Antioxidant Activity of Magnolol, Honokiol, and Related Phenolic Compounds

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ABSTRACT: The antioxidant activity of 10 Japanese and Chinese crude drugs (Kampo drugs) was determined *in vitro*. Extract of *Magnolia cortex*, which had the highest antioxidant activity, contained phenolic compounds magnolol and honokiol. However, inhibitory effects of these compounds on lipid oxidation were weaker than that of α-tocopherol as measured by thiobarbituric acid assay. The structure–activity relationship of phenolic compounds showed that antioxidant activities were in the order 4-allyl-2,6-dimethoxyphenol ≥ *p,p′*-biphenol > eugenol > 2-allyl-6-methylphenol > honokiol > magnolol > caffeic acid > *p*-ethylphenol > guaiacol. As expected, these results showed that an electron donor and/or bulky groups at the *ortho*- or *para*-position of the phenol were required for inhibition of lipid oxidation. Electron spin resonance spin trapping experiments showed that phenol compounds with an allyl substituent on their aromatic rings directly scavenged superoxide (O_2^-), and that only eugenol trapped hydroxyl radicals. These findings suggest that phenolic compounds that contain allyl groups may be effective antioxidants because of the scavenging ability of O_2^- or hydroxyl radical, whereas other phenols, without an allyl moiety such as α-tocopherol, may play a role in the termination of free radical chain reactions.
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KEY WORDS: Allyl group, antioxidant, electron spin resonance, eugenol, free radical, honokiol, lipid oxidation, magnolol, oxidation, phenolic compound.

Lipid oxidation and nonenzymatic oxidation of proteins and nucleic acids, caused by free radicals, may be related to aging and diseases, such as atherosclerosis, diabetes, cancer, and ischemia–reperfusion injury (1–6). Highly reactive free radicals, especially oxygen-derived radicals, are formed by exogenous chemicals or endogenous metabolic processes and then are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Consequently, oxygen, while essential for animals and human life, can be toxic to living tissues. Almost all organisms are well protected against free-radical damage by either enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, or compounds, such as ascorbic acid, α-tocopherol, and glutathione (7). When the mechanism of antioxidant protection becomes unbalanced by factors such as aging, deterioration of physiological functions can occur, resulting in diseases or accelerated aging.

Consequently, it is important to find compounds that prevent oxidation. Many attempts have been made to find highly active antioxidants that prevent oxidation *in vivo*. Naturally occurring phenolic compounds, such as flavonoids and tannins, have been shown to scavenge active oxygen species and to effectively prevent oxidative cell damage (8); however, the mechanism for their scavenging reaction *in vivo* is still unclear.

Traditional Chinese drugs (Kampo drugs) are used for treatment and prevention of many diseases in China and Japan and are formulated from several crude drugs composed of dried plants or insects. Because it has been shown that the Kampo drug *Juzen Daiho Toh* has antioxidant activity (9), much attention has been focused on the antioxidant activities of Kampo drugs. Although Kampo drugs are a potential source for active antioxidants, few reports have found new compounds with antioxidant activity (9). It has not, however, been reported whether these compounds have potent activity against lipid oxidation.

In the present work, we examined the antioxidant activity of several Kampo drugs under conditions of *in vitro* oxidation. In addition, the structure–antioxidant activity relationship was investigated by using several phenolic compounds isolated from either Kampo drugs or plants.

MATERIALS AND METHODS

Crude drugs. Ten crude drugs from *Magnolia cortex, Rehmannia radix, Zizyphi fructus, Mentha herba, Forsythia fructus, Ephedra herba, Cimicifuga rhizoma, Schizonepeta spica, Saussurea radix*, and *Artemisia capillaris* were kindly supplied by Dr. T. Nishimura of Teikyo University, Tokyo, Japan. Magnolol and honokiol were purchased from Yoneyama Kagaku Yakuhinn Co. (Tokyo, Japan). Egg phosphatidylcholine was isolated from egg yolk by a published method (10). Thiobarbituric acid (TBA), L-ascorbic acid, and $FeSO_4$ were from Tokyo Kasei Kogyo Co., Daichi Kagaku

