Antioxidant Properties of Novel Tetraoxygenated Phenylindan Isomers Formed during Thermal Decomposition of Caffeic Acid

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Mild pyrolysis (228 °C, 15 min) of rosmarinic, chlorogenic, and caffeic acids increased their antioxidative efficacy in a biological rat liver membrane assay by 4-, 11-, and 460-fold, respectively. The active components in the caffeic acid pyrolysates were identified as the recently isolated novel tetraoxygenated 1,3-*cis*- and 1,3-*trans*-phenylindan isomers, which showed comparable IC_{50} values (0.041 and 0.04 μM, respectively) and were ~8-fold more active than butylated hydroxytoluene (BHT). Comparison of nonroasted, light-roasted, and dark-roasted coffee extracts showed that the degree of roasting is positively correlated to the inhibition of lipid peroxidation in rat liver membranes. The potent reducing properties of the phenylindan isomers resulted in (a) prooxidative effects at relatively higher concentrations in an ethyl linoleate peroxidation assay, and (b) promotion of hydroxylation of 2′-deoxyguanosine to afford 8-oxo-2′-deoxyguanosine. However, the results of the rat liver homogenate model system show that pyrolysis of caffeic acid and its esters chlorogenic acid and rosmarinic acid can procure potent antioxidants and underlines the potential use of heat processing to generate novel bioactive molecules.

Keywords: *Caffeic acid; phenylindan isomers; antioxidants, prooxidants; polyphenols; lipid peroxidation*

INTRODUCTION

"Activated" oxygen molecules, also referred to as "reactive oxygen species" (ROS) are generated as a consequence of normal metabolic events or exposure to oxidizing agents or ionizing radiation. Examples of biologically relevant ROS are hydroxyl (•OH), superoxide ($O_2^{•-}$), nitroxyl (NO•), R-oxyl (RO•), R-peroxyl (ROO•), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). It has been established that ROS play an important role in certain clinical diseases and in the process of aging (Harman, 1981; Halliwell and Gutteridge, 1989; Ames and Gold, 1991; Ames *et al.*, 1993), and recently emphasis has also been placed on the significance of ROS in oxygen-mediated food deterioration (Aruoma, 1991; King *et al.*, 1993).

In the past decade, focus has been on antioxidants obtained from natural sources, i.e. either endogenously present in the food raw material or generated during food processing by heating (pyrolysis) or microbial fermentation (Eriksson and Na, 1993; Löliger and Wille, 1993). In addition to the well-known antioxidant vitamins C and E and the carotenoids, much attention is directed at other potential antioxidants of plant origin. In this context, many plant phenolics have been shown to possess potent *in vitro* antioxidant properties, often surpassing those of vitamin C and vitamin E (Toda *et al.*, 1985; Kitahara *et al.*, 1992; Namiki, 1990; Matsuzaki and Hara, 1985). Furthermore, the free radical scavenging properties of many of these natural chemicals may have a beneficial impact on health (Okuda, 1993; Ames *et al.*, 1993, 1995).

There are numerous examples of natural phenolics with effective free radical scavenging properties, such as flavonoids (quercetin, rutin), hydroxycinnamic acid derivatives (caffeic, chlorogenic, and ferulic acids), catechins (epigallocatechin, epigallocatechin 3-gallate), diterpenoid phenols (carnosol, carnosic acid), and simple monocyclic compounds such as thymol, hydroxytyrosol, and sesamol (for a detailed review see Pratt, 1992). Recently, novel phenylindan-type stereoisomers were isolated and identified as the major products of caffeic acid pyrolysis (Stadler, 1994; Stadler *et al.*, 1996). Structurally, these tricyclic dimers contain two *o*-dihydroxybenzyl moieties (Figure 1), common features found in compounds with good reducing and antioxidant properties (Cuvelier *et al.*, 1992).

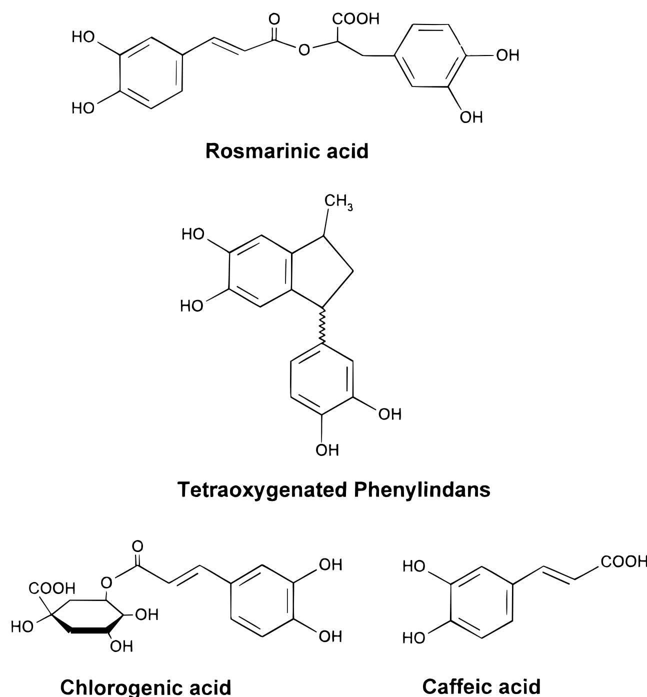

Figure 1. Structural formulas of the tetraoxygenated phenylindans and caffeic acid esters used in this study.

