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Antioxidant activity of eugenol compounds

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Summary

Antioxidant action of eugenol compounds was analyzed in relation to the role of transition metal. Eugenol and isoeugenol inhibited iron-mediated lipid peroxidation, and autooxidation of Fe^{2+} ion. Inhibitory effects of isoeugenol on lipid peroxidation and iron oxidation were more potent than those of eugenol. Eugenol and isoeugenol protected low density lipoprotein (LDL) from copper-dependent oxidation, and showed a potent copper-reducing activity to the same extent. Both compounds effectively scavenged a stable radical, 1,1'-diphenyl-2-picrylhydrazyl (DPPH). Antioxidant properties of eugenol compounds can be explained by forming complexes with reduced metals, and the potent preventive effect of isoeugenol on lipid peroxidation may be related to the decreased formation of perferryl ion or the iron-oxygen chelate complex as the initiating factor of lipid peroxidation by keeping iron at a reduced state.

Eugenol, a methoxyphenol with a short hydrocarbon chain, is found in bay leaves, allspice, and the oil of cloves that originate from the *Syzygium* species[1]. Eugenol has been used as a spice based on its strong odor, and further as a dental antiseptic because of its detergent-like effect[2, 3]. Isoeugenol, found in some vegetables such as monkey orange[4], also acts as an antioxidant[5, 6] and is used as a fragrant food additive. Recently, we reported that capsaicinoids, one of the methoxyphenolic compounds, show antioxidant properties by inhibiting LDL oxidation and lipid peroxidation[7]. Eugenol compounds have a methoxyphenolic structure similar to capsaicinoids, and are thus expected to act as antioxidants. Here we demonstrate that eugenol compounds inhibit iron-mediated lipid peroxidation and copper-dependent LDL oxidation. The antioxidant action of eugenol compounds may be due to the inhibition of perferryl ion formation. We discuss the antioxidant mechanism of eugenol and isoeugenol in relation to their structures with a short hydrocarbon chain attached to the ring.

Materials and Methods

Chemicals. Eugenol, isoeugenol, trolox, tocopherol, capsaicin, human low-density lipoprotein (LDL), bathophenanthroline disulfonate, neocuproine, and 1,1'-diphenyl-2-picrylhydrazyl (DPPH) were the products of Sigma-Aldrich-Japan (Tokyo, Japan).

Lipid peroxidation. Lipid peroxidation was determined as the formation of thiobarbituric acid-reactive substances[8] by an iron/ascorbate method with liver microsomes[9]. The reaction mixture of 1 ml contained 60 mM Tris-HCl buffer (pH 7.5), 10 μM $FeCl_3$, 0.5 mM ascorbic acid, and 0.2 mg microsomal fraction in the presence and absence of eugenol compounds. Lipid peroxides produced were determined after incubation for 10 min.

Autooxidation of Fe^{2+} ion. Interaction of eugenol compounds with iron was evaluated by the effect of these compounds on the rate of autooxidation of Fe^{2+} ion as described previously[9]. The samples of 2 mL contained 10 mM

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Tris-HCl (pH 7.1), 0.1 or 0.06 mM $FeSO_4$ and the additive. The reaction was started by the addition of $FeSO_4$. Aliquots of 0.2 mL were mixed with 0.1 mL of 1 mM bathophenanthroline disulfonate at appropriate intervals, and absorbance at 540 nm was measured.

Oxidation of human low density lipoprotein Oxidation of LDL was determined as described previously[10]. LDL was diluted to the concentration of 60 μg/mL with 10 mM potassium phosphate buffer (pH 7.4) containing 1.5 μM EDTA and 0.15 M NaCl. LDL oxidation was performed at 37℃ in 1 mL of 10 mM phosphate buffer (pH 7.4) containing 2 μM $CuSO_4$, 0.15 M NaCl, and 1 or 1.5 μM eugenol compounds or capsaisin. The progress of oxidation was monitored spectrophotometrically (Shimadzu UV1600, equipped with Peltier-thermostatted six-cell holder) by the formation of conjugated dienes at 234 nm[10].

Reduction of copper ion Copper reduction was followed by determining the cuprous ion concentration with neocuproine[10]. The samples of 0.3 ml contained 10 mM Tris-HCl (pH 7.1), 0.05 mM $CuSO_4$, various concentrations of eugenol compounds, and 0.5 mM neocuproine. The mixture was incubated at room temperature, and the absorbance at 450 nm was recorded.

Scavenging activity of DPPH radical Radical-scavenging activity was determined by the reaction of the stable radical 1,1'-diphenyl-2-picrylhydrazyl (DPPH) with eugenol compounds. Various concentrations of eugenol compounds were mixed with 0.1 mM DPPH in total volume of 1 ml ethanol. Change in the absorbance at 520 nm was recorded.

