o*-quinones. Flavonoids are powerful electron-donating antioxidants, the presence of o-catechol group (3′,4′—OH) in the B-ring being determinant for a high antioxidant activity.[20] In addition, the presence of a pyrogallol group (3′,4′,5′—OH) in the B-ring further enhances the antioxidant activity, as was demonstrated for myricetin.[20] The majority of any nitration reactions will occur on ring B, predominantly at the 2′- and the 5′- positions.[25] Myricetin, the most effective flavonoid at inhibiting the oxidation of DHR123 by peroxynitrite, is therefore unlike to undergo nitration, due to the presence of an additional hydroxyl group at the 5′ position. It appears therefore that the most effective flavonoids at protecting DHR123 oxidation by peroxynitrite, the catechol-containing flavones, will undergo oxidation reactions to their semiquinone/quinone derivatives.

The reactivity of peroxynitrite is significantly modified by bicarbonate. Since the reaction of peroxynitrite with CO_2 is fast and taking into

account that CO_2/HCO_3^- is an important plasma buffer system, the reactions of peroxynitrite *in vivo* are more likely to be mediated by reactive intermediates derived from the reaction of peroxynitrite with CO_2. As stated above, during $ONOO^-$ mediated DHR123 oxidation, in the presence of bicarbonate, several reactive species may be involved: $ONOO^-$, $CO_3^{\cdot-}$ and $^{\cdot}NO_2$. Most of the flavonoids were less effective inhibitors when bicarbonate is present, suggesting that they are less competitive with DHR123 at reacting with $CO_3^{\cdot-}$ and $^{\cdot}NO_2$, the species generated from the reaction between $CO_2/HCO3^-$ and $ONOO^-$. To fully assess the biological impact of the reactions with those radicals further studies are still needed.

The results of this work show another antioxidant property of flavonoids and indicate that their ability to protect against peroxynitrite depends on a few structural features, which are also important to scavenge oxygen free radicals and to chelate metal ions.[20,22]

Acknowledgements

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