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The objective of this study was to assess the *in vitro* antioxidant and potentially prooxidant activities of these novel tetraoxygenated phenylindan isomers using two independent lipid peroxidation assays and an oxidation assay with the purine nucleoside 2′-deoxyguanosine (2′-dG) as a substrate. This study addresses, for the first time, the impact of thermal treatment (pyrolysis) on the antioxidant activities of caffeic acid and its esters chlorogenic and rosmarinic acids.

EXPERIMENTAL PROCEDURES

Chemicals. All reagents were prepared fresh before use. Linoleic acid ethyl ester (LAEE), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), quercitrin, quercetin, and sodium dodecyl sulfate (SDS) were purchased from Sigma (Buchs, Switzerland). Hydrogen peroxide (H_2O_2) 3% wt stock solution and L-(+)-ascorbic acid (vitamin C) were from Merck (Darmstadt, Germany). Caffeic acid, chlorogenic acid, and tetrabutyl ammonium sulfate were from Fluka (Buchs, Switzerland). Malondialdehyde (MDA) bis(diethyl acetal), 2-thiobarbituric acid (TBA), vitamin E, $FeCl_2·4H_2O$ were from Aldrich Chemicals (Buchs, Switzerland). 4-Ethylpyrocatechol was from Lancaster (Strasbourg, France). Rosmarinic acid and epigallocatechin 3-gallate were from Karl Roth Chemie (Karlsruhe, Germany). Carnosic acid was prepared according to Aeschbach *et al.*, 1990. The nucleoside 8-oxo-2′-dG was synthesized according to a standard procedure (Lin *et al.*, 1985) and structural confirmation obtained by high-resolution mass spectrometry and ^{1}H-NMR analyses (purity + 98% as determined by HPLC).

Oxidation of Ethyl Linoleate (LAEE) with Fenton's Reagent. LAEE was oxidized by Fe^{2+} and H_2O_2 using a method adapted from Tamura *et al.* (1991) with some modifications. The reaction was performed in Eppendorf "Safe-Lock" tubes (2.0 mL), and the mixture contained 0.75 mg LAEE emulsified by rapid stirring in 4% SDS. To this solution was added 0.25 mmol Tris-HCl, pH 6.5, different concentrations of antioxidants or polyphenols to be tested, H_2O_2 (0.5 μmol), and finally $FeCl_2$ tetrahydrate (1.0 μmol), in a total reaction volume of 1.0 mL (supplemented with Millipore grade water). The samples were incubated in the dark at 37 °C for 16 h in a rapidly shaking (190 rpm) water bath. The reaction was terminated by addition of 20 μL of 4% BHT solution in ethanol. Aliquots (50−100 μL) of each of the samples were employed in the TBA derivatization reaction.

Reaction of LAEE Oxidation Products with TBA. A reaction mixture (total volume made up to 0.55 mL) consisting of 0.2 mL of acetate buffer (2 M, pH 3.5), 0.2 mL of 1.6% TBA solution in water, 50 μL of 8% SDS, and 50−100 μL of the testing solution was heated for 1 h in a water bath at 90 °C. After incubation, the samples were brought to room temperature and 1 mL of *n*-butanol (water saturated) added. The tightly closed tubes were shaken for 5 min (Eppendorf shaker) and subsequently centrifuged for phase separation (5 min, 14000 rpm, Eppendorf centrifuge). An aliquot of the organic layer was removed carefully and diluted 2:1 (1-butanol/methanol). Aliquots (20 μL) were directly injected onto the HPLC column as described in "HPLC Analysis of the TBA−MDA Adduct".

Preparation of the MDA Standard for the LAEE Assay. Standard solutions of MDA were prepared by acid hydrolysis of the bisdiethyl derivative (306 mg) added to 50 mL of 0.1 N HCl. The solution was heated at 40 °C for 30 min. Thereafter, the solution was brought to room temperature and adjusted to 1 mg of free MDA per milliliter by dilution with 0.1 N HCl. A standard curve was obtained by further dilution of the stock solution (1:100) to the appropriate final concentration.

HPLC Analysis of the TBA−MDA Adduct. Analytical HPLC employed a Hewlett Packard 1090 system equipped with a diode array detector and a Perkin Elmer 1046A fluorescence detector. Quantitation of the TBA−MDA adduct was done fluorometrically by reversed-phase HPLC using a Supelco LC-18 DB column (4.6 × 250 mm), with solvent methanol/water (1:1) containing 0.05% (w/v) tetrabutylammonium sulfate as ion pairing reagent (Tatum *et al.*, 1990). The flow rate was 0.8 mL/min. The TBA−MDA adduct was monitored photometrically (267 and 532 nm) and fluorometrically ($\lambda_{excitation}$ = 515 nm; $\lambda_{emission}$ = 545 nm). The retention time of the TBA−MDA adduct was 5.3 min. The standard curve showed linearity ($r^2 > 0.99$) from 1.8 to 45 pmol of MDA.

Oxidation of 2′-Deoxyguanosine (2′-dG) to 8-Oxo-2′-deoxyguanosine (8-Oxo-2′-dG) in the Presence of Fe^{3+}−EDTA. This method was employed as described previously (Kasai and Nishimura, 1986) with some modifications (Stadler *et al.*, 1994). The substrate molecule 2′-dG (1.3 mM) was incubated in a potassium phosphate buffer (0.1 M, pH 7.4), $FeCl_3$ (0.1 mM), EDTA (0.5 mM), and 1 mM of test compounds with or without the addition of H_2O_2 (4.4 mM). The reaction mix was incubated for 30 min at 37 °C and the reaction terminated by diluting 1:10 (v/v) with 10% ethanol. Aliquots were removed (5 μL) and analyzed by HPLC with UV (254 and 290 nm) and electrochemical detection (E = 0.6 eV) as described in Stadler *et al.* (1994). To quantify 8-oxo-2′-dG, standard solutions of known concentrations were injected under identical conditions and the amounts in the samples extrapolated from a standard curve.