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Yakuhin Co., and Kanto Chemical Co. Inc. (all Tokyo, Japan), respectively. 2-Allyl-6-methylphenol and 4-allyl-2,6-methoxyphenol were from Aldrich, Inc. (Tokyo, Japan), and *o*-phenylphenol, *m*-phenylphenol, *p*-phenylphenol, *o*-*n*-propylphenol, *p*-*n*-propylphenol, *p*-benzylphenol, *o,o′*-biphenol, *p,p′*-biphenol, caffeic acid, guaiacol, eugenol, *p*-ethylphenol, and phenol were from Tokyo Kasei Kogyo Co. Palmatine chloride, baicalein, baicalin, puerarin, wogonin, and *d*-catechin were purchased from Yoneyama Kagaku Yakuhinn Co. Flavanone, epigallocatechin, and epigallocatechin-gallate were purchased from Funakoshi Co. (Tokyo, Japan). Hypoxanthine (HX), xanthine oxidase (XO), and ethylenediamine tetraacetic acid (EDTA) were purchased from Wako Pure Chemical Co. (Osaka, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Labotec Co. (Tokyo, Japan). α-Tocopherol was purchased from E. Merck (Darmstadt, Germany). Chloroform and butanol were purchased from Yoneyama Kagaka Yakuhinn Co.

Fe^{2+} and ascorbic acid-induced oxidation of egg phosphatidylcholine (egg PC) and assessment of antioxidant activity. Lipid oxidation was assayed as formation of TBA-reactive substances (TBARS) (11). Mixtures of a solution of egg PC in chloroform (200 μL;10 mg/mL) and each sample (100 μL) were evaporated to dryness under nitrogen gas. Control and reference substrate with 20 mM α-tocopherol were also tested for comparison. Lipid oxidation was initiated by the addition of 0.2 mM $FeSO_4$ and 2 mM ascorbic acid. After incubation at 37°C for 30 min, the reaction was terminated by addition of 5 mM EDTA. The oxidation mixture was then mixed with 3 mL of 1% phosphoric acid and 1 mL of aqueous 0.7% TBA solution and heated at 98°C for 45 min. The mixture was extracted with 4 mL of *n*-butanol, and the absorbance was measured at 535 nm with a Hitachi 220A spectrophotometer (Hitachi Seisakusho Co. Ltd., Tokyo, Japan). Antioxidant activity was calculated as follows:

$$\text{inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \qquad [1]$$

where *A* represents absorbance.

Measurement of superoxide and hydroxyl radical scavenging activities. Superoxide and hydroxyl radical scavenging activities were measured by electron spin resonance (ESR) with DMPO as a spin trapping reagent. The HX–XO system was used as a superoxide-generating system, and hydroxyl radicals were generated by the Fenton reaction (Fe^{2+}–H_2O_2). In superoxide trapping, DMPO produces DMPO-OOH spin adducts, and DMPO-OH spin adducts are obtained when hydroxyl radicals are trapped, so these radical-scavenging activities of samples could be calculated as the decreasing rate of the DMPO-OOH or DMPO-OH signal intensities (9). ESR spectra were recorded on a Jeol-JES-RE1X spectrometer (Jeol Co. Ltd., Tokyo, Japan). The ESR conditions are shown in Table 1. The superoxide or hydroxyl radical-scavenging activities of samples were calculated as the decreasing rate of the DMPO-OOH or DMPO-OH signal intensities.

Preparation of samples for analysis of antioxidant activity of extracts of Kampo drugs. Kampo drugs (crude drugs) from 10 species, listed above in the Materials and Methods section, were used in this study . The crude drugs (2 g each) were extracted with 80 mL of $CHCl_3$/EtOH (3:1) solution at 25°C for 2 h. The extracts were filtered and concentrated *in vacuo*. The extracts were then dissolved in $CHCl_3$/EtOH (3:1) to give sample solutions (1 mg/mL). The solutions were used for the analysis of antioxidant activity.

