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Inhibitory Effects of Plant-Derived Flavonoids and Phenolic Acids on Malonaldehyde Formation from Ethyl Arachidonate

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The antioxidant activities of naturally occurring plant compounds were measured in a lipid peroxidation system consisting of ethyl arachidonate and Fenton's reagent. Inhibitory effects of 24 plant-derived flavonoids and 5 phenolic acids on malonaldehyde (MA) formation from ethyl arachidonate were examined using gas chromatography (GC) with a nitrogen−phosphorus detector (NPD). Luteolin, which showed the strongest antioxidant activity, inhibited MA formation by 94% and 97% at the levels of 0.5 and 1.0 mM, respectively. The antioxidant activities of the flavones and flavonols decreased in the following order: luteolin > rhamnetin > fisetin > kaempferol > morin > quercetin. Among the flavanones tested, hesperitin, taxifolin, and naringenin exhibited appreciable antioxidant activities (61−84%) at the 1.0 mM level. The inhibitory effect of epigallocatechin gallate (82.5% at the 1.0 mM level) was the strongest among the flavan-3-ols tested. Ferulic acid had the most potent antioxidant activity (74.6% at the 1.0 mM level) of the phenolic acids tested.

KEYWORDS: Natural antioxidants; flavonoids; lipid peroxidation; malonaldehyde

INTRODUCTION

Flavonoids are secondary metabolites present in plants and plant-based foods. They are usually responsible for the red and yellow colors and bitter and astringent tastes of flowers, fruits, and beverages (*1*). Over 4000 flavonoids have been identified, and they form the largest group of naturally occurring polyphenols (*2*). Their basic structures have a flavan nucleus (2-phenylbenzo-γ-pyran) consisting of two benzene rings combined by an oxygen-containing pyran ring. The common classes are the flavones, flavonols, flavanones, flavan-3-ols, isoflavones, and anthocyanidins, based on the degree of oxidation of the heterocyclic ring (*3*). So far, a great number of pharmacological and biological effects have been ascribed to flavonoids. The following activities have been described: anti-inflammatory, antiosteoporotic, antiallergic, antihepatotoxic, antitumor, antimicrobial, antiviral, antimutagenic, and antioxidant activity (*4*−*7*). The relationships between biological activities and chemical structure of flavonoids in various systems have also been extensively reviewed (*8*−*11*).

There is a strong need to find alternative natural antioxidants because some synthetic antioxidants have been reported to possess toxicity to humans (*12*). Natural antioxidants including flavonoids have been shown not only to prevent oxidative deterioration in foods but also to inhibit oxidative damage caused by lipid peroxidation. It is thought that many diseases, such as cancer (*13*), atherosclerosis (*14*), aging (*15*), leukemia (*16*), rheumatoid arthritis (*17*), liver diseases (*18*), and diabetes (*19*), are mediated by free radical processes. Although there have been many reports dealing with the antioxidant activity of flavonoids, the inhibitory effects of flavonoids on malonaldehyde (MA) production from lipid peroxidation are rarely reported (*20*, *21*). In the present study, flavonoids and phenolic acids derived from plants were evaluated for antioxidant activity using a lipid peroxidation system consisting of ethyl arachidonate oxidized with Fenton's reagent. MA was identified and quantified by gas chromatography/mass spectrometry (GC/MS) and gas chromatography (GC) with a nitrogen−phosphorus detector (NPD), respectively. In addition, we investigated the relationships between antioxidant activity and chemical structure of flavonoids and phenolic acids.

MATERIALS AND METHODS

Chemicals. Caffeic acid (99%), (+)-catechin (99%), chlorogenic acid (99%), *trans*-cinnamic acid (99%), epigallocatechin (99%), epigallocatechin 3-gallate (99%), ferulic acid (99%), gallic acid (99%), myricetin (85%+), ethyl arachidonate (99%+), butylated hydroxytolu-

