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Hypochlorite scavenging activity of flavonoids

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Abstract

Scavengers of hypochlorite, a highly reactive oxidant produced by activated phagocytes, could have potential therapeutic effects in diseases in which this oxidant plays a pathogenic role. Flavonoids are polyphenolic substances present in food plants and have been extensively studied for their antioxidant properties against various free radicals. Less is known about their reactivity with hypochlorite. In this study, the hypochlorite scavenging activity of flavonoids was investigated using a microplate assay recently developed in our laboratory. This method evaluates the ability of a substance to inhibit the formation of chloramines in human serum albumin upon oxidation by hypochlorite. Thirteen flavonoids were tested. Most of them inhibited human serum albumin oxidation at micromolar concentrations and appeared more active than Trolox, a water-soluble equivalent of vitamin E. It was observed that the greater the number of hydroxyl substitutions, the greater the scavenging activity. The 3-hydroxy substitution seemed to be particularly important for scavenging activity, whereas the presence of a 2,3-double bond in the C ring did not. Flavonoids were found to be good hypochlorite scavengers in-vitro and further information is provided about the chemical aspects important for scavenging activity. Thus, flavonoids could have beneficial effects in diseases such as atherosclerosis in which hypochlorite plays a pathogenic role.

Introduction

Oxidative stress induced by various reactive oxygen species (ROS) is thought to be involved in the aetiology of several diseases as well as ageing (Rice-Evans & Diplock 1993). Hypochlorite ($HOCl/OCl^-$) is the major strong ROS produced by activated neutrophils and monocytes via the reaction of H_2O_2 with Cl^- ions catalysed by the heme enzyme myeloperoxidase (Kettle & Winterbourn 1997). The production of hypochlorite is part of the host defence mechanism against microorganisms but, under certain conditions, may also cause tissue damage (Winterbourn & Kettle 2000) and is considered to be important in the progression of a number of diseases, including atherosclerosis (Hazell et al 1996), inflammatory bowel diseases (Blackburn et al 1999), rheumatoid arthritis (Wu & Pizzo 1999), chronic inflammation and some cancers (Weitzman & Gordon 1990).

Antioxidants have been proposed as a remedy against the deleterious effects of hypochlorite and other ROS. Several epidemiological studies have shown beneficial effects of vegetables and fruits (Block et al 1992), part of which has been attributed to the antioxidant effects of phenolic compounds (Hertog et al 1993). Flavonoids are a large group of naturally occurring phenolic compounds almost ubiquitous in plants, which have recently been studied for their antioxidant properties (Pietta 2000). They have been reported to be good scavengers of hydroxyl radicals ($OH\cdot$) (Hanasaki et al 1994), superoxide ($O_2\cdot^-$) (Robak & Gryglewski 1988), nitric oxide ($NO\cdot$) (van Acker et al 1995), peroxyl radicals ($ROO\cdot$) (Dugas et al 2000) and peroxynitrite ($ONOO^-$) (Haenen et al 1997), and to have protective effects against lipid peroxidation (Saija et al 1995) and low-density lipoprotein oxidation (Aviram & Fuhrman 1998). Their inhibitory effects on the enzyme myeloperoxidase (Pincemail et al 1988), degranulation of activated neutrophils (Blackburn et al 1987) and the stimulus-induced superoxide generation by phagocytic cells (Lu et al 2001) have also been studied. It has been reported that different plant

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extracts containing various flavonoids react efficiently with hypochlorite (Daels-Rakotoarison et al 2002; Valentao et al 2002). There are also some reports about the reactivity of some individual flavonoids such as quercetin (Pincemail et al 1988; Hirose et al 2002), catechin (Scott et al 1993; Sakagami et al 1995; Hirose et al 2002), silibinin (Mira et al 1994; Dehmlow et al 1996), the isoflavone genistein-8-C-glucoside (Zavodnik et al 2000), and S5682 (Daflon 500 mg), a purified flavonoid fraction composed of diosmin and hesperidin (Cypriani et al 1993) with hypochlorite. However, to our knowledge, the structure–scavenging activity relationship of flavonoids against hypochlorite has not been evaluated in a systematic evaluation of structurally different flavonoids.