Isolation of magnolol and honokiol. Pulverized *M. cortex* (16 g) was suspended in 400 mL of $CHCl_3$/EtOH (3:1) at 60°C for 2 h. After filtration, the extract was concentrated to dryness *in vacuo*. The residue was dissolved in 2 mL $CHCl_3$ and applied to a silica gel column (3 × 23 cm). The column was eluted with $CHCl_3$. Active fractions were combined and evaporated to a yellow powder (722 mg). The yellow powder was further purified by preparative reverse-phase high-performance liquid chromatography (HPLC) on a Senshu Pak ODS-5251-H column (Senshu Chemicals, Tokyo, Japan) with an ultraviolet (UV) detector [mobile phase, 80% MeOH; flow rate, 0.5 mL/min; retention times of fraction 6 (21 mg) and fraction 9 (53 mg) were 22 min and 32 min, respectively]. Fraction 6 was identified as honokiol, and fraction 9 as magnolol, based on physicochemical data from both compounds, reported previously (12).

Statistical analysis. The results were presented as mean ± standard error (SE). All were assessed by two-factor analysis of variance.

TABLE 1
Electron-Spin Resonance (ESR) Spin-Trapping Method: Reaction Mixtures and Conditions

DMPO-OH[a]			DMPO-OOH		
10 mM	$FeSO_4$	75 μL	2 mM	hypoxanthine	50 μL
1 mM	H_2O_2	75 μL	5.5 mM	DETAPAC	50 μL
92 mM	DMPO	75 μL	92 mM	DMPO	100 μL
Sample or H_2O		75 μL	0.02 U/mL	xanthine oxidase	50 μL
			Sample or H_2O		50 μL
Temperature		25°C			25°C
Power		0.8 mW			8 mW
Field		335.1 ± 5 mT/G			335.1 ± 5 mT/G
Modification		100 KHz			100 KHz
Time constant		0.03 s			0.03 s

[a]Abbreviations: DMPO: 5,5-dimethyl-1-pyrroline *N*-oxide; DETAPAC: diethylenetriamine pentaacetic acid.

RESULTS AND DISCUSSION

Extract from *M. cortex* showed the highest antioxidant activity of the drugs (Table 2). To isolate and identify active compounds, the extract from *M. cortex* was fractionated by column chromatography into gross chemical classes. For further purification of the active fraction, reverse-phase HPLC was used to yield magnolol and honokiol, which were identified by comparing their physicochemical properties—melting points and infrared, UV, ^{1}H NMR, and ^{13}C NMR spectra—with previous data (12). Magnolol and honokiol are major phenolic components of *M. cortex,* which has been reported to be an important constituent of medications for neuronal disturbances or gastrointestinal disorders in traditional Chinese medicine (13).

Inhibitory effects of magnolol and honokiol (250 μM) on lipid oxidation were 63.6 and 76.9%, respectively, compared to α-tocopherol at 97.4% (Table 3). Consequently, to determine the molecular design of strong antioxidants, we investigated the structure–activity relationship by using phenolic compounds isolated from either Kampo drugs or plants (Fig. 1). Ten phenolic compounds (Table 3) showed potent antioxidant activity and were evaluated for dose-dependent antioxidant effects (Fig. 2). Antioxidant potency decreased in the order: 4-allyl-2,6-dimethoxyphenol ≥ *p,p′*-biphenol > eugenol > *2*-allyl-*6*-methylphenol > honokiol > magnolol > caffeic acid > *p*-ethylphenol > guaiacol. These results showed that phenols with an electron donor and/or bulky groups at the *ortho* or *para* position inhibited lipid oxidation more than phenol compounds with an electron-withdrawing group or a group at the *meta* position. These findings are in agreement with a current concept of the chemical reactivity of phenol (14).