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ene (BHT), α-tocopherol, trizma hydrochloride, and trizma base were purchased from Sigma Chemical Co. (St. Louis, MO). Chrysin (95%), fisetin (99%), morin (95%), rutin (98%), quercetin (98%+), *N*-methylhydrazine (NMH), 2-methylpyrazine, sodium dodecyl sulfate (SDS), and ferrous chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Acacetin (98%+), flavanone (98%+), hesperetin (98%), hesperidin (98%), luteolin (97%), luteolin 7-*O*-glucoside (98%), naringenin (95%+), naringin (98%), and tectochrysin (95%) were obtained from Indofine Chemical Co. (Somerville, NJ). Also, 3,7-dihydroxyflavone, isorhamnetin, rhamnetin, tamarixetin, and taxifolin were kindly supplied by Dr. R. Molyneux (WRRC, USDA-ARS, Albany, CA). Hydrogen peroxide was supplied by Fisher Scientific Co., Ltd. (Fair Lawn, NJ). Solid-phase extraction (SPE) cartridges (1 g of C18 sorbent mass/6 mL column volume) were purchased from Varian Sample Preparation Products (Harbor City, CA). The standard stock solution of 2-methylpyrazine was prepared by adding 10 mg of 2-methylpyrazine to 1 mL of ethyl acetate. The solution was stored at 5 °C. Authentic 1-methylpyrazole (1-MP) was synthesized according to the method developed by Umano et al. (*22*). Sodium malonaldehyde was synthesized according to methods previously described by Lacombe et al. (*23*) and Marnett et al. (*24*).

Oxidation of Ethyl Arachidonate with Fenton's Reagent (Fe^{2+}/H_2O_2) in the Presence of Flavonoids and Phenolic Acids. An aqueous solution (5 mL) containing 10 μL of ethyl arachidonate (0.028 mmol), 0.25 mmol of trizma buffer (pH 7.4), 1 μmol of ferrous chloride, 2 μmol of hydrogen peroxide, 0.75 mmol of potassium chloride, and 0.2% of surfactant SDS was incubated with various amounts of plant-derived flavonoids and phenolic acids for 16 h at 37 °C in a 20-mL test tube. The oxidation of samples was stopped by adding 50 μL of a 4% BHT solution (*22*). The sample tubes were covered with aluminum foil during incubation to avoid any influence of light on the lipid peroxidation. The known antioxidants, α-tocopherol and BHT, were used to compare antioxidant activity to that of the compounds tested.

Analysis of MA Formed from Ethyl Arachidonate upon Oxidation. Antioxidant activity of flavonoids was determined by analyzing MA (derivatized to 1-MP with NMH) formed from lipids upon oxidation (*23*). NMH (30 μL) was added to the oxidized lipid solutions, and the solutions were allowed to react with stirring for 1 h at room temperature. Each solution was worked up using solid-phase extraction. Briefly, C18 solid-phase extraction cartridges (1.0 g of sorbent mass/6 mL column volume) were conditioned with dichloromethane, methanol, and deionized water in succession. The samples were applied to the cartridges which were attached to a vacuum manifold (Supelco, Bellefonte, PA). The cartridges were eluted with 10 mL of dichloromethane. After addition of 20 μL of 2-methylpyrazine solution (20 μg/mL) as an internal standard, the eluate was analyzed for 1-MP with a GC equipped with an NPD.

Recovery Test on MA from Ethyl Arachidonate Using Solid-Phase Extraction (SPE). Sodium malonaldehyde (200 nmol) was added to 10 μL of ethyl arachidonate. The sample was prepared and analyzed for MA as described above.

Instrumental Analysis. A Hewlett-Packard (HP) model 6890 GC equipped with an NPD and a 30-m × 0.25-mm-i.d. (d_f = 1 μm) DB-WAX bonded-phase fused silica capillary column (J&W Scientific, Folsom, CA) was used to analyze 1-MP. The detector and injector temperatures were 250 °C. The linear velocity of helium carrier gas was 30 cm/s at a split ratio of 1:23. The oven temperature was programmed from 60 to 180 °C at 4 °C /min and held for 10 min at the final temperature. GC peak areas were integrated with a Tsp SP 4400 series integrator. An HP model 6890 GC interfaced to an HP 5971 mass spectrometer was used to confirm the identity of the pyrazole derivative, *N*-methylpyrazole, in the sample. The GC/MS conditions were the same as for the GC. The mass spectra were obtained by electron impact ionization at 70 eV and an ion source temperature of 250 °C. Each experiment was repeated three times.