Vacuum Pyrolysis of Caffeic, Chlorogenic, and Rosmarinic Acids. Each of the phenolic acids (10 mg) were placed in a vacuum hydrolysis tube (5 mL volume) and suspended in water (50 μL) to create a slurry. The tubes were evacuated (0.02−0.05 bar) and water removed at room temperature. The dry residue was heated in a thermostated heater block at 228 °C for 15 min. The tubes were left standing to attain room temperature and then evacuated. The residues (pyrolysates) were dissolved in a small volume (2 mL) of methanol/water (1:1). In the case of chlorogenic acid, traces of insoluble material remained attached to the wall of the tube. The soluble material was filtered (0.2 μm) and an aliquot (100 μL) analyzed directly by HPLC. The remaining material of each of the pyrolysates was concentrated *in vacuo* (40 °C), lyophilized, dissolved in ethanol, and stored at −20 °C until usage.

Synthesis of Phenylindan Isomers. The tetraoxygenated 1,3-*cis*- and 1,3-*trans*-phenylindan isomers were obtained as described (Stadler, 1994; Stadler *et al.*, 1996) by pyrolysis of caffeic acid in a Büchi vacuum oven at 228 °C. The first purification step of the crude pyrolysis mixture was by silica gel column chromatography (Stadler *et al.*, 1996) and afforded the "semipurified" phenylindan isomer mixture (termed "phenylindan isomer mix" in the biological lipid peroxidation assay). Quantification of the phenylindans in the crude pyrolysate and in the partially purified isomer mix was determined by HPLC analysis (UV detection) and extrapolation of the peak areas of the two isomers from a standard curve recorded with the individual chromatographically pure isomers of known amount (Stadler *et al.*, 1996). Further purification by semipreparative HPLC afforded the individual chromatographically and isomerically pure 1,3-*cis* and 1,3-*trans* compounds (Stadler *et al.*, 1996).

HPLC Analysis of the Crude Pyrolysates. Aliquots of the pyrolysates were prepared as described in "Vacuum Pyrolysis of Caffeic, Chlorogenic, and Rosmarinic Acids". HPLC employed a Hewlett Packard 1090 system equipped with a diode array detector. Chromatography was done on a Macherey & Nagel column, C-18, 5 μm (250 × 10 mm), using solvents A (50 mM ammonium acetate, pH 4.5) and solvent B (methanol, at a flow rate of 1.5 mL/min). The gradient commenced with 35% B for 10 min and going to 60% B over 30 min and then increasing to 80% B over 10 min and resting and 80% B for 5 min. The pertinent reaction products were identified by comparing the retention times with authentic compounds under identical conditions and on-line UV spectra.

Preparation of Rat Liver Homogenates. Livers were obtained from male OFA rats (200 g) fed *ad libitum* with a standard lab chow. Liver tissue was homogenized in 25 volumes ice-cold Tris-HCl buffer (50 mM, pH 7.0) using a Potter−Eljevheim homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 50000g for 15 min at 4 °C. The supernatant was discarded and the pellet was resuspended

by homogenization as described above in ice-cold Tris-HCl buffer at a concentration of 4% (w/v).

Preparation of Coffee Samples Coffees were prepared from green coffee beans of Columbia Arabica (by courtesy of Dr. Löhmar, Nestlé, Ludwisburg, Germany). Briefly, light and dark roasting corresponded to 5.96% and 9.54% organic loss, respectively. Then, coffees were brewed and freeze-dried. Coffee solutions were prepared in bidistilled water at 60 °C just before use.

Biological Lipid Peroxidation Assay. Lipid peroxidation was measured by monitoring the formation of TBA reactive substances (TBARS) according to Buege and Aust (1978) with some modifications. Lipid peroxidation was initiated by the addition of 100 μL of rat liver homogenate to the following prooxidant system: $FeSO_4$ (10 μM), ascorbate (250 μM) in Tris-HCl buffer (50 mM, pH 7.0). Various concentrations of the compounds/antioxidants to be tested were dissolved in ethanol (final concentration in the assay was 2%) and added to the incubation mixture to a final volume of 1.0 mL. The tubes were placed in a water bath at 37 °C with shaking for 30 min. The reaction was terminated by addition of EDTA (2.0 mM) and BHT (0.02%). One milliliter of 0.4% (w/v) TBA in 0.2 N HCl was added to the reaction mixture which was heated in a boiling water bath for 30 min. After cooling to room temperature, 1.0 mL of 10% (w/v) trichloroacetic acid was added and the resulting precipitate was removed by centrifugation (1000*g*, 10 min). The absorbance of each supernatant (1.0 mL) was determined at 532 and 520 nm against a blank containing all the reagents except the liver homogenate. The difference between absorbancies at 532 and 520 nm were taken as the TBARS value (Uchimaya and Mihara, 1977).

Antioxidant Capacity. The inhibitory effect of various antioxidants on lipid peroxidation was defined as its antioxidant capacity (AC) calculated as

$$AC_i\ (\%) = 100[(M_i - M_{min})/(M_{max} - M_{min})]$$

where M_i corresponds TBARS formation at 30 min in the presence of antioxidant (i.e., antioxidant$_i$). M_{max} represents TBARS formation in the absence of antioxidants. Finally, M_{min} corresponds to "background peroxidation" when lipid peroxidation is fully inhibited by the addition of EDTA (2.0 mM) and BHT (0.02%).

