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In Vitro Antioxidant Profile of Phenolic Acid Derivatives

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Several caffeic acid esters isolated from propolis exhibit interesting antioxidant properties, but their *in vivo* use is compromised by hydrolysis of the ester bond in the gastrointestinal tract. Therefore, a series of caffeic acid amides were synthesized and their *in vitro* antioxidant profile was determined.

A series of hydroxybenzoic acids, hydroxycinnamic acids, and the synthesized caffeic acid amides were tested for both their 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and microsomal lipid peroxidation-inhibiting activity. Some of the highly active antioxidants were further tested by means of electron paramagnetic resonance for their hydroxyl radical scavenging activity. Since a promising antioxidant compound should show a lipid peroxidation-inhibiting activity at micromolar level and a low cytotoxicity, the cytotoxicity of the phenolic compounds was also studied. In all the assays used, the caffeic acid anilides and the caffeic acid dopamine amide showed an interesting antioxidant activity.

Keywords: Caffeic acid; Polyphenols; Antioxidant; DPPH; Lipid peroxidation; EPR spectroscopy

INTRODUCTION

There is currently intense research interest in polyphenolic compounds, such as flavonoids, proanthocyanidins, and phenolic acids. Several epidemiological studies suggest that a high consumption of these polyphenols is inversely related to the risk of cardiovascular diseases[1–3] and certain types of cancer.[4,5] Similarly, a moderate consumption of red wine, which is rich in polyphenols, has been associated with a low risk of coronary heart diseases.[6,7] However, in contrast to certain vitamins, it is difficult to make quantitative recommendations for health-promoting phytochemicals, such as the polyphenols.[8,9] In the past years, the *in vitro* antioxidant activity of flavonoids have been studied in detail, resulting in several structure–activity relationships.[9–12] An important but often overlooked group of polyphenols are the hydroxycinnamic acid (C_6–C_3) derivatives, which are found in almost every plant and are therefore an integral part of our diet.[13] The major representative of hydroxycinnamic acids is caffeic acid, which occurs in food mainly as an ester with quinic acid, called chlorogenic acid. Another class of polyphenols fairly common in plants are the hydroxybenzoic acids (C_6–C_1), such as gallic acid. Propolis from honeybee hives has been used for centuries as an anti-inflammatory medicine. Several caffeic acid esters were isolated from propolis and were found to have anticarcinogenic, anti-inflammatory, and immunomodulatory properties.[14,15] However, the ester bond of caffeic acid esters can be easily hydrolyzed by cinnamoyl esterases, which are produced by a wide range of bacteria and fungi present in the human digestive tract, compromising their use.[16] Therefore, in a previous study a series of caffeic acid amides were synthesized and tested as inhibitors of lipid peroxidation.[17] The study showed that caffeic anilides were strong inhibitors of lipid peroxidation, which prompted us to investigate further their *in vitro* antioxidant profile. In order to carry out a structure–antioxidant activity relationship study, a

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series of hydroxybenzoic acids, hydroxycinnamic acids, and the synthesized caffeic acid amides were tested for both their DPPH scavenging and microsomal lipid peroxidation-inhibiting activity. Some of the highly active antioxidants were further tested for their hydroxyl radical scavenging activity. Since a promising antioxidant compound should show a lipid peroxidation-inhibiting activity at micromolar level and a low cytotoxicity, the cytotoxicity of the phenolic compounds was also studied.

MATERIALS AND METHODS

Reagents

Bovine serum albumin, caffeic acid, (+)-catechin, *trans*-cinnamic acid, *p*-coumaric acid, diethylenetriaminepentaacetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), ferrous sulfate, ferulic acid, gallic acid, hydrogen peroxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protocatechuic acid, quercetin, sinapic acid, sodium dodecyl sulfate, 2- thiobarbituric acid (TBA), and trolox were purchased from Sigma-Aldrich. Coomassie blue G250, ethanol (analytical grade), rutin, trichloroacetic acid, and L(+)-ascorbic acid were obtained from Merck, while chlorogenic acid was obtained from Acros (Belgium). RPMI 1640 with L-glutamine, fetal bovine serum, and trypsin-EDTA solution were from Life Technologies.

Synthesis and Chemistry

The amides were synthesized from the free acid and the amine as described previously.[17]

Cytotoxicity on Fibroblasts

The cytotoxicity was determined by evaluating the cell growth inhibition caused by the test compounds as described previously.[18]

Cell Cultures

Human skin fibroblasts from normal donors were obtained from the laboratory of Genetics, University of Antwerp, Antwerp, Belgium.