RESULTS AND DISCUSSION

Flavonoids and cinnamic acids are known as primary antioxidants and act as free radical acceptors and chain breakers (*25*). Rice-Evans et al. (*26*) stated that the antioxidant activity of polyphenols is determined by (1) its reactivity as a hydrogen- or electron-donating agent (related to its reduction potential), (2) the fate of the resulting antioxidant-derived radical (governed by its ability to stabilize and delocalize the unpaired electron), (3) its reactivity with other antioxidants, and (4) its transition metal chelating potential. Our model test system used Fenton's reagent to generate highly reactive hydroxy radicals.

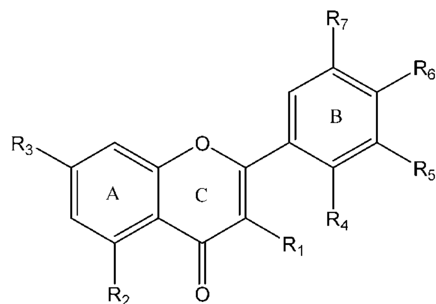
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

$${}^{\bullet}OH + LH \text{ (ethyl arachidonate)} \rightarrow H_2O + L^{\bullet}$$

$$L^{\bullet} \rightarrow \rightarrow \rightarrow \text{malonaldehyde}$$

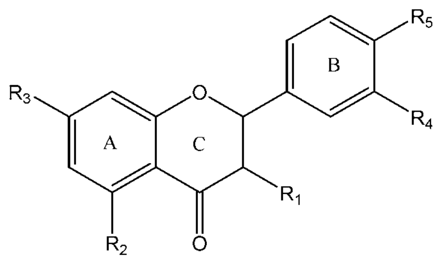
Flavonoids can scavenge hydroxy radicals and other reactive species produced by the reaction of Fe^{2+}, H_2O_2, and O_2 by acting as hydrogen donors. Flavonoids can also act as free radical quenchers for $L^{\bullet}$, which has a direct effect on the formation of malonaldehyde. A competing reaction is the redox cycling of the iron ($Fe^{3+} \rightarrow Fe^{2+}$) by flavonoids to further catalyze the Fenton reaction. The ability of certain flavonoids to chelate metal ions, particularly iron and copper, supports their role as antioxidants since metal-catalyzed free radical formation is inhibited. Flavonoids are known to chelate metal ions at the 3′,4′-dihydroxy positions in the B ring, at the 3-hydroxy, 4-keto group and at the 5-hydroxy (A ring), 4-keto group (*26*). It has been demonstrated that flavonoids can either enhance or inhibit the formation of ${}^{\bullet}OH$ by Fenton-type reactions (*27*). With ferric–EDTA chelate present, flavonoids enhanced the formation of hydroxy radicals in Fenton-type reactions, while when ATP and citrate were used as iron chelators, the addition of flavonoids did not influence the level of hydroxy radicals formed. In the presence of the reducing agent, ascorbate, flavonoids had little or no effect on hydroxy radical formation with EDTA chelate, while with ATP or citrate chelates, flavonoids inhibited the formation of hydroxy radicals.