Results

We examined the effect of eugenol compounds on the microsomal lipid peroxidation. The incubation of microsomes with iron and ascorbic acid produced thiobarbituric acid-reactive substances as a marker of lipid peroxidation. The antioxidant effect was determined as the inhibition of the formation of the thiobarbituric acid-reactive substances by eugenol compounds. Isoeugenol acted as a markedly potent antioxidant, and the inhibitory action on lipid peroxidation was more effective than eugenol and capsaicin, an antioxidant reported previously[7]. The concentrations required for 50% inhibition of the peroxidation were about 10 and 100 μM for isoeugenol and eugenol, respectively (Fig. 1).

Addition of 2 μM copper caused the oxidation of LDL with the concomitant formation of conjugated dienes (Fig. 2). Isoeugenol and eugenol inhibited copper-dependent LDL oxidation by prolonging the lag phase and by suppressing the propagation rate.

Radical-scavenging activity was evaluated by the reaction of eugenol compounds with the stable radical, 1,1'-diphenyl-2-picrylhydrazyl (DPPH). Both compounds reacted with DPPH, and may thus show the activity of scavenging oxygen radical (Fig. 3). However, the reaction of isoeugenol with DPPH was considerably faster than that of eugenol.

Oxidation and reduction of transition metals are closely related to antioxidant and prooxidant action. We then examined the effect of eugenol compounds on the autooxidation of Fe^{2+} ion. Eugenol and isoeugenol did not affect the oxidation of ferrous ion, while quercetin, an antioxidant, stimulated iron oxidation effectively (Fig. 4A). On the other hand, isocitrate-mediated stimulation of Fe^{2+} oxidation was completely inhibited by isoeugenol, whereas the inhibitory effect of eugenol on the isocitrate-mediated iron oxidation was less potent (Fig. 4B). These results indicate that the eugenol compounds, in particular isoeugenol, can keep iron as a reduced form.

The reducing activity of eugenol compounds was analyzed. Eugenol and isoeugenol reduced cupric ion to cuprous ion effectively, and the copper-reducing activity of eugenol compounds was comparable to that of ascorbate,

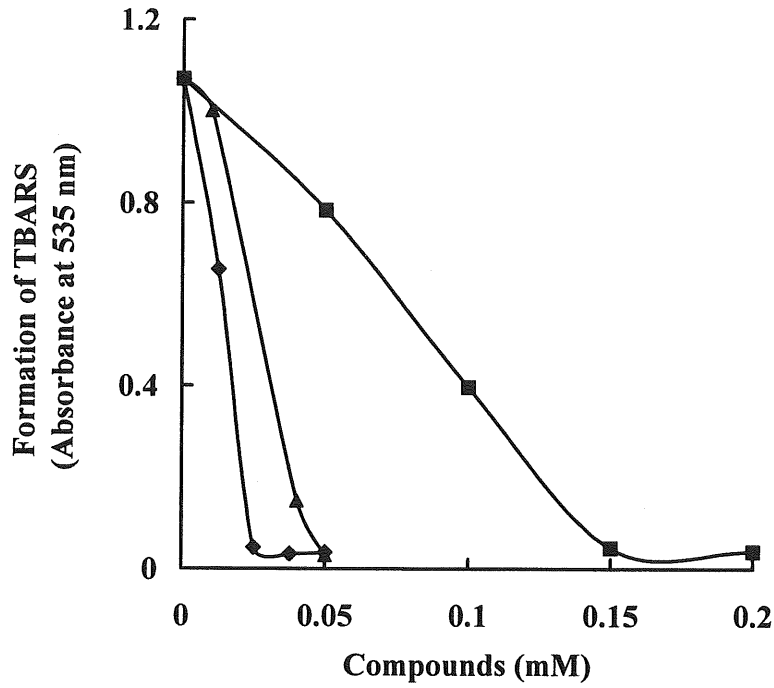

Fig. 1 Effects of eugenol, isoeugenol and capsaicin on the iron-mediated lipid peroxidation of rat microsomes. Lipid peroxidation was induced by 10 μM $FeCl_3$ and 0.5 mM ascorbic acid, microsomal fraction of 0.2 mg protein in the absence and presence of eugenol compounds. The mixture was incubated at 37℃ for 10 min, and the reaction was stopped by addition of 100% trichloroacetic acid. Lipid peroxides produced were determined as the thiobarbituric acid-reactive substances (TBARS)[8]. ■, eugenol; ◆, isoeugenol; ▲, capsaicin.