Preparation of Test Solutions

Test solutions were freshly prepared by dissolving test compounds in DMSO, followed by dilution with growth medium, i.e. RPMI1640 medium supplemented with 15% fetal bovine serum. For each compound tested, the following dilutions were prepared and tested: 100, 50, 20, 10, 5, and 1 μg/ml.

Cytotoxicity Test

Fibroblasts were seeded into 96-well plates at a density of 10^4 cells per well in growth medium. The plates were incubated at 37°C under a humidified atmosphere containing 5% CO_2. After 24 h the medium was discarded and test solutions were added giving final concentrations of 100, 50, 20, 10, 5 and 1 μg/ml. Four wells were used for each concentration and the last two rows of each plate for appropriate cell controls. After 72 h incubation at 37°C the medium was removed and 20 μl of MTT solution (5 mg MTT dissolved in 1 ml PBS) was added to each well. Four hours at 37°C later the formazan product was solubilized by the addition of 200 μl 10% (w/v) sodium dodecyl sulfate in acidified DMSO (0.6 ml acetic acid per 100 ml solvent). The optical density of each well was measured using an automatic plate reader (Multiscan MCC/340) with a test wavelength of 570 nm and a reference wavelength of 690 nm. The absorbance is directly proportional to the number of living cells. The maximal non-toxic dose (MNTD) was defined as the highest concentration of the test compound which did not affect cell growth compared with cell controls.

Microsomal Lipid Peroxidation

The lipid peroxidation inhibiting activity was determined as described previously.[18] Briefly, reaction mixtures contained 80 μM potassium phosphate buffer (pH = 7.4), microsomes (350–500 μg protein/ml), and test products in various concentrations. Peroxidation was started by adding vitamin C at a final concentration of 200 μM. The reaction mixture was incubated in an open Eppendorf tube for 90 min at 37°C. The reactions were terminated by addition of 250 μl of a 20% trichloroacetic acid solution followed by centrifugation at 10,000*g* for 3 min. The supernatant of 600 μl was boiled with 250 μl of a 0.67% thiobarbituric acid solution for 20 min. At room temperature, the amount of lipid peroxidation was determined by measuring the absorbance of the pink chromogen at 535 nm.

The antioxidant activity of each compound was expressed as IC_{50} value, i.e. the concentration in μM able to inhibit 50% of the TBARS, and was calculated from the corresponding log-dose inhibition curve. For each compound concentration specific sample blanks were taken into account to calculate the IC_{50} value. The test was conducted in triplicate and the data were collected as mean ± SD. Different concentrations of quercetin were measured in multiplicate on separate days and the reproducibility was found to be 7%. Statistical significance between IC_{50} values of test products were evaluated with an unpaired two tailed student *t*-test. A *p* level of 0.05 was

TABLE I DPPH scavenging, lipid peroxidation-inhibiting, and cytotoxic effects of hydroxybenzoic acids, hydroxycinnamic acids, and reference antioxidants

	Compound	DPPH IC_{50} (μM) ± SD	Cytotoxicity MNTD* (μM)	Lipid peroxidation IC_{50} (μM) ± SD	ASI†
Hydroxybenzoic acids					
1	Gallic acid	9.4 ± 0.4	6.0	1.5 ± 0.06	4.0
2	Protocatechuic acid	54.2 ± 6.4	≥648.8	11.5 ± 0.01	≥56.4
Hydroxycinnamic acids					
3	Caffeic acid	19.8 ± 0.5	55.5	3.3 ± 0.08	16.8
4	Chlorogenic acid	22.8 ± 1.5	≥282.2	10.5 ± 0.3	≥26.9
5	*trans*-Cinnamic acid	>100	≥674.8	>100	>>6.8
6	*p*-Coumaric acid	>100	≥609	>100	>>6.1
7	Ferulic acid	61.9 ± 0.01	≥515	>100	>>5.1
8	Sinapic acid	32.1 ± 0.1	≥446	>100	>>4.5
Reference antioxidants					
9	(+)-Catechin	14.3 ± 0.007	≥344.5	3.4 ± 0.07	≥101.3
10	Quercetin	9.7 ± 0.8	29.6	0.95 ± 0.04	31.2
11	Rutin	>100	150.5	26.5 ± 0.9	5.7
12	Trolox	24.0 ± 1.5	≥400	2.8 ± 0.2	≥142.9

*MNTD: maximal non-toxic dose. †ASI: antioxidant selectivity index = MNTD/IC_{50}.

considered to be statistically significant. The water-soluble vitamin E derivative trolox was used as a positive control in the microsomal lipid peroxidation assay. The IC_{50} value of trolox was found to be 2.8 ± 0.2 μM.

