Antioxidant Activity of Eugenol and Related Monomeric and Dimeric Compounds

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Since the inhibitory effect of eugenol (a), which was isolated as an antioxidative component from plant, *Caryopylli flos*, on lipid peroxidation was less than that of α-tocopherol, we synthesized the eugenol-related compounds dieugenol (b), tetrahydrodieugenol (c), and dihydroeugenol (d), to find new strong antioxidants and assessed them for their inhibitory effect on lipid peroxidation and scavenging ability for superoxide and hydroxyl radicals. The antioxidative activities were in the order: (b)>(c)>(d)>(a) for the thiobarbituric acid reactive substance (TBARS) formation. These results suggest that the dimerized compounds have higher antioxidant activities than that of the monomers. Electron spin resonance (ESR) spin trapping experiments revealed that eugenol and its dimer, having allyl groups in the structure, scavenged superoxide, and that only eugenol trapped hydroxyl radicals under the conditions used. These finding suggest that eugenol and dieugenol have a different mechanism of antioxidation, *i.e.* eugenol may inhibit lipid peroxidation at the level of initiation, however, the related dimeric compounds may inhibit lipid peroxidation at the level of propagation of free radical chain reaction like α-tocopherol.

Key words   eugenol; dimer; lipid peroxidation; electron spin resonance; dieugenol

Peroxidation of cell membrane lipid results in membrane destabilization and change of transport characteristics, which eventually leads to cell death. Many studies have demonstrated that lipid peroxidation, caused by free radicals, may be related to aging and several diseases, such as atherosclerosis, diabetes, cancer, and ischemia-reperfusion injury.[1—5] The polyunsaturated fatty acids in the cell membrane are protected against lipid peroxidation through endogenous antioxidants such as α-tocopherol.[6] 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*. A large number of naturally occurring molecules having antioxidant properties are known to be phenolic compounds: acid-phenols or flavonoids and their esters.[7] The antioxidative potency of phenolic compound depends on the chemical structure, in particular, electron delocalization on the aromatic nucleus.[8] However, the mechanism of their scavenging reaction *in vivo* is still obscure.

In order to find antioxidants from Chinese drugs (Kampo drugs or crude drugs), we have recently showed that CHCl$_3$: EtOH (3 : 1) extracts from the bark of *Magnolia cortex* have potent antioxidant activity, and demonstrated that the antioxidative components are magnolol and honokiol.[9] In the present work, the identification of the antioxidative components from *Caryophylli Flos* and the characterization of their inhibitory effects on lipid peroxidation are described.

Materials and Methods

Chemicals   *Caryophylli Flos* was kindly supplied by Dr. T. Nishimura of Teikyo University, Tokyo, Japan. Eugenol was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). α-Tocopherol was purchased from E. Merck (Darmstast, Germany). Thiobarbituric acid (TBA), L-ascorbic acid, and FeSO$_4$ were purchased from Tokyo Kasei Kogyo Co., Daiichi Kagaku Yakuhin Co., and Kanto Chemical Co. Inc. (Tokyo, Japan), respectively. Hypoxanthine (HX), xanthine oxidase (XO), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ethylenediaminetetraacetic acid disodium salt (EDTA) were from Wako Pure Chemical Co. (Osaka, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was from Labotec Co. (Tokyo, Japan). CHCl$_3$, EtOH, and BuOH were from Yoneyama Yakuhin Kogyo Co. (Osaka, Japan).

Fe^{2+} and Ascorbic acid Induced Oxidation of Egg Phosphatidyl-

choline (Egg PC) and Assessment of Antioxidant Activity   Lipid peroxidation was assayed as formation of thiobarbituric acid reactive substance (TBARS).[10] 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 2 mM α-tocopherol were also tested for comparison. Lipid peroxidation 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% thiobarbituric acid 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., Japan). Antioxidant activity was calculated as follows:

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

where A represents absorbance.

Measurement of Superoxide, Hydroxyl Radical and DPPH Scavenging Activities   Superoxide and hydroxyl radical scavenging activities were measured by electron spin resonance (ESR) with DMPO as a spin trapping reagent.[11] The HX–XO system was used as a superoxide generating system, and hydroxyl radicals were generated by the Fenton reaction (Fe^{2+}–H$_2$O$_2$). 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] DPPH (2 mM) was dissolved in ethyl alcohol. Each sample (50 μl) in chloroform was evaporated to dryness under nitrogen gas. The DMPO–OOH, DMPO–OH and DPPH spin adducts were measured after exactly 60 seconds. ESR spectra were recorded on a JEOL-JES-RE1X spectrometer (JEOL Co. Ltd., Tokyo, Japan). The ESR conditions are shown in Table 1.