To understand the mechanism of antioxidant effects of these phenolic compounds, we analyzed *in vitro* lipid oxidation by using ESR spectrometry to determine whether they scavenged free radicals directly or acted as free-radical chain-breaking antioxidants, such as α-tocopherol. Relative signal intensities of both the spin adduct DMPO-OOH, which is produced by O_2^- generated from HX–OX systems, and the spin adduct DMPO-OH, produced by hydroxyl radical through the Fenton reaction, decreased upon the addition of phenolic compounds with antioxidant activity (Table 4). The values are expressed as the concentration of phenolic compound that causes a 50% decrease in the level of O_2^- or hydroxyl radicals [50% inhibitory concentration (IC_{50}) values]. Five phenolic compounds—magnolol, honokiol, 2-allyl-6-methylphenol, 4-allyl-2,6-dimethoxyphenol, and eugenol—were able to trap O_2^-, and no decrease in the signal was observed following addition of other phenols under the experimental conditions used.These findings showed that an allyl moiety at the *ortho* or *para* position on phenols increased the O_2^- scavenging activity. Among the five phenols, only eugenol scavenged hydroxyl radicals under the conditions used. Although the mechanism of inhibiting lipid oxidation by eugenol is still unclear, our results suggest that eugenol may inhibit oxidation by trapping active oxygen species, such as O_2^- or hydroxyl radicals, rather than by breaking the free-radical chain reaction.

We also investigated antioxidant activities of 10 other phenolic compounds with antioxidant properties in the flavonoid and tannin classes (Fig. 3) because magnolol and its related phenols had weaker antioxidant activities than α-tocopherol. The antioxidant activities decreased in the order epigallocatechin-gallate > baicalein > epigallocatechin > baicalin > catechin >puerarin > flavanone > palmatin chloride > wogonin (Table 5). Epigallocatechin-gallate, baicalein, and epigallocatechin showed strong antioxidant activities. All had highly potent antioxidant activities at low (15 μM) concentration. Baicalein and wogonin are flavanoid components of *Scutellaria radix*. Antioxidant activity of baicalein which has two OH groups at the *ortho* position of phenol was greater than that of wogonin with two OH groups at the *meta* position. Epigallocatechin-gallate and epigallocatechin also have OH groups at neighboring positions and had strong antioxidant activities. These results suggest that the presence of OH groups at neighboring positions is essential for strong antioxidant activity, and that the number of phenolic OH groups is not critical. Because baicalein, epigallocatechin, and epigallocatechin-gallate also have electron donor substituents at the

TABLE 2
Inhibitory Effects of Crude Drugs on Lipid Oxidation by Thiobarbituric Acid-Reactive Substances (TBARS)

Crude drugs[a]	Inhibition of lipid oxidation (%)[b]
Magnolia cortex	73.15 ± 0.1
Schizonepeta spica	20.15 ± 4.2
Rehmannia radix	51.70 ± 0.1
Mentha herba	0.00
Forsythia fructus	51.85 ± 0.4
Cimicifuga rhizoma	60.10 ± 0.8
Artemisia capillaris	62.55 ± 1.3
Ephedra herba	56.05 ± 0.1
Zizyphi fructus	53.90 ± 0.5
Saussurea radix	56.05 ± 0.3

[a]Concentration of extract was 1 mg/mL.
[b]Mean ± SE; *n* = 4.

TABLE 3
Inhibition of Lipid Oxidation by Phenolic Compounds Using TBARS Assay

Compounds	Inhibition of lipid oxidation (%) 62.5 μM	250 μM
α-Tocopherol (control)	97.2 ± 0.1	97.4 ± 0.1
4-Allyl-2,6-dimethoxyphenol	78.8 ± 2.0	91.3 ± 0.1
p,p¢-Biphenol	81.7 ± 1.3	88.3 ± 1.0
Eugenol	63.2 ± 0.2	85.0 ± 0.5
Honokiol	32.7 ± 1.3	76.9 ± 0.4
2-Allyl-6-methylphenol	38.7 ± 3.0	75.7 ± 0.6
Magnolol	21.6 ± 0.4	63.6 ± 0.1
Caffeic acid	53.7 ± 3.0	60.6 ± 1.4
p-Ethylphenol	14.8 ± 0.6	53.9 ± 0.8
Guaiacol	6.6 ± 2.0	35.9 ± 0.5

[a]Mean ± SE, *n* = 4. For abbreviation, see Table 2.