DPPH Scavenging Activity

The stable free radical DPPH was dissolved in ethanol to give a 100 μM solution; 0.5 ml of a test compound in ethanol (or ethanol itself as control) was added to 3.0 ml of the ethanolic DPPH solution. For each test compound, different concentrations were tested. The mixtures were shaken vigorously and left to stand in the dark for 20 min. The decrease in DPPH absorption was measured at 517 nm and the actual decrease in absorption induced by the test compound was calculated by subtracting that of the control. The antioxidant activity of each test compound was expressed as an IC_{50} value ± SD, i.e. the concentration in μM that inhibits DPPH absorption by 50%, and was calculated by linear regression analysis. Different concentrations of quercetin were measured in multiplicate on separate days and the reproducibility was found to be 8%. Trolox was used as a positive control and its IC_{50} value was found to be 24.0 ± 1.5 μM. The statistical significance between IC_{50} values of test products was evaluated with an unpaired two tailed student's *t*-test. Statistical significance was set at a p level of 0.05.

Hydroxyl Radical Scavenging Activity

The $HO^{\cdot}$ scavenging activity was measured according to the method described by Taira and coworkers[19] and further adapted in our laboratory. Hydroxyl radicals were generated in a Fenton type reaction and were visualized by DMPO in an EPR instrument. The spin trap DMPO was checked for radical and colored impurities. No EPR signal was detected and a single absorption peak of DMPO at 227 nm was measured with an UV spectrophotometer. The final reaction mixture contained the following reagents: 10 mM DMPO, 0.1 mM ferrous sulfate, 0.1 mM DETAPAC, 50 mM phosphate buffer pH = 7.4 and test compounds in various concentrations. The reaction mixture was transferred to a quartz flat cell with a volume of 200 μl. After two minutes the EPR spectrum was recorded at room temperature using a Magnettech miniscope MS 100 instrument (Magnettech, Berlin, Germany). Scan conditions were as follows: microwave frequency: 9.4 GHz; microwave power: 10 mW; modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; scan time: 42 s; center field: 336.6 mT and sweep width: 6 mT.

Different concentrations of a test compound were analyzed and then the half-maximal inhibitory concentration (IC_{50}) was calculated by linear regression analysis. The test was conducted in quadruplicate and the data were collected as mean ± SD.

RESULTS AND DISCUSSION

In this study, the *in vitro* antioxidant profile of more than twenty synthesized caffeic acid amides was investigated. In order to carry out a structure–antioxidant activity relationship study, it is important to test a whole battery of compounds originating from one or more different types. It must also be emphasized that a compound may not just be classified as an antioxidant on the basis of a single antioxidant experiment.[9] Therefore, a series of hydroxybenzoic acids, hydroxycinnamic acids, caffeic acid amides, and the flavonoids (+)-catechin, quercetin and rutin, and trolox as reference antioxidants were tested for both their DPPH scavenging and lipid peroxidation-inhibiting

TABLE II DPPH scavenging, lipid peroxidation-inhibiting, and cytotoxic effects of caffeic acid amides

