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Hypochlorite scavenging activity of hydroxycinnamic acids evaluated by a rapid microplate method based on the measurement of chloramines

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Abstract

Scavengers of hypochlorite (XOCl) could have beneficial effects in diseases in which this oxidant plays a pathogenic role. It has been reported that ferulic acid and chlorogenic acid, the quinic ester of caffeic acid, are good hypochlorite scavengers, but a systematic evaluation of the naturally occurring hydroxycinnamic acids (HCAs), which these substances belong to, has not been performed yet. Thus, in this work we studied, by two different in-vitro methods, the antioxidant activity of five HCAs: *p*-coumaric acid, ferulic acid, sinapinic acid, caffeic acid and chlorogenic acid. The methods applied in this study were based on the oxidation of human serum albumin (HSA) by XOCl, a new microplate method based on the measurement of chloramines and a previously described carbonyl assay. Firstly, lysine-derived chloramines, in the presence or absence of the HCAs, were detected using 5-thio-2-nitrobenzoic acid (TNB), measuring the absorbance at 415 nm by a microplate reader. To remove excess XOCl, Trolox, a known XOCl scavenger, was added before TNB. Secondly, lysine-derived carbonyls, in the presence or absence of the HCAs, were detected by using 2,4-dinitrophenylhydrazine. Hydroxycinnamic acids appeared active (caffeic $\geq$ sinapinic $>$ chlorogenic $\cong$ ferulic $>$ *p*-coumaric acid) by both methods, suggesting possible pharmacological applications for these compounds, which are present at high concentrations in the plant kingdom.

Introduction

It is well known that oxidative stress plays a significant role in aging and several diseases. Among the most important reactive oxygen species (ROS) is hypochlorite (XOCl), a powerful oxidant produced by activated neutrophils via the reaction of H_2O_2 and Cl^-, catalysed by the heme enzyme myeloperoxidase (Kettle 1996), which can promote oxidative damage and has been implicated in the pathogenesis of various diseases such as atherosclerosis (Heinecke 1997), rheumatoid arthritis (Babior 2000) and inflammatory bowel diseases (Blackburn et al 1999). Its negative effects in these pathological conditions include oxidation of lipids and various macromolecules: in particular, oxidation by XOCl of protein lysine residues, leading to chloramine and carbonyl groups, was implicated in the pathogenesis of atherosclerosis (Hazell et al 1999).

Thus, XOCl-scavenging substances may be considered possible drugs for the treatment of aforementioned diseases. Among the known scavengers of XOCl, there are the flavonoids (de Groot & Rauen 1998), polyphenolic compounds well known for their antioxidant activity. Surprisingly, less attention has been directed to the antioxidant properties of simple phenolic acids like hydroxycinnamic acids (HCAs) against XOCl, which are present in the plant kingdom in higher concentrations than flavonoids (Rice-Evans et al 1997). To our knowledge, only ferulic acid and chlorogenic acid, the quinic ester of caffeic acid, have been studied from this point of view (Scott et al 1993; Kono et al 1995).

Hence, we decided to study the XOCl-scavenging activity of the main naturally occurring HCAs and chlorogenic acid. Based on our previous experience (Grippa et al 2000; Gatto et al 2002), we developed a new microplate assay to study the ability of these compounds to inhibit the formation of chloramines in human serum albumin

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(HSA) by XOCl (Hawkins & Davies 1998). Data obtained by this new method were confirmed by a carbonyl assay, performed according to the methods of other authors (Levine et al 1990; Yan et al 1996), with some modifications.