Statistical Analyses. The results of the LAEE model system were compared using the Student's *t*-test with pooled variances. For lipid peroxidation in rat liver membranes, a logistical transformation of the sigmoidal curves for each antioxidant was performed according to logit (AC) = log [AC/(1 − AC)]. The relationship logit (AC) vs log (concentration) was used to calculate IC_{50} values. Furthermore, an empirical estimation for the standard deviation (SD) of IC_{50} values was obtained from the SD values from each curve: log (SD) = −0.9541 + 0.9215 log (IC_{50}); $r^2 = 0.9066$, $n = 23$. Finally, the 95% confidence interval (CI) of IC_{50} values from triplicates was calculated according to CI = ±1.15 SD.

RESULTS

Vacuum Pyrolysis of Caffeic, Chlorogenic, and Rosmarinic Acids. Caffeic acid and its two phenolic acid esters were subjected to thermal treatment as described under Experimental Procedures. HPLC analysis with UV detection (280 nm) of the pyrolysis products of the three catechols revealed a multitude of additional compounds. In the case of rosmarinic acid, peak detection or identification of the products was not possible due to extreme peak broadening under the HPLC conditions used (profile not shown).

Chromatographic analysis of thermally treated chlorogenic acid (Figure 2A) enabled discernment of individual peaks, but did not show the formation of one or two dominant products of sufficiently high yield to enable isolation and identification. In contrast, however, HPLC of the crude caffeic acid pyrolysate (Figure 2B) revealed the presence of a number of products of isolable yield, among them the 1,3-*cis*- and 1,3-*trans*-phenylindan isomers recently described (Stadler, 1994; Stadler *et al.*, 1996). The phenylindan isomers were quantified by HPLC analysis (UV detection) as described in the Experimental Procedures, contributing ~10% (w/w) to the total solids in the crude pyrolysate. Further purification by silica gel column chromatography afforded the *cis*/*trans* isomer mixture of ~70% purity (Figure 2C). Notably, the tetraoxygenated phenylindans were detected in neither the rosmarinic nor the chlorogenic acid pyrolysates, indicating the importance of the free carboxylic group in the dimerization process (Stadler *et al.*, 1996).

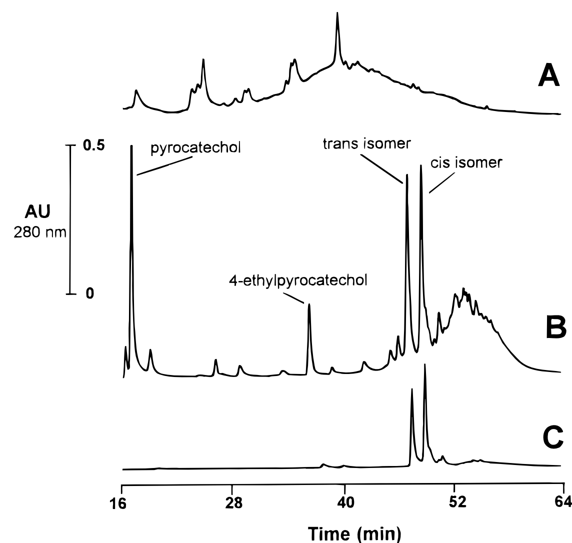

Figure 2. Reversed-phase HPLC analysis with UV detection of the crude pyrolysates of (A) chlorogenic acid, (B) caffeic acid, and (C) phenylindan isomer mix. HPLC conditions are as described in the Experimental Procedures.

Artificial LAEE Peroxidation Model. This model lipid peroxidation system, in which a synthetic lipid substrate is emulsified in SDS/buffer and oxidation initiated by an Fe^{2+}/H_2O_2 mixture, has been used in a number of investigations to assess the antioxidant activity of natural compounds (Tamura *et al.*, 1991; Kitta *et al.*, 1992; Osawa *et al.*, 1992; Stadler *et al.*, 1994).

As shown in Table 1, the common dietary antioxidants vitamin C and vitamin E showed a dose-dependent inhibition of LAEE peroxidation. However, vitamin C did not inhibit peroxidation at the relatively low concentration of 20 μM, whereas all of the other compounds investigated were clearly inhibitory at those levels. The most potent inhibitory effects were observed with the simple monomeric phenols thymol, sesamol, and BHA. Low doses (20 μM) of the tetraoxygenated phenylindan isomers, isolated from the caffeic acid pyrolysate as described in the Experimental Procedures, also suppressed LAEE peroxidation to an extent comparable to that of caffeic acid and even better than vitamins E and C on a per weight basis.

The crude caffeic acid pyrolysis mixture itself, which contained significant amounts of the phenylindan isomers (Figure 2), was also active in suppressing LAEE peroxidation at the lowest concentration employed. However, as is evident from the data in Table 1, a prooxidant effect was recorded at the highest concentration of caffeic acid (200 μM). This effect was also evident in the pyrolyzed caffeic acid reaction mixture as well