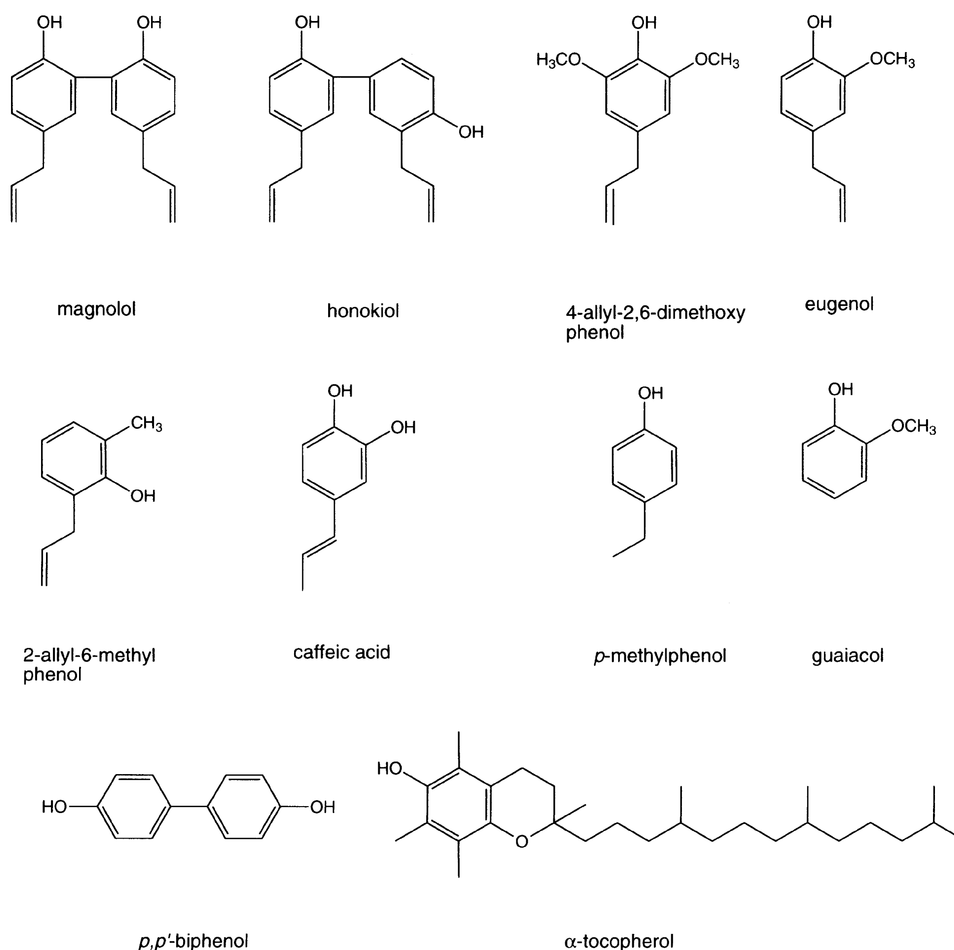

FIG. 1. Structures of phenolic compounds.