Isolation of Eugenol   *Caryophyllia Flos* (50 g) was suspended in 800 ml of CHCl$_3$/EtOH (3 : 1) at room temperature overnight. After filtration, the extract was concentrated to dryness *in vacuo*. The residue was dissolved in CHCl$_3$ and applied to a silica gel column (3×23.5 cm). The column was eluted with CHCl$_3$, and fractions were combined and evaporated to give a yellow oil (1 g). The yellow oil was further purified by Sephadex LH-20 column (3×15 cm) (Pharmacia Biotech, Sweden). FAB-MS *m/z*: 165 (M+H)$^+$. HR-FAB-MS *m/z*: 165.1061 (Calcd for C$_{10}$H$_{13}$O$_2$: 165.1130). The column was developed with MeOH, and active fractions (320 mg) were identified as eugenol, based on physico-chemical date reported previously.[12]

Synthesis of Dieugenol   Eugenol (10 g) was dissolved in pyridine (10 g) and mixed with FeSO$_4$ (100 mg) and 31% H$_2$O$_2$ (20 g×3) at 60 °C for 24 h. The reaction product was extracted with AcOEt and distilled by steam distil-

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Table 1. Electron Spin Resonance (ESR): Reaction Mixtures and Conditions

DMPO–OOH		DMPO–OH		DPPH	
2 mM hypoxanthine	50 μl	10 mM FeSO$_4$	75 μl	2 mM DPPH	200 μl
5.5 mM DETAPAC	50 μl	1 mM H$_2$O$_2$	75 μl	Sample[a] or H$_2$O	50 μl
92 mM DMPO	100 μl	92 mM DMPO	75 μl		
0.02 U/ml Xanthin oxidase (phosphate buffer, pH 7.2)	50 μl	Sample[a] or H$_2$O	75 μl		
Sample[a] or H$_2$O	50 μl				
Temperature	25 °C		25 °C		25 °C
Power	0.8 mW		8 mW		0.8 mW
Field	335.1+5 mT/G		335.1+5 mT/G		335.1+5 mT/G
Modification	100 kHz		100 kHz		100 kHz
Time constant	0.03 s		0.03 s		0.03 s

Abbbrevitions: DMPO: 5,5-dimethyl-1-pyrroline N-oxide; DETAPAC: diethylenetriamine pentaacetic acid. *a*) Each sample in CHCl$_3$ was evaporated to dryness under nitrogen gas. H$_2$O was used as a control.

lation. The residue was recrystallized from ethanol to give dieugnol (3.56 g, 35.6%) as a white solid, mp 93 °C.[13] FAB-MS *m/z*: 327 (M+H)$^+$. HR-FAB-MS *m/z*: 327.1985 (Calcd for C$_{20}$H$_{23}$O$_4$: 327.2022).

Synthesis of Dihydroeugenol Eugenol (100 mg) was dissolved in EtOH and hydrogenated on 10% Pd–C at room temperature for 5 h. After filtration, the reaction mixture was evaporated to dryness *in vacuo*. The residue was distilled by steam distillation to give dihydroeugenol as a yellow oil (98 mg, 98%). NMR (CDCl$_3$) *δ*: 0.93 (t, 3H, *J*=7.3 Hz, 9-H), 1.61 (m, 2H, *J*=7.3, 7.9 Hz, 8-H), 2.51 (t, 2H, *J*=7.9 Hz, 7-H). FAB-MS *m/z*: 167 (M+H)$^+$. HR-FAB-MS *m/z*: 167.1320 (Calcd for C$_{10}$H$_{15}$O$_2$: 167.1289).

Synthesis of Tetrahydrodieugenol Dieugenol (300 mg) was dissolved in EtOH and hydrogenated on 10% Pd–C at room temperature for 5 h. After filtration, the reaction mixture was evaporated to dryness *in vacuo*. The residue was recrystallized from EtOH to give tetrahydrodieugenol as a white solid (289 mg, 96%), mp 113 °C. NMR (CDCl$_3$) *δ*: 0.93 (t, 3H, *J*=7.3 Hz, 9,9′-H), 1.62 (m, 2H, *J*=7.3, 7.9 Hz, 8,8′-H), 2.52 (t, 2H, *J*=7.9 Hz, 7,7′-H). FAB-MS *m/z*: 331 (M+H)$^+$. HR-FAB-MS *m/z*: 331.2385 (Calcd for C$_{10}$H$_{25}$O$_2$: 331.2340).