The level of MA formed from the oxidation of 10 μL of ethyl arachidonate without addition of flavonoids, BHT, or α-tocopherol was 676.8 ± 3.1 nmol. It had been previously demonstrated that the amount of MA formed from oxidation of ethyl arachidonate was higher than that from ethyl linoleate or ethyl linolenate (*20*). The recovery of MA (as 1-MP) from ethyl arachidonate using SPE cartridges was 95.6 ± 0.9%. The values are mean ± standard deviation (n = 3). **Figure 1** shows the structures of plant-derived flavonoids tested for antioxidant activity in the present study. Among the 24 flavonoids tested (**Table 1**), luteolin showed the strongest antioxidant activity, inhibiting MA formation by 94% and 97% at the levels of 0.5 and 1.0 mM, respectively. The antioxidant activity of this flavone was similar to that of the synthetic antioxidant 2,6-bis-(1,1-dimethylethyl-4-methylphenol) (BHT). **Figure 2** shows the inhibitory activity of luteolin, BHT, and α-tocopherol toward MA formation from ethyl arachidonate oxidized with Fenton's reagent. BHT and α-tocopherol were much more effective antioxidants (IC_{50} values of 0.84 ± 0.14 and 2.14 ± 0.8 μM, respectively) than luteolin at levels below 2.0 μmol. In contrast, luteolin had an IC_{50} value of 223 ± 10 μM. The IC_{50} values were expressed as the mean of 50% inhibitory concentration of triplicate determinations, obtained by interpolation of the concentration–inhibition curves. There was little change in the activity of BHT and α-tocopherol at doses above 0.5 μmol, while the inhibitory activity of luteolin steadily increased up to 2.5 μmol. At that level, its activity exceeded that of α-tocopherol. At the 5.0 μmol level, the activity of luteolin was very similar to that of BHT. Luteolin had been previously been



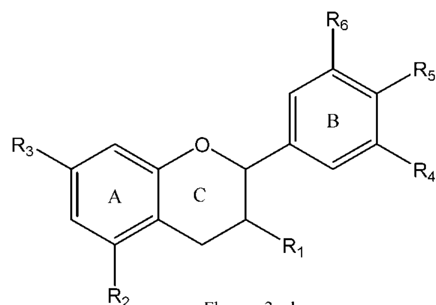
Flavonols and Flavones

$R_1=R_4=R_7=H$; $R_2=R_3=R_5=R_6=OH$ (luteolin)
$R_1=R_2=R_5=R_6=OH$; $R_3=OCH_3$; $R_4=R_7=H$ (rhamnetin)
$R_1=R_3=R_5=R_6=OH$; $R_2=R_4=R_7=H$ (fisetin)
$R_1=R_2=R_3=R_6=OH$; $R_4=R_5=R_7=H$ (kaempferol)
$R_1=R_2=R_3=R_4=R_6=OH$; $R_5=R_7=H$ (morin)
$R_1=R_2=R_3=R_5=R_6=OII$; $R_4=R_7=II$ (quercetin)
$R_1=R_4=R_7=H$; $R_2=R_5=R_6=OH$; $R_3=O$-glu (luteolin-7-*O*-glucoside)
$R_1=R_2=R_3=R_5=OH$; $R_6=OCH_3$; $R_4=R_7=H$ (tamarixetin)
$R_1=R_4=R_5=R_7=H$; $R_2=R_3=OH$; $R_6=OCH_3$ (acacetin)
$R_1=O$-rut; $R_2=R_3=R_5=R_6=OH$; $R_4=R_7=OH$ (rutin)
$R_1=R_2=R_3=R_5=R_6=R_7=OII$; $R_4=OII$ (myricetin)
$R_1=R_2=R_3=R_6=OH$; $R_5=OCH_3$; $R_4=R_7=H$ (isorhamnetin)
$R_1=R_4=R_5=R_6=R_7=OH$; $R_2=OH$; $R_3=OCH_3$ (techochrysin)
$R_1=R_3=H$; $R_2=R_4=R_5=R_6=R_7=H$ (3,7-dihydroxyflavone)
$R_1=R_4=R_5=R_6=R_7=H$; $R_2=R_3=OH$ (chrysin)



Flavanones

$R_1=H$; $R_2=R_3=R_4=OH$; $R_5=OCH_3$ (hesperetin)
$R_1=R_2=R_3=R_4=R_5=OH$ (taxifolin; a dihydroflavonol)
$R_1=R_4=H$; $R_2=R_3=R_5=OH$ (naringenin)
$R_1=H$; $R_2=R_4=OH$; $R_3=O$-rut; $R_6=OCH_3$ (hesperidin)
$R_1=R_2=R_3=R_4=R_5=H$ (flavanone)
$R_1=R_4=H$; $R_2=R_5=OH$; $R_3=O$-neohesp (naringin)



Flavan-3-ols

R_1=O-gallate; $R_2=R_3=R_4=R_5=R_6=OH$ (epigallocatechin 3-gallate)
$R_1=R_2=R_3=R_4=R_5=OH$; $R_6=H$ (catechin)
$R_1=R_2=R_3=R_4=R_5=R_6=OH$ (epigallocatechin)