Table 1. Effect of Antioxidants and Phenolics on MDA Formation (nmol/mg of LAEE) in an Artificial Detergent Dispersed LAEE Peroxidation Assay[a]

compound	concentration (μM) [control[b] = 8.9 ± 0.54] 20	100	200
vitamin E	6.87 ± 0.76	3.55 ± 0.81	2.79 ± 0.48
vitamin C	8.83 ± 0.37*	6.86 ± 0.78	5.60 ± 0.16
quercitrin	6.23 ± 0.07	10.96 ± 0.26	18.23 ± 1.33
sesamol	3.41 ± 0.37	1.45 ± 0.37	2.84 ± 0.41
thymol	2.13 ± 0.14	1.01 ± 0.12	0.91 ± 0.27
BHA	3.48 ± 0.21	0.78 ± 0.13	0.79 ± 0.10
5-aminosalicylic acid	4.6 ± 0.72	15.3 ± 0.87	25.99 ± 1.43
4-ethylpyrocatechol	5.53 ± 0.38	3.70 ± 0.10	7.78 ± 0.73
pyrocatechol	4.96 ± 0.66	10.34 ± 0.76	19.34 ± 1.22
caffeic acid	6.65 ± 0.1	11.79 ± 0.56	20.1 ± 0.53
pyrolyzed caffeic acid[c]	4.96 ± 0.66	10.34 ± 0.76	19.24 ± 1.22
1,3-*trans*-phenylindan	4.55 ± 0.65	7.63 ± 0.63	16.95 ± 1.39
1,3-*cis*-phenylindan	4.52 ± 0.63	8.98 ± 0.93	20.33 ± 1.8

[a] Data published in part in Stadler *et al.* (1994) and presented here for comparative purposes. All values represent means ± SD (n = 3 or 4) and were significantly different from the control (P < 0.0035; Student's *t*-test with pooled variances) except for those marked with an asterisk. MDA was measured as its TBA adduct by HPLC/fluorometric detection as described in the Experimental Procedures. [b] Conditions with only Fenton's reagent ($Fe^{2+}-H_2O_2$) and LAEE. [c] Pyrolysis under vacuum of equimolar equivalents of caffeic acid. For experimental details see the Experimental Procedures.

as in incubations with the individual 1,3-*cis*- and 1,3-*trans*-phenylindan isomers.

Similar prooxidant effects of antioxidants at high concentrations have been previously reported using the LAEE assay (Osawa *et al.*, 1992; Stadler *et al.*, 1994). Such reactions are probably due to the reduction of the inactive ferric iron to the catalytically active ferrous state either directly or indirectly via the superoxide anion radical (Marklund and Marklund, 1974). In addition, many polyphenols have the ability to generate H_2O_2 via reduction of oxygen in the presence of transition metals such as Fe and Cu (Tyson and Martell, 1972; Hanham *et al.*, 1983; Inoue *et al.*, 1992). The homolytic cleavage of H_2O_2 by reduced transition metals then generates the deleterious hydroxyl radical, leading to lipid oxidation.

Hydroxylation of 2′-dG to 8-Oxo-2′-dG. Ascorbic acid is known to effectively mediate C-8 hydroxylation of guanosine in the presence of chelated iron salts and H_2O_2 (Kasai and Nishimura, 1984). This model system thus reflects the hydroxylation activity of the chemicals tested. Oxidation is initiated by Fe^{3+}−EDTA with or without the addition of H_2O_2. After a 30 min incubation period the major product, 8-oxo-2′-dG, was quantified by HPLC with UV and electrochemical detection. As depicted in Table 2, substituted catechols readily catalyze the formation of 8-oxo-2′-dG. The amount of the 8-oxo-2′-dG adduct formed due to the phenylindan isomers was higher than the simple catechols 4-ethylpyrocatechol and pyrocatechol. Moreover, hydroxylation is potentiated by the addition of exogenous H_2O_2, and incubations with the 1,3-*cis*- and 1,3-*trans*-phenylindans reached 15% turnover of the substrate to 8-oxo-2′-dG.

The 1,3-*cis*- and 1,3-*trans*-phenylindan isomers showed higher hydroxylating activity without exogenous H_2O_2 in the incubation mixes than their progenitor caffeic acid, i.e. factor 3−3.5 times greater. These augmented levels of 8-oxo-2′-dG reflect, in part, the H_2O_2 generating capacity of the tetraoxygenated phenylindans (Stadler *et al.*, 1996).

Biological Lipid Peroxidation Model. Lipid peroxidation in this membrane homogenate was initiated

Table 2. Hydroxylation of 2′-dG to 8-Oxo-2′-dG by Phenolics and Antioxidants[a]

condition	8-oxo-2′-dG (%)
control[b]	0
4-ethylpyrocatechol	0.9
pyrocatechol	0.2
caffeic acid	0.87
pyrolyzed caffeic acid[c]	1.8
1,3-*trans*-phenylindan	2.8
1,3-*cis*-phenylindan	3.0
1,3-*trans*-phenylindan + H_2O_2	15.4
1,3-*cis*-phenylindan + H_2O_2	14.8
vitamin C + H_2O_2	5.5

[a] All values represent means (n = 4) with SD < 10%. Unless otherwise stated, all incubations without exogenous H_2O_2 and with Mn^{2+} (88 μM). [b] Incubation with 2′-dG, buffer and Fe^{3+}−EDTA only. [c] Pyrolysis under vacuum of equimolar equivalents of caffeic acid. For experimental details see the Experimental Procedures.

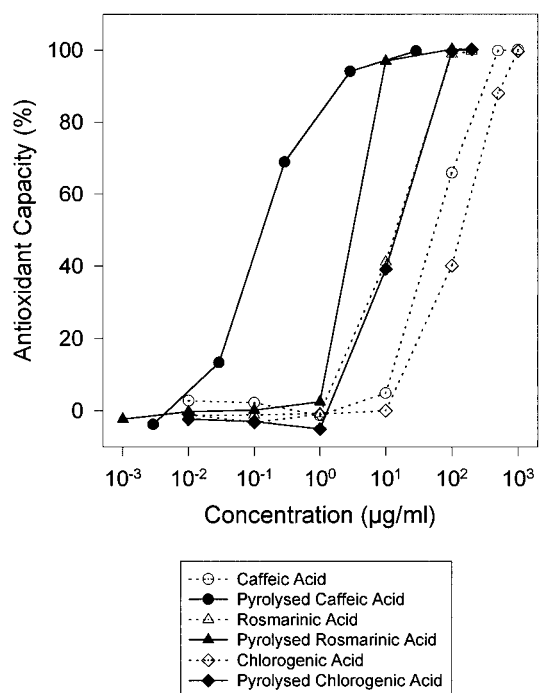

Figure 3. Effect of different concentrations of caffeic, chlorogenic, and rosmarinic acids and their pyrolyzed counterparts on the suppression of lipid peroxidation initiated by Fe^{2+}−ascorbate in a biological membrane assay system. The antioxidant capacity (AC) is depicted as a function of concentration (per weight basis) and was evaluated as described in the Experimental Procedures.