	Caffeic acid amides	DPPH IC_{50} (μM) ± SD	Cytotoxicity MNTD* (μM)	Lipid peroxidation IC_{50} (μM) ± SD	ASI†
13	3-Methylbut-2-enyl amine	20.0 ± 0.3	4.0	3.4 ± 0.02	1.2
14	Ammonia	15.8 ± 0.2	111.7	2.2 ± 0.02	50.8
15	Hydroxylamine	–	102.6	2.1 ± 0.07	48.9
16	Methylamine	31.8 ± 0.4	103.6	6.0 ± 0.3	17.3
17	Ethylamine	19.5 ± 0.5	96.6	2.7 ± 0.09	35.8
18	Isopropylamine	25.4 ± 0.1	90.5	3.9 ± 0.2	23.2
19	Isobutylamine	19.2 ± 0.6	85.1	2.2 ± 0.07	38.7
20	Isopentylamine	19.5 ± 0.02	20	1.4 ± 0.07	14.3
21	Allylamine	23.5 ± 0.06	45.7	2.2 ± 0.02	11.1
22	Aniline	18.7 ± 0.1	39.2	0.38 ± 0.01	103.2
23	2-Aminophenol	15.1 ± 0.3	36.9	0.29 ± 0.007	127.2
24	3-Aminophenol	18.8 ± 0.4	73.8	0.37 ± 0.03	199.5
25	4-Aminophenol	–	73.8	0.63 ± 0.01	117.1
26	Benzylamine	17.8 ± 0.1	18.6	1.02 ± 0.08	18.2
27	Phenethylamine	22.1 ± 0.08	17.7	0.85 ± 0.007	20.8
28	Dopamine	9.5 ± 0.2	63.5	0.59 ± 0.08	107.6
29	Tyrosine-OCH_3	19.0 ± 0.06	140	3.2 ± 0.06	43.8
30	Diethylamine	17.7 ± 0.2	85.1	4.1 ± 0.06	20.8
31	Pyrrolidine	23.0 ± 0.2	43	2.4 ± 0.09	17.9
32	Piperidine	23.2 ± 0.5	4.0	3.6 ± 0.04	1.1
33	Morpholine	–	200.8	6.1 ± 0.2	32.9
34	3-Methyl-2-Butenylamine‡	>100	86.6	29.1 ± 3.7	3.0
35	3-Methyl-2-butenol¶	19.0 ± 0.2	4.0	3.5 ± 0.2	1.2

*MNTD: maximal non-toxic dose. †ASI: antioxidant selectivity index = MNTD/IC_{50}. ‡*p*-Coumaric amide. ¶Caffeic acid ester.

activities. Their half-maximal inhibitory concentrations (IC_{50} values) are listed in Tables I and II.

From all the compounds tested the hydroxybenzoic acid gallic acid (**1**), the flavonol quercetin (**10**), and the caffeic acid dopamine amide (**28**) showed the highest DPPH scavenging activity, with IC_{50} values lower than 10 μM. All these compounds possess a catechol moiety, which is generally accepted as the major radical target site for phenolic compounds.[20] From all the hydroxycinnamic acids tested, caffeic acid (**3**) showed the highest DPPH scavenging activity, whereas *p*-coumaric acid (**6**) and *trans*-cinnamic acid (**5**) were not active even at concentrations up to 100 μM. Esterification of caffeic acid with quinic acid decreased slightly the scavenging activity ($p < 0.05$). *O*-Methoxylation of the phenyl group of *p*-coumaric acid increased the activity, as shown by the lower IC_{50} values for ferulic acid (**7**) and sinapic acid (**8**) compared to that of *p*-coumaric acid. Introduction of a dopamine amide function on caffeic acid doubled the antioxidant activity, indicating once again the importance of a catechol moiety for the DPPH scavenging activity.

TABLE III $HO^{\cdot}$ Scavenging activity of some highly active antioxidants

	Compound	$HO^{\cdot}$ Scavenging activity IC_{50} (μM)
Hydroxybenzoic acid		
1	Gallic acid	191 ± 22
Hydroxycinnamic acid		
3	Caffeic acid	572 ± 19
Reference antioxidants		
9	(+)-Catechin	693 ± 27
10	Quercetin	391 ± 11
11	Rutin	1177 ± 38
12	Trolox	1243 ± 59
Caffeic acid amides		
23	2-Aminophenol	582 ± 67
28	Dopamine	379 ± 16

In contrast to DPPH, $HO^{\cdot}$ are highly reactive radicals that react at the site of formation. Therefore, some of the antioxidants were further tested for their $HO^{\cdot}$ scavenging activity (Table III). $HO^{\cdot}$ were generated in a Fenton type reaction and were visualized by the spin trap DMPO in an EPR instrument. The EPR signal of the DMPO-$OH^{\cdot}$ adduct is inhibited by the presence of $HO^{\cdot}$ scavengers, who compete with DMPO for $HO^{\cdot}$. Since DMPO is present in the reaction mixture at a final concentration of 10 mM, the IC_{50} values of the $HO^{\cdot}$ scavenging assay are much higher than those of the DPPH assay. The most active $HO^{\cdot}$ scavenger was the pyrogallol-containing gallic acid, while rutin and trolox showed the lowest activity. Caffeic acid and caffeic acid 2-aminophenol amide (**23**) had similar IC_{50} values, which were significantly higher than that of quercetin and caffeic acid dopamine amide (**28**). These results are in agreement with those from the DPPH assay.