We have recently developed a number of in-vitro methods for measuring the antioxidant activity of different compounds against hypochlorite (Grippa et al 2000; Gatto et al 2002; Firuzi et al 2003).

In the present study, we applied a microplate method based on the measurement of chloramines in human serum albumin (HSA) (Firuzi et al 2003) to determine the hypochlorite scavenging activity of structurally different flavonoids.

Materials and Methods

Reagents

Apigenin (cat. no. A-3145), baicalein (cat. no. 46,511-9), (±) catechin (cat. no. C-1788), 5,5′-dithiobis(2-nitrobenzoic acid) (cat. no. D-8130), ethylenediaminetetraacetic acid (cat. no. 25,404-5), ferulic acid (cat. no. F-3500), fisetin (cat. no. F-4043), hesperetin (cat. no. H-4125), human serum albumin (HSA) (96–99% pure, Cohn's fraction V, cat. no. A-1653), 5-hydroxyflavone (cat. no. H-4405), 7-hydroxyflavone (cat. no. H-4530), (±) naringenin (cat. no. N-5893), quercetin dihydrate (cat. no. Q-0125), resveratrol (cat. no. R-5010), rutin hydrate (cat. no. R2303), sodium hypochlorite solution 6–14% (Fluka, cat. no. 13440), sodium phosphate dibasic (cat. no. S-7907), sodium phosphate monobasic (cat. no. S-9638), (±) taxifolin (cat. no. T-4512) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich, cat. no. 23, 881-3) were purchased from Sigma-Aldrich. Hydrochloric acid (cat. no. 403871) and methanol (cat. no. 412383) were obtained from Carlo Erba Reagenti. 3-Hydroxyflavone (cat. no. 6213) and hesperidin (cat. no. 086-07342) were purchased from Lancaster Synthesis and Wako Pure Chemical Industries, respectively.

All reagents were of analytical grade and were used without further purification. The concentration of the sodium hypochlorite solution was determined spectrophotometrically at pH 12 (ϵ_{292}: 350 $M^{-1}\,cm^{-1}$).

Hypochlorite scavenging activity of flavonoids

Hypochlorite/hypochlorous acid (XOCl; the pK_a of hypochlorous acid is 7.5, thus at pH 7, OCl^- and HOCl are both present and so we chose the abbreviation XOCl to refer to both chemical species) scavenging activity of structurally different flavonoids was evaluated by a colorimetric microplate-based method (Firuzi et al 2003). Briefly, in 96-well microplates, HSA (5 μM in 50 mM phosphate buffer, pH 7 at room temperature) was incubated in triplicate for 5 min with the substance under investigation, dissolved at different concentrations over the range 20 μM to 2 mM (1.3–130 μM, final concentration). Hesperidin was dissolved in 0.01 M NaOH, 5-hydroxyflavone and 7-hydroxyflavone were dissolved in acetone, and all other substances were dissolved in methanol. The organic solvent to HSA solution ratio was always less than 1/15. XOCl was added to these solutions at a final concentration of 45 μM and, after 10 min, Trolox (0.3 mM) was added to remove excess XOCl, followed by 5-thio-2-nitrobenzoic acid (TNB) addition at a final concentration of 75 μM. At 5 min after TNB addition, the absorbance was measured at 415 nm using a microplate reader (model 3550; Bio-Rad). Proper blanks were analysed for each tested concentration.

The scavenging activity (s) at each concentration (C) of the test substance X ($s_{(X,\ C)}$) was calculated according to the formula: $s_{(X,\ C)} = (A_C - A_O)/(A_N - A_O) \times 100$, where A is the absorbance of the non-oxidized (A_N) or oxidized samples, in the presence (A_C) or absence (A_O) of the scavenger. The samples analysed to obtain A_N and A_O were prepared by adding only the solvent instead of the antioxidant.