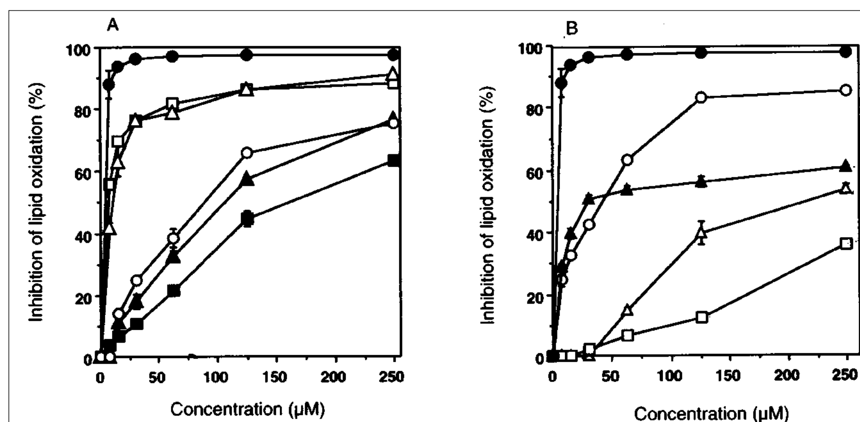

FIG. 2. Inhibition of lipid oxidation by phenolic compounds by thiobarbituric acid-reactive substance measurement: (A) ●, α-tocopherol; △, 4-allyl-2,6-dimethoxyphenol; □, *p,p′*-biphenol; ○, 2-allyl-6-methylphenol; ▲, honokiol; ■, magnolol. (B) ●, α-tocopherol; ○, eugenol; ▲, caffeic acid; △, *p*-ethylphenol; □, guaiacol. Each point and error bar represent the mean ± SE; $n = 4$.

ortho position, we concluded that the presence of electron donor substitutes at the *ortho* position was necessary for strong antioxidant activity of these phenolic compounds. This conclusion is supported by a previous report that the antioxidant activities of catechol derivatives with phenolic OH at the *ortho* position are much higher than those at the *meta* position. The effect of catechol substituents correlated with their redox potentials, indicating that antioxidant activities of cate-

TABLE 4
Inhibition of Superoxide and Hydroxyl Radical Production by Phenolic Compounds[a]

Compounds	Superoxide (IC_{50}, mM)	Hydroxyl radical (IC_{50}, mM)
Magnolol	5.3	<10
Honokiol	3.5	<10
4-Allyl-2,6-dimethoxyphenol	0.7	<10
2-Allyl-6-methylphenol	3.5	<10
Eugenol	5.3	1.88

[a]Expressed as IC_{50}, the concentration causing a 50% decrease in O_2^- or hydroxyl radical levels.

chol derivatives are controlled by their electron donor activities (14).

The ability of α-tocopherol and 10 plant compounds to trap the active oxygen species O_2^- and hydroxyl radicals was investigated by ESR spectrometry (Table 6). O_2^-–trapping ability agreed with the results of the inhibition of lipid oxidation by TBARS. No compound showed the ability to trap hydroxyl radicals under these experimental conditions. Antioxidant activities of flavonoids are mainly *via* scavenging of O_2^- (15), and double bonds and/or OH groups on the C ring of flavonoids and tannins are essential for O_2^- trapping (16). Because an allyl substituent (double bond) exists in the structure of magnolol and its related compounds, the findings obtained in this study are compatible with this theory. On the other hand, flavanone and allylbenzene trapped neither O_2^- nor hydroxyl radicals in this system (data not shown), suggesting that these active oxygen species did not react directly at the C ring or allyl groups. Although Kampo drugs, especially *M. cortex,* have excellent antioxidant activity, when extracts including magnolol and honokiol were purified and the compounds were isolated, the antioxidant activity was less than that in the crude extract. Strong antioxidant activity of Kampo drugs on lipid oxidation is probably caused by additive and/or synergistic effects of other antioxidant components in these drugs. In experiments that investigated the molecular structure of strong phenolic antioxidants, the presence of both an allyl substituent and an electron donor or bulky group was necessary for strong antioxidant activity. Inhibitory effects of