Figure 1. Structures of the flavonoids tested.

reported to possess lower radical scavenging activity than quercetin (*11*). Glycosylation in the C-7 position (luteolin 7-*O*-glucoside) resulted in a small reduction in antioxidant activity (96.6% vs 73.1%). Slightly lower activity, though still high, was observed for rhamnetin, fisetin, kaempferol, morin, and quercetin, a group of flavonols with a free hydroxyl group at the C-3 position. Similar results were reported by Burda and Oleszek (*7*), who suggested that the flavonol C-3 hydroxyl group was responsible for the high inhibitory activity to β-carotene oxidation. Loss of a hydroxyl group of quercetin at C-5 (fisetin) or C-3′ (kaempferol) had little effect on the inhibitory activity. Similarly, the arrangement of the two hydroxyl groups on the B ring, ortho (quercetin) versus meta (morin), did not influence the antioxidant activity, in contrast to an earlier report that found that quercetin had significantly higher radical [2,2′-azinobis-

Table 1. Inhibitory Effects of Flavonoids toward MA Formation from Ethyl Arachidonate Oxidized by Fenton's Reagent

sample name	inhibition of MA formation (%)[a] at 0.5 mM concn	at 1.0 mM concn
Flavones and Flavonols		
luteolin	93.8 ± 1.9	96.6 ± 2.1
rhamnetin	88.3 ± 2.2	92.4 ± 1.7
fisetin	81.1 ± 2.3	86.2 ± 6.1
kaempferol	25.4 ± 3.8	80.9 ± 5.5
morin	52.7 ± 7.6	78.9 ± 7.9
quercetin	56.2 ± 4.8	76.1 ± 7.6
luteolin 7-*O*-glucoside	61.0 ± 3.1	73.1 ± 6.3
tamarixetin	49.8 ± 10.4	42.8 ± 1.9
acacetin	14.0 ± 10.6	40.3 ± 7.3
rutin	14.6 ± 2.5	24.3 ± 3.7
myricetin	−46.0 ± 32.9	21.1 ± 2.5
isorhamnetin	−18.3 ± 24.3	15.6 ± 23.8
tectochrysin	−30.7 ± 17.8	−5.2 ± 10.9
3,7-dihydroxyflavone	−20.7 ± 15.4	−8.1 ± 11.5
chrysin	0.27 ± 8.5	−18.9 ± 13.8
Flavanones		
hesperetin	55.7 ± 6.0	83.7 ± 8.9
taxifolin	46.1 ± 7.7	77.8 ± 8.8
naringenin	28.8 ± 2.8	61.0 ± 4.0
hesperidin	37.1 ± 9.3	25.2 ± 3.7
flavanone	−8.9 ± 7.8	14.4 ± 7.1
naringin	−29.0 ± 34.7	−18.4 ± 12.7
Flavan-3-ols		
epigallocatechin 3-gallate	59.9 ± 6.2	82.5 ± 2.4
(+)-catechin	32.3 ± 1.6	38.7 ± 1.4
epigallocatechin	7.8 ± 2.9	21.6 ± 1.1
BHT	96.4 ± 0.2	97.5 ± 1.0
α-tocopherol	87.1 ± 6.2	90.5 ± 6.8

[a] Values represent means ± SD of three independent experiments.