Materials and Methods

Reagents

Benzoic acid (Sigma, cat. no. B7521), caffeic acid (Sigma, cat. no. C-0625), chlorogenic acid (Sigma, cat. no. C-3878), ferulic acid (Sigma, cat. no. F-3500), p-coumaric acid (Sigma, cat. no. C-9008), 2,4-dinitrophenylhydrazine (DNPH, Fluka, cat. no. 42210), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma, cat. no. D-8130), ethanol (Fluka, cat. no. 02870), human serum albumin (HSA, Sigma, 96–99% pure, Cohn's fraction V, cat. no. A-1653), α-lipoic acid (Sigma, cat. no. T-5625), methionine (Sigma, cat. no. M-9625), sinapinic acid (Sigma, cat. no. D-7927), S-methylglutathione (Sigma, cat. no. M-4139), hypochlorite, 6–14% (XOCl, Fluka, cat. no. 13440), dibasic sodium phosphate (Sigma, cat. no. S-7907), monobasic sodium phosphate (Sigma, cat. no. S-9638), trichloroacetic acid (Fluka, cat. no. 91240), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich, cat. no. 23, 881-3) were purchased from Sigma-Aldrich (www.sigma-aldrich.com). Hydrochloric acid (HCl, cat. no. 403871) and methanol (cat. no. 412383) were obtained from Carlo Erba (www.carloerbareagenti.com). Ethyl acetate (cat. no. 2513) was purchased from Lab-Scan (Dublin, Ireland).

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

Chloramine assay

Oxidation of HSA was quantified by measuring the chloramines using the reagent 5-thio-2-nitrobenzoic acid (TNB), as described by other authors (Kettle & Winterbourn 1994) with several significant modifications.

Oxidation of albumin

In 96-well microplates, HSA (5×10^{-6} M in 50 mM phosphate buffer, pH 7 at 22 °C) was oxidised by various concentrations of XOCl (0–7.5×10^{-5} M) for 10 min at room temperature, and Trolox was added up to a concentration of 3.1×10^{-4} M to remove excess XOCl.

Preparation of TNB

DTNB was dissolved in phosphate buffer at a concentration of 1 mM. The pH was adjusted to 12 with NaOH and, after 5 min, the pH was brought back to 7 with HCl and the solution was kept at 4 °C until use.

Measurement of chloramines

Five minutes after the addition of Trolox to the oxidised HSA, TNB was added up to a concentration 7.5×10^{-5} M and the samples were incubated for 5 min. Then, the absorbance of the samples was measured at 415 nm, using a microplate reader (Bio-Rad, model 3550).

Stability of chloramines

In another set of experiments, HSA was oxidised as described above by XOCl 4.5×10^{-5} M and TNB was added 5–30 min after the addition of Trolox.

Hypochlorite scavenging activity of hydroxycinnamic acids and chlorogenic acid

Putative scavengers, dissolved in methanol at different concentrations, were added to HSA and the samples were incubated at room temperature for 5 min. Then, the protein was oxidised by adding XOCl (4.5×10^{-5} M) and the samples were analysed as described above. For coloured substances, such as p-coumaric acid, sinapinic acid, caffeic acid and chlorogenic acid, proper blanks were assayed for each tested concentration. The scavenging activity was calculated according to the formula $[(A_{HCA} - A_O)/(A_N - A_O)] \times 100$, where A is the absorbance of the non-oxidised (A_N) or oxidised samples, in the presence (A_{HCA}) or absence (A_O) of the scavenger.

Moreover, the ratios between the chloramine concentrations in the presence and in the absence of HCAs in oxidised samples ($[\text{chloramines}]_O/[\text{chloramines}]_{HCA}$) were calculated according to the formula: $(A_N - A_O)/(A_N - A_{HCA})$ and plotted against [HCA]/[HSA]. According to the formula:

$$[\text{chloramines}]_O/[\text{chloramines}]_{HCA} = 1 + [HCA]/[HSA] \; (K\ HCA + XOCl/K\ HSA + XOCl) \quad (1)$$

the slope of each curve represents the reaction rate of HCA and XOCl, divided by the reaction rate of HSA and XOCl. Furthermore, the ratio of the slopes of HCA and that of Trolox provides a measure to compare the reaction rates:

$$\text{slope}_{HCA}/\text{slope}_{Trolox} = K\ HCA + XOCl/K\ Trolox + XOCl \quad (2)$$

Interferences between the tested substances and the assay system

The substances, dissolved in methanol (p-coumaric acid, ferulic acid, sinapinic acid, caffeic acid, chlorogenic acid, Trolox and benzoic acid) or in phosphate buffer (methionine, α-lipoic acid and S-methylglutathione), were mixed with the TNB solution, incubated for 15 min at room temperature and the absorbance was measured.