The ratios between the chloramine concentrations in the presence and absence of flavonoids in oxidized samples ($[\text{chloramines}]_O/[\text{chloramines}]_{\text{flavonoid}}$) were calculated according to the formula: $A_N - A_O/A_N - A_{\text{flavonoid}}$ and plotted against [flavonoid]/[HSA]. According to the formula: $[\text{chloramines}]_O/[\text{chloramines}]_{\text{flavonoid}} = 1 +$ [flavonoid]/[HSA] (K flavonoid + XOCl/K HSA + XOCl), the slope of each curve represents the reaction rate of the flavonoid and XOCl divided by the reaction rate of HSA and XOCl (K flavonoid + XOCl/K HSA + XOCl).

Furthermore, the ratio of the slopes of the flavonoids and that of Trolox provides a measure to compare the reaction rates of different flavonoids: relative rate constant = $\text{slope}_{\text{flavonoid}}/\text{slope}_{\text{Trolox}}$ = K flavonoid + XOCl/K Trolox + XOCl.

Precision of the assay

Ferulic acid was analysed at a final concentration of 5 mM for quality control purposes and the inter- and intra-assay coefficients of variation were calculated.

Statistical analysis

The regression analyses of the scavenging activity versus the concentration of the substance under investigation were obtained using SigmaPlot version 8.0 for Windows (SPSS Inc., Chicago, IL, USA). The concentrations capable of inducing 25% inhibition of HSA oxidation (25% effective concentration, EC25) were calculated by the same software using the sigmoidal concentration–activity regression

curves. The slopes of the sigmoidal curves were also calculated by the same software. Multiple comparisons were performed by one-way analysis of variance, followed by Fisher's LSD test using the program SigmaStat version 3.00 for Windows (SPSS Inc.). Data were considered statistically different at $P < 0.05$. Linear regression analyses were performed using the same software.

Results

Hypochlorite scavenging activity of flavonoids

The reactivity of the flavonoids with chloramines and TNB was evaluated as previously described (Firuzi et al 2003). They did not react with chloramines or TNB at the tested concentrations, indicating that flavonoids did not interfere with the assay (data not shown).

The scavenging activity at different concentrations of the test substance was calculated according to the above given formula and plotted against the concentration. A representative concentration–activity curve is shown in Figure 1. The EC25 and the slopes of the sigmoidal concentration–activity curves were calculated and are reported in Table 1. Furthermore, $[\text{chloramines}]_O/[\text{chloramines}]_{\text{flavonoid}}$ (chloramine concentrations in the absence and presence of the test flavonoid, respectively) was plotted against [flavonoid]/[HSA] and curves with an intercept of about 1 and different slopes were obtained. The ratios of these slopes and that of Trolox (relative rate constants) are reported in Table 1.

The EC25 of the tested flavonoids increased in the order: catechin, quercetin, taxifolin, baicalein, hesperetin, naringenin, apigenin, rutin, fisetin, 3-hydroxyflavone, hesperidin and 7-hydroxyflavone. 5-Hydroxyflavone was not active. The EC25 of the tested flavonoids was slightly higher than that of resveratrol, a non-flavonoid polyphenolic antioxidant, but much lower than that of Trolox, a water-soluble equivalent of vitamin E (Table 1). A good inverse correlation was observed between relative rate constants and EC25 (data not shown). The slopes of the sigmoidal concentration–activity curves are also reported in Table 1, but no correlation was observed between these data and the EC25 and relative rate constants.

When the flavonoids were divided into two groups, with or without a hydroxyl group in position 3 in the C ring, an inverse correlation was observed between the total number of hydroxyl groups and the EC25 (Figure 2).

The intra- and inter-assay coefficients of variation were 5.9 % (n = 6) and 9.6% (n = 45), respectively.