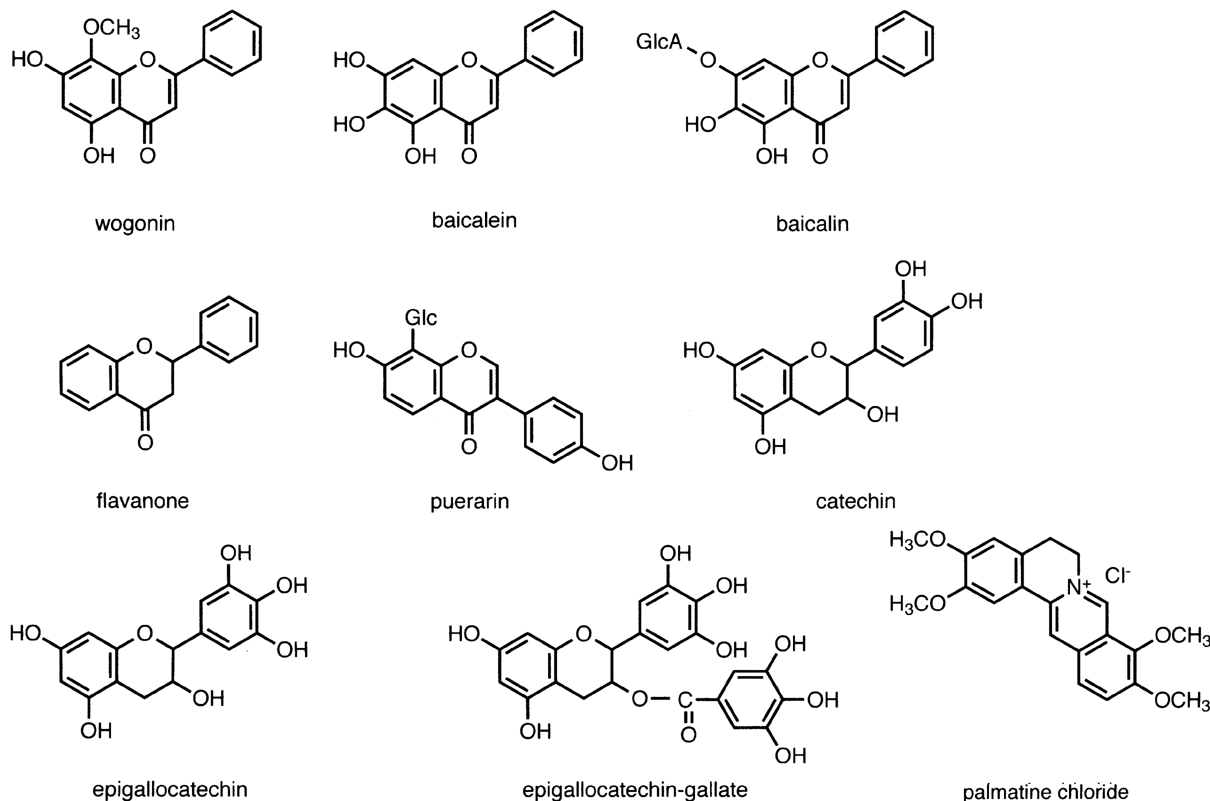

FIG. 3. Structures of plant compounds.

TABLE 5
Inhibitory Effects of Compounds on Lipid Oxidation by TBARS Assay

Compounds	Inhibition of lipid oxidation (%) 15 μM	250 μM
α-Tocopherol (control)	88.0 ± 0.3[a]	97.4 ± 0.1
Epigallocatechin-gallate	84.1 ± 0.8	96.1 ± 1.1
Baicalein	83.4 ± 1.0	92.2 ± 0.3
Epigallocatechin	72.1 ± 0.4	88.9 ± 1.2
Baicalin	—[b]	76.0 ± 1.1
Catechin	—	66.1 ± 0.6
Puerarin	—	59.5 ± 0.4
Flavanone	—	29.0 ± 1.2
Palmatine chloride	—	20.2 ± 2.0
Wogonin	—	16.6 ± 0.8

[a]Mean ± SE; $n = 4$.
[b]Not tested. See Table 2 for abbreviation.

TABLE 6
Inhibition of Superoxide and Hydroxyl Radical Production by Plant Extract Compounds[a]

Compounds	Superoxide (IC_{50}, nM)	Hydroxyl radical (IC_{50}, μM)
Epigallocatechin-gallate	1.40	<1
Epigallocatechin	5.65	<1
Baicalein	17.60	<1
Catechin	17.60	<1
Baicalin	282.70	<1
Palmatine chloride	353.30	<1

[a]Expressed as IC_{50}, the concentration causing a 50% decrease in O_2^- or hydroxyl radical levels.

phenolic compounds containing allyl groups were similar to those of flavonoids; that is, these compounds scavenged oxygen-derived free radicals directly, whereas other phenols without allyl groups, such as α-tocopherol, may act as free-radical chain-breaking antioxidants.

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