To determine possible interactions between tested substance and chloramines, the substance was added to oxidised protein after the addition of Trolox. These solutions were left at room temperature for 15 min, then TNB was added and absorbance was measured subsequently.

Precision of the chloramine assay

Ferulic acid was analysed at a final concentration of 5×10^{-3} M for quality control purposes (QC) and the inter- and intra-assay coefficients of variation (CV) were calculated.

Carbonyl assay

Following the oxidation of HSA with XOCl in the presence or absence of a putative scavenger, the carbonyl content of HSA was measured according to Levine (Levine et al 1990), with some modifications.

Measurement of carbonyls in oxidised albumin
HSA 2 mg mL^{-1} (3×10^{-5} M in phosphate buffer) was oxidised by XOCl in the range 0–1.2×10^{-3} M (0–6.2×10^{-7} M mg HSA)$^{-1}$) for 15 min at room temperature. Then, DNPH (dissolved at a concentration of 15 mM in HCl 2 M) was added up to a final concentration of 4.3 μM (mg HSA)$^{-1}$, and the samples were incubated for 30 min in the dark, mixing them every 10 min. Then, trichloroacetic acid was added up to a concentration of 10% w/v, and the samples were incubated for 10 min in the dark and centrifuged at 16 000 g for 5 min. The supernatants were discarded, the precipitates were washed with a mixture of ethanol–ethyl acetate (1:1), incubated in the dark for 10 min and centrifuged again. Following two additional washes, the precipitates were dissolved by vortexing for 20 min in 100 mM sodium phosphate buffer pH 7 (Yan et al 1996), and the absorbance was measured at 360–370 nm by a Hewlett Packard diode array spectrophotometer (model 8452A). The carbonyl content was calculated using the molar absorption coefficient (ϵ) of 22 000 (Levine et al 1990).

Determination of hypochlorite scavenging activity
Methanolic solutions of p-coumaric acid, ferulic acid, sinapinic acid, caffeic acid, chlorogenic acid, Trolox and benzoic acid at different concentrations were added to HSA (3×10^{-5} M) and oxidised by XOCl 6.2×10^{-4} M (3.1×10^{-7} M (mg HSA)$^{-1}$) and the samples were analysed as described above. For sinapinic acid, blank samples were run in parallel in which distilled water was added instead of DNPH solution. The antioxidant activity was calculated using the formula described above.

Statistical analysis

The competition kinetics curves and sigmoidal regression analysis of the scavenging activity versus the concentration of antioxidants were obtained using the software SigmaPlot version 8.0 for Windows (SPSS Inc., Chicago, IL).

Multiple comparisons were performed by one-way analysis of variance, followed by Fisher's LSD test using the program SigmaStat (version 2.03 for Windows; SPSS Inc. Chicago, IL). Regression analyses were performed using the same software. Data were considered statistically different at $P < 0.05$.

Results

Chloramine assay

Development of the assay
When human serum albumin (HSA) was oxidised by hypochlorite (XOCl) at different concentrations in the range 0–7.5×10^{-5} M and the reagent 5-thio-2-nitrobenzoic acid (TNB) was added to quantify the chloramines, an inverse linear correlation was observed between the absorbance of the samples and the concentration of XOCl (Figure 1).

When samples of oxidised HSA were incubated at room temperature for 5–30 min before the addition of TNB, no major absorbance change was observed, indicating a good stability of the chloramines for at least 30 min in the described conditions (data not shown).

Hypochlorite scavenging activity of hydroxycinnamic acids and chlorogenic acid
The antioxidant activity at different concentrations of the substance was calculated according to the formula reported in Materials and Methods and the effective concentration 25 (EC25) was calculated for each substance and reported in Table 1.