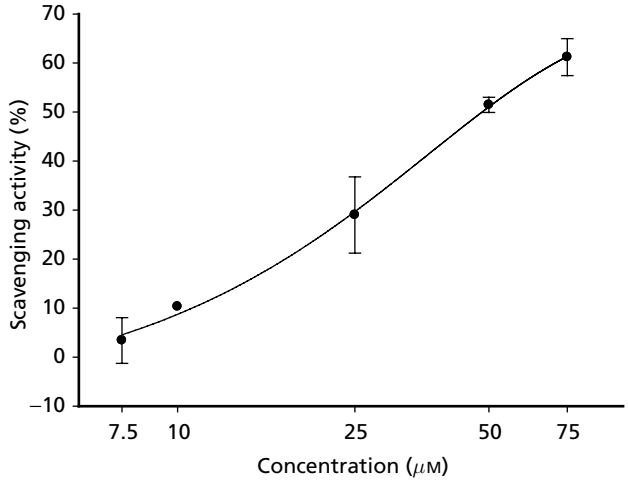

Figure 1 Concentration–activity curve of rutin. The scavenging activity was calculated as described in the text and plotted against the concentration of rutin. The values are the mean ± s.d. of triplicates.

Discussion

The hypochlorite scavenging activity of structurally different flavonoids belonging to the four major classes (flavones, flavonols, flavanones and flavanols) was evaluated by a method recently developed in our laboratory (Firuzi et al 2003) (Table 1). This method measures the ability of a substance to inhibit the hypochlorite-mediated oxidation of HSA, quantified by the measurement of chloramines, which are formed by the reaction of hypochlorite with protein amine groups (Hawkins & Davies 1998).

The oxidation of various proteins by hypochlorite has been implicated in different diseases (Blackburn et al 1999; Hazell et al 1999). Methionine and cysteine residues in proteins are the most readily oxidized (Arnhold et al 1990), but amine groups such as ε-amino groups of lysine residues can also be oxidized by hypochlorite, giving rise to chloramines (Hawkins & Davies 1998). Indeed, halogenation of amine groups is the most favoured chlorinating reaction of hypochlorite (Pullar et al 2000). Also, in oxidative modification of low-density lipoprotein molecules, which is thought to contribute to atherogenesis (Berliner & Heinecke 1996), the major site of attack of hypochlorite seems to be the protein component (apoprotein B-100) and the ε-amino groups of lysine residues (Hazell et al 1999).

Except for 5-hydroxyflavone, all the flavonoids tested in this study were able to protect HSA against oxidation by hypochlorite, with EC25 values much lower than that of Trolox, a water-soluble equivalent of vitamin E. Some of them had EC25 values comparable with resveratrol, a non-flavonoid polyphenolic antioxidant (Table 1). The relative rate constants of the flavonoids, a measure of the rate of their reaction with hypochlorite, were much higher than that of Trolox. A good inverse correlation was observed between effective concentrations and relative rate constants and these two parameters were used as indexes of antioxidant activity. The slopes of the sigmoidal concentration–activity curves were also reported to provide more information about the shapes of the concentration–activity curves, but no correlation was seen with the indexes of activity (Table 1). Flavonoids have been shown to be potent antioxidants against different ROS in-vitro (Pietta 2000). There are also reports about the hypochlorite scavenging activity of some individual

Table 1 Chemical structures of the flavonoids tested in this study and their hypochlorite scavenging activity.