Results and Discussion

Extract from *Caryophylli flos* with organic solvents showed excellent antioxidant activity (Table 2). Especially, the extract with a mixture of chloroform : ethanol (3 : 1) had potent antioxidant activity, *i.e.*, an inhibition of TBARS formation, and scavenging ability for superoxide and hydroxyl radicals. In order to identify the active compound in the extract, the purification was carried out using column chromatography, and we identified the potent compounds as eugenol based on its physico-chemical properties, IR, UV, ^{1}H- and ^{13}C-NMR spectra. Eugenol is a major component of the oils from clove, cinnamon, basil and nutmeg.[14,15] Since Nagababu *et al*. revealed that eugenol might inhibit lipid peroxidation at the stage of the initiation, propagation, or both, many attempts for elucidation of the mechanism of antioxidant activity of eugenol have been carried out.[16] Nevertheless, the mechanism details are still obscure. In order to define the mechanism, and to search for related compounds having a stronger antioxidant activity, we synthesized some related compounds; dieugenol, which is an oxidized compound of eugenol,[13] dihydroeugenol and tetrahydrodieugenol, which were synthesized to understand the antioxidant effects of the allyl group (Fig. 1). As shown in Table 3 and Fig. 2, Fe^{2+}-ascorbic acid induced lipid peroxidation was inhibited significantly in a dose-dependent manner by these compounds. Antioxidant potency decreased in the order: dieugenol>tetrahydrodieugenol>dihydroeugenol>eugenol. The inhibitory effect of dimerized compounds is higher than that of monomeric compounds, eugenol itself. These results

Table 2. Inhibition of Lipid Peroxidation, Superoxide and Hydroxyl Radicals by Extract with Various Solvents

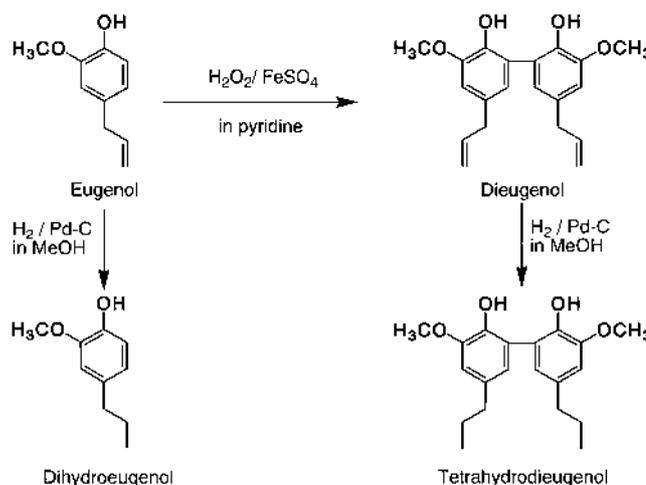| Solvent	Inhibition of lipid peroxidation (%)	DMPO–OH IC$_{50}$ (mg/ml)	DMPO–OOH IC$_{50}$ (mg/ml)
CHCl$_3$	93.2±0.1	1.0	>2.0
EtOH	93.8±0.6	>2.0	0.6
CHCl$_3$: EtOH (3:1)	94.3±0.1	1.0	1.0
H$_2$O	50.4±2.0	>2.0	>2.0

Mean±S.E., n=4.

Fig. 1. Structures of Eugenol and Related Assessed Compounds

suggest that bulky substituents are necessary at the neighboring position of the hydroxyl group in eugenol. An organic radical as a model of a lipid carbon-centered radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was trapped remarkably by the dimerized compounds, dieugenol and tetrahydrodieugenol (Table 3). The trapping potency of dieugenol was almost 5 times higher than that of eugenol. Interestingly, the order of the potency was similar to that of the inhibitory effect on TBARS formation. Although it is not obvious at present why the trapping effect of the monomeric dihydroeugenol is two times higher than that of eugenol, it is likely that the allyl group at the *para* position of the phenolic hydroxyl moiety is not critical for inhibition of lipid peroxidation.