Furthermore, $[\text{chloramines}]_0/[\text{chloramines}]_{HCA}$ (where $[\text{chloramines}]_0$ and $[\text{chloramines}]_{HCA}$ are chloramine concentrations in the absence and the presence of the hydroxycinnamic acids (HCAs), respectively) was plotted against [HCA]/[HSA] and curves with an intercept of about 1 and different slopes were obtained. The ratio of HCA slopes and that of Trolox are reported in Table 1.

The observed order of effectiveness, in decreasing order, was as follows: caffeic acid, sinapinic acid, chlorogenic acid, ferulic acid and p-coumaric acid (Table 1). Benzoic acid did not show any activity.

Interfering substances
While HCAs, chlorogenic acid, benzoic acid, Trolox, methionine and S-methylglutathione did not react directly with TNB, as evaluated by spectrophotometric analysis, α-lipoic acid increased the TNB absorbance and therefore could not be tested with this assay. S-methylglutathione and

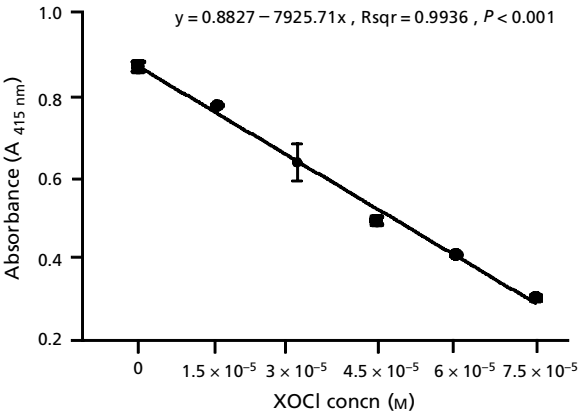
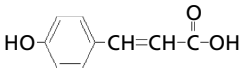
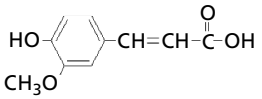
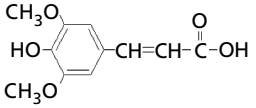
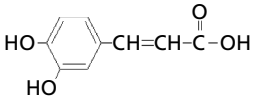
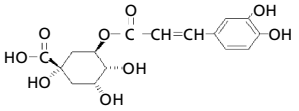


Figure 1 Chloramine assay. Oxidation of human serum albumin (HSA) by hypochlorite (XOCl). In 96-well microplates, HSA (5×10^{-6} M) was oxidised by XOCl (0–7.5×10^{-5} M) for 10 min at room temperature, then Trolox was added up to the concentration of 3.1×10^{-4} M to remove the excess of XOCl. Finally, TNB (7.5×10^{-5} M) was added and the absorbance of the samples was measured at 415 nm, using a microplate reader. Values are means ± standard deviations of triplicate analyses.

Table 1 Hypochlorite scavenging activity of hydroxycinnamic acids and chlorogenic acid.

	S_{max}(mM)	Chloramine assay		Carbonyl assay EC25 (mM)
		EC25 (mM)	Relative rate constant	
p-Coumaric acid 	300	7.2 ± 0.4 d	0.5 ± 0.1 d	5.6 ± 1.2 c
Ferulic acid 	300	2.4 ± 0.4 b	1.2 ± 0.2 bc	1.8 ± 0.4 b
Sinapinic acid 	150	1.5 ± 0.1 a	1.5 ± 0.1 b	1.1 ± 0.2 ab
Caffeic acid 	300	1.2 ± 0.1 a	2.4 ± 0.2 a	0.8 ± 0.1 a
Chlorogenic acid 	150	2.3 ± 0.2 b	1.4 ± 0.5 bc	1.8 ± 0.1 b
Trolox	300	3.9 ± 0.9 c	1 ± 0.1 c	0.9 ± 0.2 a

S_{max}, maximum concentration at which the substance was tested. EC25, effective concentration 25. Values are the means ± s.d. of at least 3 different experiments. Values with different letters (a,b,c,d) are significantly different at $P < 0.05$. Relative rate constant is the slope of competition kinetics plot, divided by the slope of Trolox (see text).

methionine but not HCAs, chlorogenic acid, benzoic acid or Trolox, reacted directly with chloramines before the addition of TNB, therefore could not be tested by this method.