by Fe^{2+}−ascorbate. The TBARS accumulation was monitored spectrophotometrically after the MDA color reaction. The antioxidative capacity (AC) of the pyrolyzed phenolic acids (i.e., caffeic, chlorogenic, and rosmarinic acids) was assessed on a per weight basis (micrograms per milliliters) to enable direct comparison between the complex mixtures.

As shown in Figure 3, thermal treatment of all three compounds resulted in a positive shift of the AC in the order of increasing activity as follows: chlorogenic acid < rosmarinic acid <<< caffeic acid. In addition, IC_{50} values (Table 3) of the chlorogenic and rosmarinic pyrolysates—obtained by logit transformation analysis (see "Statistical Analyses")—were 11- and close to 4-fold higher, respectively, as compared to their nontreated progenitors. Most interestingly, a 460-fold lower IC_{50} value was obtained for the caffeic acid pyrolysate compared to its unheated counterpart. On a molar

Table 3. IC_{50} Values of Pyrolyzed[a] and Nonpyrolyzed Phenolic Acids and Coffee on a per Weight Basis (μg/mL)

compound	IC_{50} (μg/mL)	CI (μg/mL)
chlorogenic acid	131.00	11.40
pyrolyzed chlorogenic acid	11.70	1.20
rosmarinic acid	11.90	1.20
pyrolyzed rosmarinic acid	3.28	0.38
caffeic acid	64.60	5.90
pyrolyzed caffeic acid	0.14	0.02
green coffee	142.60	12.30
coffee (light roasted)	38.90	3.70
coffee (dark roasted)	14.40	1.50

[a] Pyrolysis under vacuum of 10 mg each of the phenolic acids. For experimental details see the Experimental Procedures. Values were obtained after logistical transformation of the individual curves of Figures 3 and 4, logit (AC) vs log (concentration); CI represents the 95% confidence interval. For details of statistical analysis see the Experimental Procedures.

Table 4. IC_{50} Values of Phenolic Compounds Expressed on a Molar Basis (μM) and Obtained after Logistical Transformation of the Individual Curves of Figure 5, logit (AC) vs log (concentration)[a]

compound	IC_{50} (μM)	CI (μM)
chlorogenic acid	370	25.8
caffeic acid	358	25.1
rosmarinic acid	33.1	2.8
quercetin	17.9	1.80
sesamol	11	1.20
epigallocatechin 3-gallate	3.2	0.37
BHT	0.3	0.04
carnosic acid	0.17	0.02
1,3-*cis*-phenylindan	0.041	0.007
1,3-*trans*-phenylindan	0.04	0.007
phenylindan isomer mixture[b]	0.06	0.006

[a] Containing approximately equivalent amounts of the 1,3-*cis* and 1,3-*trans* isomer as well as 30% impurities as determined by HPLC analysis. For details see the Experimental Procedures. CI represents the 95% confidence interval. For details of statistical analysis see the Experimental Procedures.

basis, the untreated chlorogenic, caffeic, and rosmarinic acids showed IC_{50} values of 370, 358, and 33.1 μM, respectively (Table 4). Furthermore, none of the compounds tested nor their crude pyrolysis reaction products showed any prooxidative effects under the concentration ranges employed in this biological assay. Thus, among the three nontreated phenolic acids tested, rosmarinic acid was the strongest inhibitor of Fe^{2+}–ascorbate-initiated lipid peroxidation. However, caffeic acid showed far greater antioxidative activity than rosmarinic acid or chlorogenic acid after subjection to the same thermal treatment.

Chlorogenic acids and their isomers are the major polyphenols found in green coffee beans and, depending on the variety of the bean, can account for 5–10% of total solids (Clifford, 1985). Upon roasting, the levels of chlorogenic acids are depleted significantly (8–10% total chlorogenic acids lost for 1% reduction in dry matter) due to a number of thermally driven reactions, which can lead to the formation of more complex high molecular weight structures (Clifford, 1985). Therefore, for comparative purposes, different concentration ranges of extracts of green, light roasted, and dark roasted coffees were also tested on a per weight basis in this peroxidation system. As shown in Figure 4, a dose-dependent suppression of lipid peroxidation was recorded for all the crude coffee extracts. Moreover, the AC increased with the degree of heat treatment (i.e. roasting) of the coffee beans. Therefore, as in the case

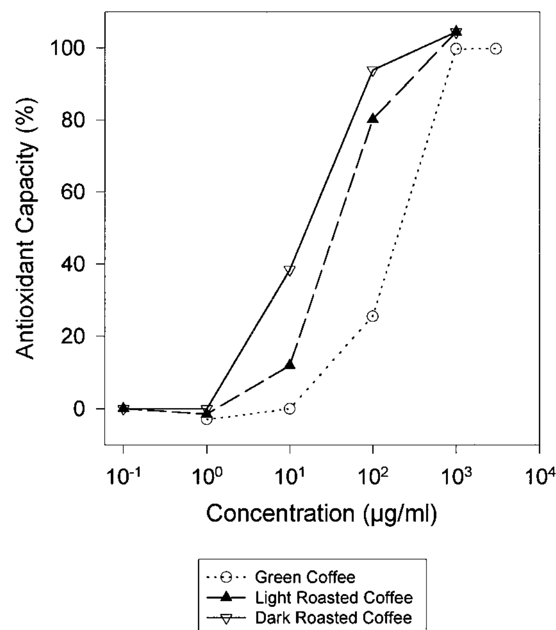

Figure 4. Effect of degree of roasting of coffee on the inhibition of lipid peroxidation induced by Fe^{2+}–ascorbate in a biological membrane assay system. The antioxidant capacity (AC) is depicted as a function of concentration (per weight basis) and was calculated as described in the Experimental Procedures.