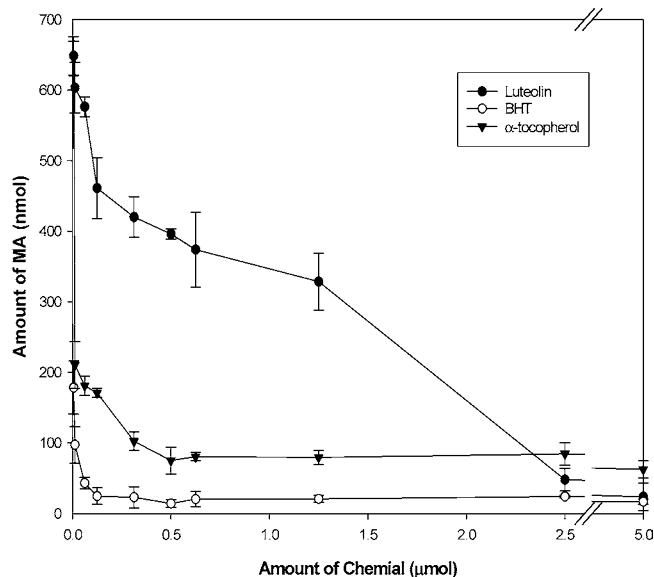


Figure 2. Effect of luteolin, BHT, and α-tocopherol on MA formation from ethyl arachidonate oxidized by Fenton's reagent. Values are the average of three replications.

(3-ethylbenzothiazoline)-6-sulfonate radical cation, $ABTS^{\bullet +}$] scavenging activity than morin (*11*). Morin and quercetin were reported to have high DPPH radical scavenging activity (*7*). Methylation of the C-7 hydroxyl group of the A ring of quercetin to form rhamnetin caused an increase in antioxidant activity (92.4% vs 76.1%). The importance of free hydroxyls in the B ring was noted, as methylation of the C-4′ hydroxyl group of quercetin to form tamarixetin resulted in a moderate loss of antioxidant activity (76.1% vs 42.8%), while methylation of the C-3′ hydroxyl group to form isorhamnetin had a greater effect (76.1% vs 15.6%). Glycosylation of the C-3 hydroxyl group of quercetin to form rutin (quercetin 3-*O*-rhamnoglucoside) caused a large loss of inhibitory activity (76.1% vs 24.3%). It had been previously observed that glycosylation or methylation of the C-3 hydroxyl group of flavonols resulted in the loss of antioxidant activity (*7*, *11*, *28*). The low activity of myricetin and its high standard deviation at the 0.5 mM level may reflect its high susceptibility to oxidation, that resulted in its rapid oxidation and partial decomposition during the antioxidant assay (*7*). The pro-oxidative effects of myricetin possibly arise from the enhanced production of reactive species such as $^{\bullet}OH$, O_2^-, and H_2O_2. It has been reported that myricetin enhanced the production of $^{\bullet}OH$ in the Fenton system (*29*). Hodnick et al. (*30*) reported that flavonoids having a pyrogallol configuration produce O_2^- and H_2O_2 during their autoxidation. It has been noted that, while myricetin exhibits high peroxyl radical absorbing activity ($ORAC_{ROO^\bullet}$, 4.3 μM Trolox equivalents/μM), its hydroxyl radical absorbing activity ($ORAC_{OH^\bullet}$, 0.25 μM Trolox equivalents/μM) is much weaker (*31*). Since we measured the latter activity in our assay, the low activity observed for this flavonoid seemed reasonable. We did not determine the fate of myricetin, but it is probably worthwhile to examine it to clarify the reason for its low activity. Tectochrysin, 3,7-dihydroxyflavone, and chrysin, flavones lacking B ring hydroxyl groups, did not exhibit antioxidant activity. Among the flavanones tested in this study, hesperitin, taxifolin, and naringenin exhibited appreciable antioxidant activities (61−84%) at the 1.0 mM level. In agreement with previous studies (*11*), glycosylation of the C-7 hydroxyl group of hesperetin to form hesperidin (hesperetin 7-*O*-rutinoside), or the C-7 position of naringenin to form naringin (naringenin 7-*O*-neohesperidoside), resulted in the large or complete loss of antioxidant activity. The inhibitory effect of epigallocatechin 3-gallate was the strongest among the flavonols tested. Removal of the gallic acid moiety from epigallocatechin 3-gallate resulted in a large loss in antioxidant activity (82.5% vs 21.6%). Saturation of the 2,3-double bond and removal of the 4-oxo group in the B ring of quercetin forms catechin, which caused about a 50% reduction in antioxidant activity. Similar effects of structure on radical scavenging activity were observed by Rice-Evans et al. (*11*).