Precision of the chloramine assay

The intra- and inter-assay coefficients of variation (CV) were 5.9% (n = 6) and 10.5% (n = 55), respectively.

Carbonyl assay

Development of the method

Oxidation of HSA by increasing concentrations of XOCl caused an increase in the carbonyl content of the protein with a linear correlation up to 6×10^{-7} M XOCl (mg HSA)$^{-1}$ (Figure 2A).

Increasing the quantity of 2,4-dinitrophenylhydrazine (DNPH) up to 3×10^{-6} M DNPH (mg HSA)$^{-1}$ caused an increase in the final carbonyl content detected in HSA, while further increase in DNPH did not change the measured carbonyl content (Figure 2B). Consequently, we chose 4.3×10^{-6} M DNPH (mg HSA)$^{-1}$ as a suitable amount of DNPH for the experiments.

When we examined the effect of the incubation time of oxidised HSA with DNPH, 30 min appeared to be a suitable time (Figure 2C).

The reaction between XOCl and HSA seemed to be rapid, as indicated by the the considerable amount of carbonyls measured after 5 min (Figure 2D). No major changes were observed after 15 min and the latter was chosen as incubation time for the assay (Figure 2D).

Hypochlorite scavenging activity of hydroxycinnamic acids and chlorogenic acid

The antioxidant activity was reported in Table 1 as effective concentration 25 (EC25). Caffeic acid was the most active substance, followed by sinapinic acid, chlorogenic acid, ferulic acid and p-coumaric acid. Benzoic acid did not show any activity.

Correlation between the chloramine and the carbonyl assays

When the EC25 of the HCA and chlorogenic acid obtained by the chloramine assay were plotted against