In a previous study, we investigated the spin population of the caffeic acid methylamide (**16**) and anilide (**22**) to illustrate the effect of the *N*-substituent on the caffeoyl group on the unpaired spin density distribution.[17] An additional mesomeric effect with the second aromatic ring was demonstrated for compound **22**, resulting in a more effective spin delocalisation compared to the *N*-methyl compound

(**16**).[17] This could partially explain the higher DPPH free radical scavenging activity of compound **22** compared to compound **16**.

Besides the free radical scavenging assays, all the compounds were tested in the microsomal lipid peroxidation assay, which is one of the most biomimetic antioxidant tests. Lipid peroxidation was initiated in rat liver microsomes by the addition of vitamin C and was measured spectrophotometrically with the TBA-test. The role of vitamin C and Fe^{3+} ions in starting microsomal lipid peroxidation was examined by measuring the IC_{50} and IC_{100} values of the powerful Fe^{3+} chelator desferrioxamine. The IC_{50} and IC_{100} value of desferrioxamine was found to be, respectively, 0.03 and 0.1 μM, indicating clearly the importance of both Fe^{3+} ions and vitamin C to start microsomal lipid peroxidation.

Of all hydroxycinnamic acids tested, caffeic acid exhibited the highest lipid peroxidation-inhibiting activity, while chlorogenic acid was about three times less active. All the other hydroxycinnamic acids were not active even at concentrations up to 100 μM. The lipid peroxidation-inhibiting activity of caffeic acid is comparable with that of the flavanol (+)-catechin, but was much lower than the flavonol quercetin.

Caffeic acid (**3**) and ester (**35**) appeared as active as amide (**13**), while the *p*-coumaric amide (**34**) is about ten times less active compared to the corresponding caffeic acid amide (**13**), proving that the caffeoyl group is the most important moiety for the antioxidant activity. However, the lipid peroxidation-inhibiting activity of the caffeic acid amides, with aromatic amines (**22**–**29**) was significantly higher than the amides with aliphatic amides (**13**–**21**, **30**–**33**). The most interesting caffeic acid amides are the anilides (**22**–**25**) with IC_{50} values in the higher nanomolar range.

Besides the DPPH scavenging and microsomal lipid peroxidation-inhibiting activities, the cytotoxicity of the phenolic compounds was also investigated. The cytotoxicity of a compound can be determined by measuring cytotoxic parameters, such as the integrity of the cell and the effect on cell growth. Since the latter is a more sensitive cytotoxic parameter and more appropriate for pharmacological work, the cytotoxicity of the phenolic compounds was investigated on growing fibroblasts using an MTT assay.[18] The maximal non-toxic dose (MNTD) was defined as the highest concentration of the compound that did not affect cell growth compared with cell controls. Except for caffeic acid, all other hydroxycinnamic acids showed a low cytotoxicity, indicating that a catechol moiety in a hydroxycinnamic acid increases the cytotoxicity, while the introduction of a sugar moiety decreases the cytotoxicity. In the case of the hydroxybenzoic acids tested, the catechol-containing protocatechuic acid exhibited a significantly lower cytotoxicity compared to the pyrogallol-containing gallic acid. The MNTD of the synthesized caffeic acid amides varied strongly from 4.0 to 200.8 μM compared to 55.5 μM for caffeic acid.

Since a promising antioxidant compound should show a lipid peroxidation-inhibiting activity at micromolar level and a low cytotoxicity, an antioxidant selectivity index (ASI), i.e. the maximal non-toxic dose divided by the IC_{50} value for lipid peroxidation, was introduced.[18] Compounds with an ASI higher than 100 would have an interesting safety/antioxidant activity profile and merit therefore further investigation. It must be emphasized that the well-known antioxidant quercetin exhibited a low ASI, while (+)-catechin and trolox, which had in both assays a lower antioxidant activity compared to quercetin and myricetin, showed an ASI of more than 100. None of the hydroxycinnamic acids and hydroxybenzoic acids exhibited an ASI higher than 100 at a maximal concentration tested of 100 μg/ml, while the caffeic acid anilides (**22**–**25**) and the caffeic acid dopamine amide (**28**) showed an ASI higher than 100.

In conclusion, the caffeic acid anilides and the caffeic acid dopamine amide showed an interesting antioxidant activity. In addition they also exhibited an ASI higher than 100. Therefore, these compounds need further investigation to evaluate their *in vivo* antioxidant activity.

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