Substances	R_3	R_5	R_6	R_7	$R_{3'}$	$R_{4'}$	EC25 (μM)[1]	Relative rate constant[2]	Slope of the concentration–activity curve ($1/\mu$M)[3]
FLAVONES									
Apigenin	H	OH	H	OH	H	OH	15.0 (±4.6)[c]	127.9 (±9.8)[c]	27.9 (±5.2)
Baicalein	H	OH	OH	OH	H	H	9.1 (±1.2)[ab]	282.9 (±36.2)[b]	52.4 (±10.3)
5-OH Flavone	H	OH	H	H	H	H	Not active	Not active	–
7-OH Flavone	H	H	H	OH	H	H	246.3 (±59.9)[h]	16.0 (±0.4)[g]	27.1 (±4.3)
FLAVONOLS									
3-OH Flavone	OH	H	H	H	H	H	72.3 (±7.0)[f]	45.2 (±2.4)[e]	16.9 (±2.3)
Fisetin	OH	H	H	OH	OH	OH	29.4 (±2.3)[e]	107.4 (±1.6)[d]	65.7 (±1.7)
Quercetin	OH	OH	H	OH	OH	OH	8.8 (±0.6)[ab]	449.6 (±24.5)[a]	68.2 (±10.1)
Rutin	OR[4]	OH	H	OH	OH	OH	22.7 (±3.6)[d]	134.1 (±9.4)[c]	58.8 (±1.1)
FLAVANONES									
Hesperetin	H	OH	H	OH	OH	OM[5]	13.7 (±4.1)[bc]	167.4 (±30.1)[c]	23.8 (±4.9)
Hesperidin	H	OH	H	OR[4]	OH	OM[5]	100.3 (±16.7)[g]	31.4 (±0.6)[f]	22.4 (±2.3)
Naringenin	H	OH	H	OH	H	OH	14.5 (±4.4)[c]	139.3 (±1.0)[c]	29.8 (±0.3)
Taxifolin	OH	OH	H	OH	OH	OH	9.0 (±2.1)[ab]	476.2 (±5.0)[a]	33.4 (±6.3)
FLAVANOLS									
Catechin	OH	OH	H	OH	OH	OH	8.6 (±0.9)[a]	460.4 (±30.1)[a]	55.7 (±0.8)
OTHER ANTIOXIDANTS									
Resveratrol							5.2 (±0.6)[a]	454.7 (±19.4)[a]	28.5 (±1.5)
Trolox							3900 (±900)[i]	1 (±0.1)[h]	49.6 (±13.4)

[1]25% effective concentration against 45 μM hypochlorite. [2]Relative rate constant was calculated dividing the slope of the competition kinetics plot of the substance by that of Trolox. [3]The slope of the sigmoidal concentration–activity curve. [4]*O*-Rutinose. [5]*O*-Methyl. Values are the mean ± s.d. of at least three experiments. Multiple comparisons were performed by one-way analysis of variance followed by Fisher's LSD test. Values within a column with different letters are significantly different at $P < 0.05$.

flavonoids (Pincemail et al 1988; Hirose et al 2002). However, there has been no systematic evaluation of their reactivity against hypochlorite and related structure–activity relationships.

Our results showed that catechin, quercetin and taxifolin had similar activities (similar effective concentrations and relative rate constants) and were the most active of the tested flavonoids (Table 1). Their common structural

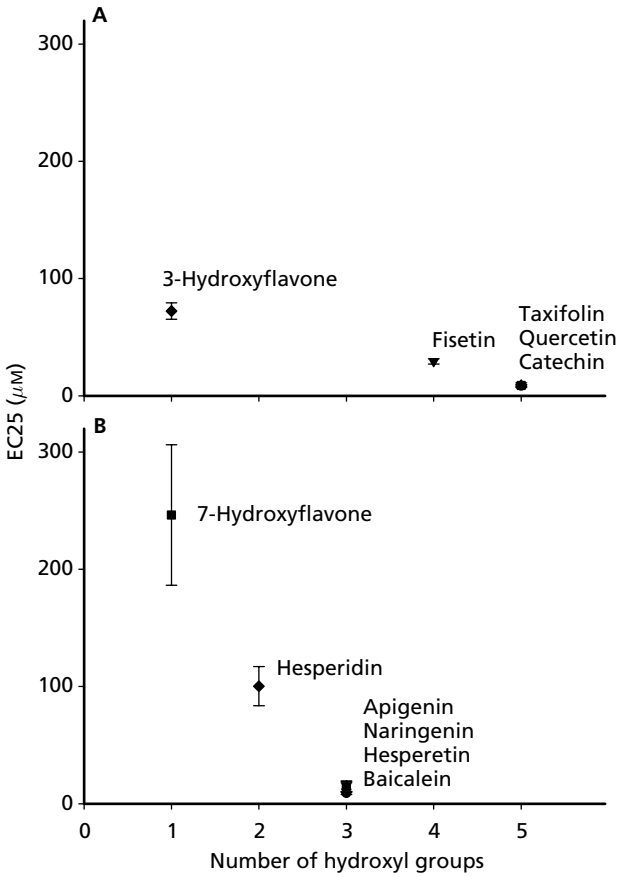

Figure 2 Correlation between the number of hydroxyl substitutions of different flavonoids and their 25% effective concentrations (EC25). EC25 values of flavonoids with (A) and without (B) a hydroxyl group in position 3 in the C ring were plotted against their total number of hydroxyl groups. Good inverse correlations were observed in both groups. The values are the mean ± s.d. of at least three different experiments.