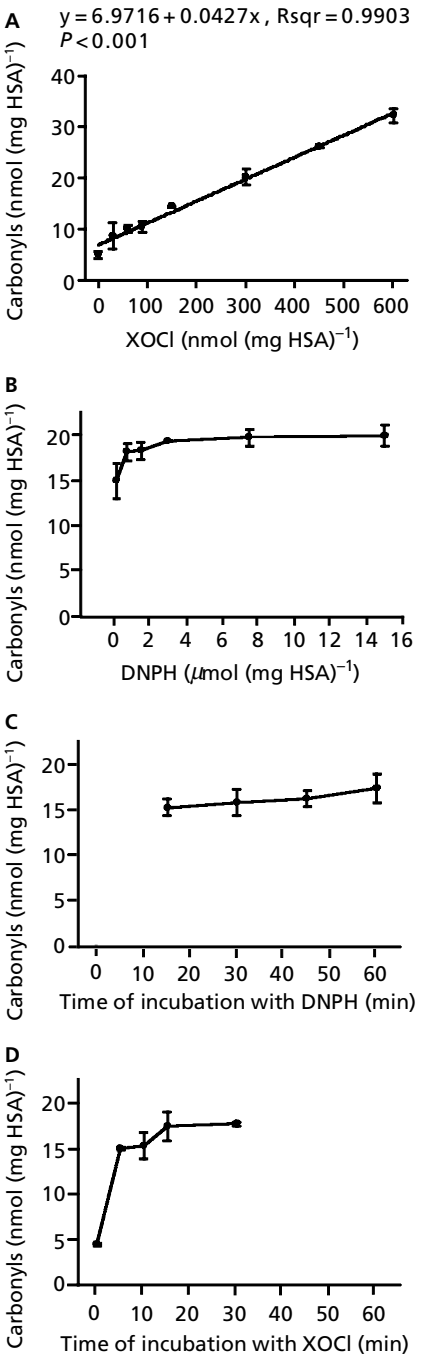


Figure 2 Carbonyl assay. A. Oxidation of human serum albumin (HSA) by hypochlorite (XOCl). HSA (3×10^{-5} M) was oxidised by different amounts of XOCl and the carbonyls were measured as described in Materials and Methods. B. Choice of the optimum DNPH–HSA ratio. HSA was oxidised by XOCl and various quantities of DNPH were used for carbonyl measurement. C. Choice of the optimum DNPH incubation time. Oxidised samples were incubated with DNPH for 15–60 min. D. Choice of the optimum hypochlorite incubation time. Oxidised samples were incubated after XOCl addition for 5–30 min. In B–D, the concentration of XOCl was 3.2×10^{-7} M (mg HSA)$^{-1}$.

those obtained by the carbonyl assay, a good correlation was observed (Figure 3)

Discussion

Hypochlorite (XOCl) scavenging activity of hydroxycinnamic acids (HCAs) and chlorogenic acid was evaluated by two different methods, based on their capability of inhibiting the formation of chloramines and carbonyls in human serum albumin (HSA) following its oxidation by XOCl. It is well known that the ϵ-amino group of lysine side chains can be oxidised by XOCl to form chloramines, some of which can then decompose to carbonyls (Hawkins & Davies 1998). Indeed, lysine residues are among the most susceptible amino acids to oxidation by XOCl (Nightingale et al 2000), a phenomenon that could have a pathological relevance in atherosclerosis (Hazell et al 1999) and other diseases.

Chloramine assay

The reagent 5-thio-2-nitrobenzoic acid (TNB) was used by other authors to quantify the chloramine content of proteins (Kettle & Winterbourn 1994; Hawkins & Davies 1998). In the conditions described in Materials and Methods, the amount of TNB, measured spectrophotometrically at 415 nm, decreased linearly due to the reaction with the chloramines produced by XOCl in the range 0–7.5×10^{-5} M (Figure 1). These absorbance changes were really due to the oxidation of TNB by chloramines, as demonstrated by the ability of methionine, a compound capable of reacting efficiently with chloramines (Hawkins & Davies 1998), to prevent them

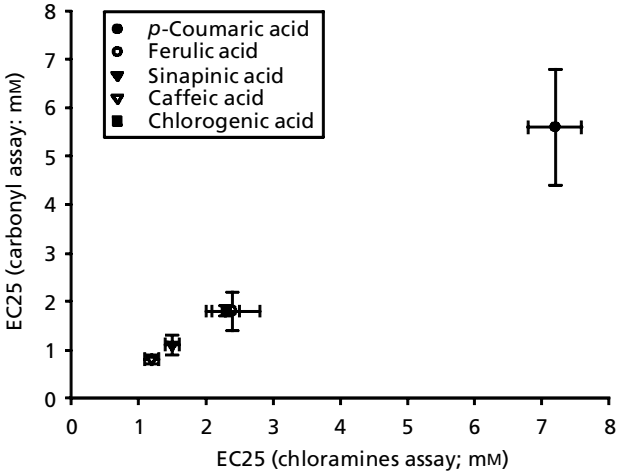


Figure 3 Correlation between the scavenging activity of the hydroxycinnamic acids and chlorogenic acid, evaluated by the chloramine and the carbonyl assay. Effective concentrations reported in Table 1 obtained by two methods were plotted against each other. A good accordance was observed between two methods. The values are the means ± standard deviations of at least 3 experiments.

when added to the oxidised HSA before TNB addition (data not shown).

The main problem encountered during the development of the method was the removal of the excess of XOCl, which could further react with TNB, thus interfering with the assay. To solve this, we used Trolox, a well-known antioxidant, which reacted with neither TNB nor chloramines, as demonstrated by us (data not shown) and other authors (Hawkins & Davies 1999). Moreover, the small quantity of XOCl used in this assay (XOCl–HSA molar ratio: 9) obviated the need for a large amount of the scavenger.

Since chloramines are considered to be semi-stable (Hawkins & Davies 1998), we verified that, in the described experimental conditions, they were stable for at least 30 min (data not shown), a time longer than the assay time.