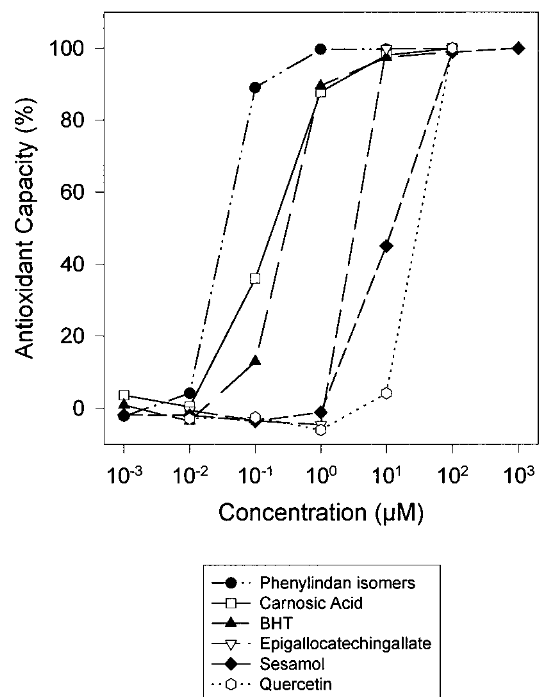

Figure 5. Effect of different concentrations of a number of phenolic compounds on the suppression of lipid peroxidation initiated by Fe^{2+}–ascorbate in a biological membrane assay system. The antioxidant capacity (AC) is depicted as a function of concentration (on a molar basis) and was evaluated as described in the Experimental Procedures.

of caffeic acid and its esters, pyrolysis significantly enhances the AC of a complex chemical mixture such as coffee.

The AC of the phenylindan isomeric mixture was compared to the AC of a number of known antioxidant molecules on a molar basis (Figure 5). It should be noted here that the purity of the phenylindan isomeric mixture, which was prepared as described in Experi-

mental Procedures, was estimated to be ~70% (as determined by HPLC), thus containing 30% hitherto unidentified compounds.

Of the compounds tested, the phenylindan isomer mixture at a concentration range of 0.01−10 μM showed the strongest inhibitory effect on lipid peroxidation, surpassing the AC of the common food antioxidant BHT and the diterpenoid phenol carnosic acid. As shown in Table 4, the isomerically pure 1,3-*cis*- and 1,3-*trans*-phenylindan isomers revealed almost identical IC_{50} values, also comparable to that of the isomeric mixture. Taking into account the chemical purity of the isomeric mixture (70%), an extrapolation of the results to achieve a 100% contribution by only the phenylindans in the semipurified mix then affords a theoretical IC_{50} value (0.042 μM), comparable to that of the individual chromatographically pure 1,3-*cis* and 1,3-*trans* isomers. Therefore, the results strongly suggest that the potent inhibitory effect of the crude caffeic acid pyrolysate as well as the phenylindan isomer mix on lipid peroxidation is mainly due to the oxygenated phenylindan isomers, with no significant contribution by chemical impurities.

The potent increase in antioxidative activity of the phenylindan isomers compared to their progenitor caffeic acid is above all reflected in the individual IC_{50} values (μM), giving a ratio (i.e. IC_{50} caffeic acid/IC_{50} 1,3-*cis*−1,3-*trans* phenylindans) close to 4 orders of magnitude.

DISCUSSION

The antioxidative activities of caffeic acid and its esters chlorogenic and rosmarinic acids have been studied in various *in vitro* oxidation models (Sharma, 1976; Graf, 1992; Halliwell *et al.*, 1995). However, this is the first report on the effects of thermal processing and how this treatment is related to antioxidative efficacy. The data presented here reveals that the AC of caffeic acid and related molecules can be increased by several orders of magnitude upon vacuum pyrolysis. Recent chemical analysis of the caffeic acid pyrolysates showed the presence of 1,3-*cis* and 1,3-*trans* tetraoxygenated phenylindan isomers as the major reaction products (Stadler, 1994; Stadler *et al.*, 1996). Furthermore, we demonstrated in this study that the enhanced inhibition of lipid peroxidation by the caffeic acid pyrolysate was due to the novel caffeic acid dimers (i.e., phenylindan isomers).

Analysis by HPLC of the thermally treated chlorogenic and rosmarinic acids did not reveal the presence of these oxygenated phenylindan structures, indicating the importance of a free carboxylic function in the dimerization process. Furthermore, these polyhydroxylated tricyclic dimers are formed by acid or thermally catalyzed decarboxylation of caffeic acid monomers, which rapidly condense to form the phenylindan structure (Taylor *et al.*, 1977; Stadler *et al.*, 1996). The potent antioxidative properties of the phenylindan molecule is probably attributable to its two catecholic nuclei harboring a tetrahydroxylated substitution pattern which contributes to free radical stabilization of the phenoxy radical after hydrogen abstraction by increased electron delocalization (Cuvelier *et al.*, 1992; Graf, 1992). In addition, a high degree of lipophilicity is conveyed upon the molecule by fusion of the two catechols to a methyl-substituted pentacyclic ring. In fact, even though the oxygenated phenylindans exhibited good solubility in water, they could be extracted efficiently into ether (99.8%).