To understand the antioxidant mechanism of these phenolic compounds, we analyzed *in vitro* lipid peroxidation using ESR spectrometry to see whether they scavenged active oxygen species directly or whether they acted as free-radical chain breaking antioxidants, such as α-tocopherol. Relative signal intensities for both the spin adduct DMPO–OOH, which is produced by O_2^- generated from HX–XO 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 3). The scavenging values are expressed as the concentration of phenolic compounds that causes a 50% decrease in the level of O_2^- or hydroxyl radicals (50% inhibitory concentration (IC_{50}) values). Eugenol and dieugenol were able to trap O_2^-, and no decrease in the signal was observed following addition of other phenolic compounds under the experimental conditions used (Table 3). An allyl moiety at the *ortho* or *para* position of the phenols increased the O_2^- scavenging activity.[9] Among the four phenols, only eugenol scavenged hydroxyl radicals under the conditions used. Our results suggest that eugenol may inhibit lipid peroxidation by trapping active oxygen species, such as O_2^- or hydroxyl radicals, rather than by breaking the free radical chain reaction.[9]

In summary, it is indicated that the inhibition mechanism of eugenol to lipid peroxidation has two steps; 1) it interferes with the chain reactions by trapping the active oxygen, 2) it is metabolized to dimer, and the dimeric compound (dieugenol) inhibits lipid peroxidation at the level of propagation of free radical chain reaction like α-tocopherol. Although the activities of eugenol itself are less than that of α-tocopherol *in vitro*, it is anticipated that the protective effect of eugenol is comparative to α-tocopherol in organisms injured by many oxidative stresses, because of its inhibition mechanism of monomeric and dimeric stage (Fig. 3).

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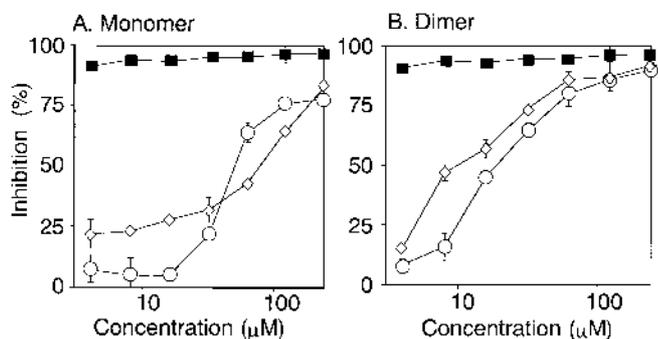16) Nagababu E., Lakshmaiah N., *Biochem. Pharamacol.*, **43**, 2393—2400 (1992).

Fig. 2. Inhibition of Lipid Peroxidation by Phenolic Compounds through TBARS Measurement

(A) ■, α-tocopherol; ◇, dihydroeugenol; ○, eugenol. (B) ■, α-tocopherol; ◇, tetrahydrodieugenol; ○, dieugenol. Each point and bar represent the mean±S.E., $n=4$.
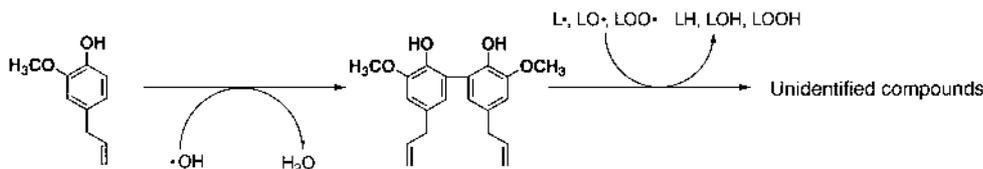

Fig. 3. Antioxidant Mechanisum of Eugenol

Table 3. Inhibition of Lipid Peroxidation, DPPH, Superoxide and Hydroxyl Radicals by Eugenol and Its Related Compounds

Samples	TBA method		DPPH IC_{50} (μM)	DMPO–OOH IC_{50} (mM)	DMPO–OH IC_{50} (mM)
	62.5 μM	250 μM			
Eugenol	43.0±1.2	76.0±0.5	>800	5.3	1.88
Dihydroeugenol	63.7±0.1	77.0±3.2	421	>10	>10
Dieugenol	86.0±0.2	92.5±0.1	160	3.3	>10
Tetrahydrodieugenol	78.9±0.1	89.8±0.6	164	>10	>10
α-Tocopherol	97.2±0.1	97.4±0.1	80	>10	>10

Mean±S.E., $n=4$.