Carbonyl assay

It is well known that the oxidation of proteins by XOCl and some other reactive oxygen species (ROS) generates carbonyl groups (Levine et al 1990). Indeed, the measurement of protein carbonyls is considered a good indicator of protein damage by free radical reactions both in-vitro and in-vivo. Based on Yan's (Yan et al 1996) and Levine's (Levine et al 1990) works, we set up an assay based on the ability of XOCl scavengers to inhibit the formation of carbonyls in HSA. The best experimental conditions (concentrations and incubation times of XOCl and 2,4-dinitrophenylhydrazine (DNPH)) were chosen based on specific experiments (Figure 2A–D).

Hypochlorite scavenging properties of hydroxycinnamic acids and chlorogenic acid

HCAs and chlorogenic acid were able to protect HSA against oxidation by XOCl with an efficiency comparable with that of Trolox (Table 1). When the EC25 of the HCAs and chlorogenic acid obtained by the chloramine assay were plotted against those obtained by the carbonyl assay, a good correlation was observed (Figure 3). Also, the relative reaction rates of HCA with XOCl, estimated as described in Materials and Methods, are in good accordance with effective concentrations. Such systematic evaluation of the XOCl scavenging activity of these substances is new; however, the XOCl scavenging properties of some of these compounds are described by a few authors. Kono (Kono et al 1995) reported that CGA protected Escherichia coli against the bactericidal action of XOCl in a concentration-dependent fashion. Ferulic acid scavenged XOCl at a rate sufficient to protect α_1-antiproteinase against inactivation (Scott et al 1993).

The observed order of activity of HCAs (caffeic acid $\geq$ sinapinic acid $>$ chlorogenic acid $\cong$ ferulic acid $>$ p-coumaric acid) (Table 1) is in agreement with their ability of inhibiting lipid peroxidation induced by metmyoglobin (Castelluccio et al 1995) or by Cu^{2+} and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (Nardini et al 1995), scavenging peroxynitrite (Pannala et al 1998) and 2,2-diphenyl-1-picrylhydrazyl radicals (Chen & Ho 1997).

It is known that the antioxidant activity of HCAs is mainly due to the 4-hydroxy group and that the presence of additional hydroxy or methoxy groups does affect it significantly (Rice-Evans et al 1997). Thus, we speculate that caffeic acid is the most active HCA (Castelluccio et al 1995; Nardini et al 1995; Pannala et al 1998; Son & Lewis 2002; and Table 1), due to the presence of a second hydroxy group in ortho-position, which would increase resonance stabilization and quinone formation (Graf 1992), also lowering the O-H bond dissociation enthalpy and increasing the rate of H-atom transfer to free radicals (Son & Lewis 2002). In agreement with this hypothesis, the presence of a methoxy instead of a hydroxy group in ortho-position in ferulic acid would decrease the efficiency of the scavenging reactions (Graf 1992; Rice-Evans et al 1997; Table 1). However, the presence of an additional methoxy group (electron-donator) would increase the stability of aryloxyl radical through electron delocalisation after hydrogen donation by hydroxy group (Rice-Evans et al 1997), thus explaining the higher activity of sinapinic acid compared with ferulic acid (Table 1). For the same reason, ferulic acid would be more active than p-coumaric acid (Castelluccio et al 1995; Nardini et al 1995; Pannala et al 1998; and Table 1). Finally, esterification of caffeic acid with quinic acid, to generate chlorogenic acid, would decrease its antioxidant activity (Chen & Ho 1997; Pannala et al 1998; Table 1).

Conclusions

We evaluated the hypochlorite (XOCl) scavenging properties of hydroxycinnamic acids (HCAs) and chlorogenic acid using two different methods based on the measurement of protein chloramines and carbonyls which yielded similar activity values. Since the pharmacokinetic properties of these compounds are favourable (Choudhury et al 1999; Olthof et al 2001), we propose to further study HCAs and chlorogenic acid with a view to their possible pharmacological applications in diseases in which XOCl plays a significant pathological role.

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