Ferulic acid had the highest antioxidant activity of the phenolic acids tested (**Figure 3**, **Table 2**). Ferulic acid has two features which contribute to its antioxidant efficiency. First, its para-substituted hydroxyl group allows the phenoxy radical to be delocalized across the entire molecule and therefore stabilized (*32*). Second, ortho substitution with an electron-donating methoxy group increases the stability of the phenoxy radical and therefore increases its antioxidant activity (*32*, *33*). The presence of a second hydroxyl group in the ortho position (caffeic acid) is known to increase antioxidant activity due to the additional resonance stabilization and *o*-quinone formation (*33*, *34*). There are conflicting reports on the relative activities of ferulic acid and caffeic acid which probably reflect the

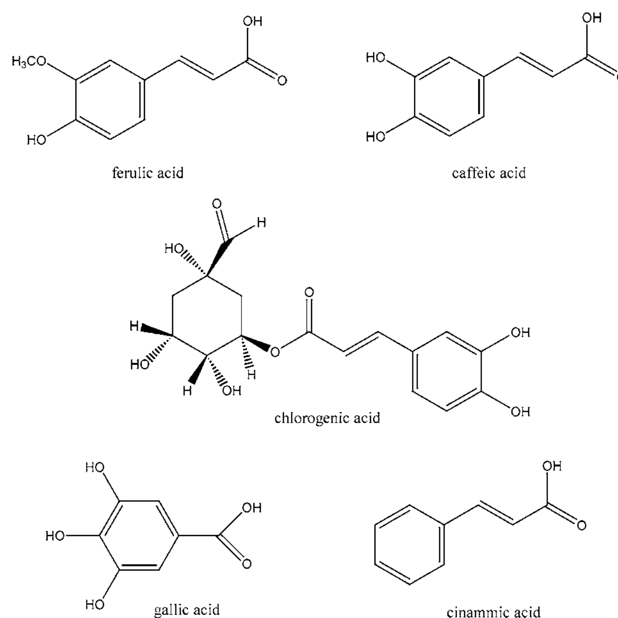


Figure 3. Structures of the phenolic acids tested.

Table 2. Inhibitory Effects of Phenolic Acids toward MA Formation from Ethyl Arachidonate Oxidized by Fenton's Reagent

sample name	inhibition of MA formation (%)[a] at 0.5 mM concn	inhibition of MA formation (%)[a] at 1.0 mM concn
ferulic acid	21.4 ± 21.6	74.6 ± 6.4
caffeic acid	29.9 ± 11.8	39.4 ± 16.8
chlorogenic acid	9.3 ± 13.4	34.2 ± 3.9
gallic acid	−67.9 ± 5.1	1.4 ± 18.2
trans-cinnamic acid	−2.2 ± 26.2	−6.4 ± 6.9
BHT	96.4 ± 0.2	97.5 ± 1.0
α-tocopherol	87.1 ± 6.2	90.5 ± 6.8

[a] Values represent means ± SD of three independent experiments.

different assays used. Chen and Ho (*35*) reported that caffeic acid had higher antioxidant activity than ferulic acid in three test models: Rancimat test [similar results were also reported by Shahidi and Wanasundara (*28*)], oil-in-water emulsion oxidation, and DPPH radical scavenging activity. It was also reported that caffeic acid is more effective than ferulic acid against lipid peroxyl radicals generated in the lipophilic phase of low-density lipoproteins (LDL) and in protecting LDL cholesterol from oxidation (*36*). However, our results were consistent with those of Rice-Evans et al. (*11*), who found that ferulic acid had an enhanced antioxidant effectiveness compared to that of caffeic acid. Esterification of the carboxylate group of caffeic acid with quinic acid (chlorogenic acid) had no influence on the activity. The pro-oxidant activity of gallic acid at the 0.5 mM level and its nonactivity at the 1.0 mM level were surprising in light of previous reports of its antioxidant efficiency (*11*, *28*). Gallic acid may have a high reactivity in the assay; it may have readily oxidized and generated reactive species, as was suggested for myricetin. It was not surprising that cinnamic acid did not exhibit antioxidant activity, since the compound does not have any hydroxyl or methoxy groups on the aromatic ring.

Future studies are planned to investigate the fate of myricetin and gallic acid in the antioxidant assay to elucidate if their low activity is due to their high reactivity leading to decomposition during the assay.

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