feature is the presence of 5 hydroxyl groups in their diphenylpropane structure (Table 1), indicating that the presence of more hydroxyl groups confers greater activity against hypochlorite. When the tested flavonoids were divided into two groups, according to the presence or absence of the hydroxyl group in position 3 in the C ring, an inverse correlation was observed between the number of OH substitutions and the EC25 in each group (Figure 2). Rutin was excluded because its 3-hydroxy group is occupied by a sugar moiety (Table 1). This observation also confirmed the importance of the number of OH substitutions in hypochlorite scavenging. The impact of the number of OH groups on antioxidant activity has also been observed by others evaluating the activity of flavonoids against other ROS (Cao et al 1997; de Groot and Rauen 1998; Silva et al 2002).

We observed that 3-hydroxyflavone is more active than 7-hydroxy- and 5-hydroxyflavone (Table 1). Thus, among mono-hydroxyflavones, the OH group in position 3 has probably the greatest effect on hypochlorite scavenging activity. In addition, blocking the 3-OH group of quercetin with a glycoside, as in rutin, greatly decreases the activity. The influence of 3-OH on antioxidant activity (Sichel et al 1991; Rice-Evans et al 1996; Haenen et al 1997) and in particular on hypochlorite scavenging efficiency (Hirose et al 2002) has also been suggested by others.

Comparing apigenin, hesperetin and naringenin, which have similar activities and in their chemical structures differ in the presence of a 2,3-double bond (Table 1), it could be deduced that the presence of a 2,3-double bond does not influence the hypochlorite scavenging activity. The same notion can be applied to catechin, quercetin and taxifolin, which have similar activity (Table 1). Other authors using different antioxidant methods reported the importance of unsaturation in the C ring, which allows electron delocalization across the molecule for stabilization of the aryloxyl radical that forms after the reaction with free radicals. They also indicated that in the absence of a catechol-like structure in the B ring as in apigenin, hesperetin and naringenin, the structural characteristics in the C ring do not influence the antioxidant activity (Rice-Evans et al 1996). Our results demonstrated that unsaturation in the C ring does not influence hypochlorite scavenging activity either in the presence or absence of a catechol-like structure in the B ring.

Flavonoid glycosides, rutin (quercetin 3-rutinoside) and hesperidin (hesperetin 7-rutinoside) were shown to be much less active than their corresponding aglycones (Table 1). This is supported by other reports that the glycosylation of flavonoids reduces their activity (Sichel et al 1991; Shahidi & Wanasundara 1992: Rice-Evans et al 1996; Arora et al 1998). Baicalein, hesperetin, naringenin and apigenin, which lack the catechol-type structure (3′,4′-*o*-dihydroxy) in the B ring, had lower effective concentrations than fisetin and rutin, which possess this structure (Table 1). Although it has been shown that a catechol-type structure gives a high contribution to the antioxidant activity against various ROS (Sichel et al 1991; Haenen et al 1997), it did not seem to have an important influence on hypochlorite scavenging activity in our study.

Conclusion

We have further elucidated the hypochlorite scavenging activity of flavonoids. They were shown to inhibit HSA oxidation at micromolar concentrations. Flavonoids have been shown to be absorbed to human plasma (Moon et al 2000) and their oral administration has proved useful in the treatment of certain diseases (Jantet 2002). Thus, considering their high reactivity against hypochlorite and their bioavailability in humans, they may have potential beneficial effects in diseases in which hypochlorite plays a pathogenic role.

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