Numerous model systems have been employed to assess the prooxidative action of chemicals. These include (a) the deoxyribose assay, where oxidation is initiated by a mixture of Fe^{3+}−EDTA/H_2O_2/ ascorbate; (b) bleomycin−Fe assay, oxidation initiated by ascorbate/O_2; and (c) oxidized DNA base measurement, oxidation is initiated by Fenton reagents (Gutteridge and Xaio-Chang, 1981; Aruoma *et al.*, 1990; Aruoma, 1991; 1994). A common feature of the aforementioned *in vitro* systems is that compounds capable of reducing Fe to the active ferrous state may act as prooxidants. In fact, such prooxidative effects have been reported for a number of dietary antioxidants and polyphenols, including ferulic acid (Scott *et al.*, 1993), hydroxytyrosol (Aeschbach *et al.*, 1994), (−)-epicatechin (Scott *et al.*, 1993), vitamin C (Mahoney and Graf, 1986; Fischer-Nielsen, 1992; Halliwell, 1994), and vitamin E (Mahoney and Graf, 1986; Maiorino *et al.*, 1993). The phenylindan isomers described here also showed prooxidative effects in both the artificial LAEE assay, albeit at relatively high concentrations, and the 2′-dG hydroxylation assay. This effect is probably attributable to the potent reducing power of the molecule (Stadler *et al.*, 1996), a characteristic shared by many *o*-dihydroxy phenols (Laranjinha *et al.*, 1995). Such prooxidant pathways are favored because high oxygen tension and nonphysiological availability of iron promotes rapid electron transfer from the polyphenol to molecular oxygen, thus generating the superoxide anion radical and H_2O_2 (Marklund and Marklund, 1977). The latter molecule can be reductively split by catalytically active ferrous iron (Fenton reaction) resulting in the formation of highly reactive oxygen species such as the hydroxyl radical. The phenylindan isomers act as efficient antioxidants at low concentrations in the LAEE system, probably due to peroxyl radical scavenging which concomitantly depletes the antioxidants due to phenol coupling and radical-induced polymerization reactions. The antioxidant/prooxidant balance is thus in favor of the former and does not leave a surplus of the phenylindan molecule for oxygen reduction as would higher concentrations. Furthermore, the prooxidative action of the 1,3-*cis*- and 1,3-*trans*-phenylindan isomers can be demonstrated by incubation with Mn^{2+} (2.2 ppm relative to the volume) in a buffered aqueous solution, which results in rapid depletion/polymerization of the compounds over time with concomitant H_2O_2 formation (Stadler *et al.*, 1996). This reductive effect is also corroborated in the 2′-dG assay, which reflects the ability of the phenols to generate H_2O_2 and reduce the ferric iron chelate. Furthermore, the results presented corroborate earlier observations that *in vitro* test systems with relatively high metal iron contents and oxygen tension will not adequately simulate physiological conditions, making extrapolation of data to *in vivo* conditions difficult (Halliwell, 1990; Halliwell *et al.*, 1995).

However, the phenylindan isomers tested with the biological membrane system resulted in a dose-dependent suppression of peroxidation of the cellular lipids. The AC of the phenylindan isomers was greater than that of the other antioxidants tested such as carnosic acid, BHT, and epigallocatechin 3-gallate. The phenylindan isomers seemed to act as radical scavengers rather than as iron chelators. In metal ion-dependent systems, an added antioxidant can also act by binding iron salts and stopping them from accelerating the peroxidation reaction (Halliwell *et al.*, 1995). Since the IC_{50} value for

phenylindans (0.04 μM) was much lower than the iron concentration used in the biological membrane peroxidation assay (10 μM), these isomers most likely behave as peroxyl radical scavengers. Finally, one could speculate about possible synergistic effects between phenylindan isomers and vitamin E. Indeed, it was shown in preliminary experiments using liver membranes from vitamin E-deficient rats, that a 10-fold higher phenylindan concentration was needed to produce an inhibition of lipid peroxidation similar to that obtained with liver membranes from rats not deficient in vitamin E (unpublished observations). This would suggest some physiologically relevant interactions between the phenylindan isomers and vitamin E.

It is worth mentioning that in this rat liver membrane system, oxidation is also initiated by nonchelated iron, which can generate free radicals in close proximity to the substrate molecules. However, none of the antioxidants investigated within the concentration ranges tested mediated prooxidant effects in this biological peroxidation model. This demonstrates the importance of *in vitro* testing using more than one model system in order to comprehend the overall action of antioxidants (Halliwell, 1990; Halliwell *et al.*, 1995).

These preliminary investigations using artificial and biological lipids for the assay of antioxidant activity demonstrate the potential of heat processing in the generation of novel antioxidant compounds. Previous studies have shown that roasted sesame oil possesses higher antioxidative activity than the untreated sesame oil. This stronger AC was attributed to the production of new antioxidant compounds during roasting of sesame seeds such as sesamol from sesamolin (Namiki, 1995). Maillard reaction products also frequently display greater free radical scavenging activity than their individual precursors (Kroyer *et al.*, 1989; Macku and Shibamoto, 1991). Further studies are now required to establish whether the potent antioxidant properties of the pyrolysis products of caffeic acid which have been recorded *in vitro* in biological membranes can also be observed *in vivo*.

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