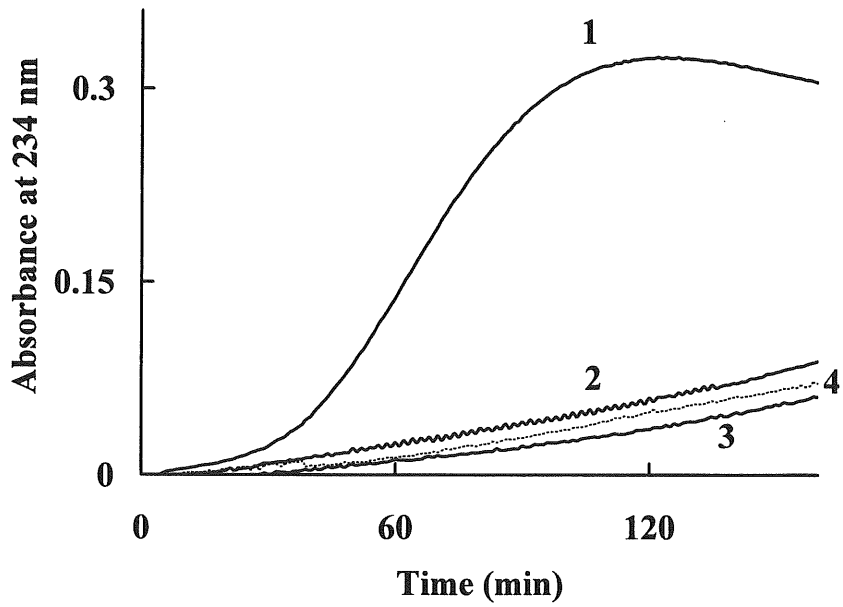

Fig. 2 Effects of eugenol compounds on the copper-dependent oxidation of low density lipoprotein (LDL). LDL of 60 μg/ml containing 1.5 μM EDTA and 0.15 M NaCl was incubated with 2 μM $CuSO_4$, 10mM phosphate buffer (pH 7.5) containing 0.15 M NaCl in the absence and presence of 1.5 μM eugenol or isoeugneol and 1 μM capsaicin in a total volume of 1 ml. The progress of oxidation was monitored spectrophotometrically (Shimadzu UV1600, equipped with Peltier-thermostatted six-cell holder) by the formation of conjugated dienes at 234 nm[10]. Curve 1, none; Curve 2, 1.5 μM eugenol; Curve 3, 1.5 μM isoeugenol; Curve 4, 1 μM capsaicin.

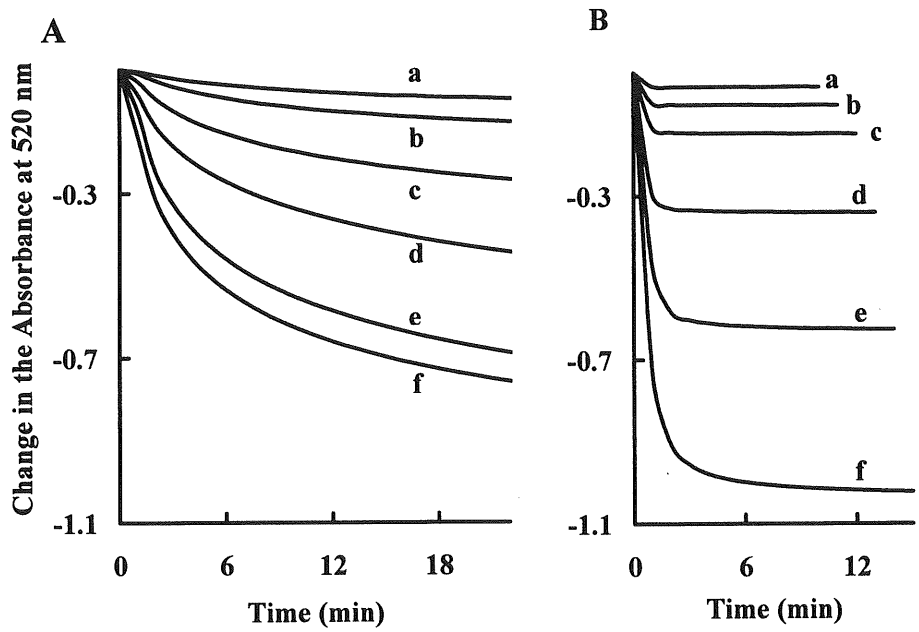

Fig. 3 Scavenging activity of eugenol compounds on the DPPH radical. Various concentrations of eugenol (A) and isoeugenol (B) were mixed with 0.1 mM DPPH in a total volume of 1 ml ethanol. Changes in the absorbance at 520 nm were recorded. Concentrations of eugenol (A) and isoeugenol (B) were as follows: Curve a, 4.7 μM; Curve b, 9.4 μM; Curve c, 18.8 μM; Curve d, 37.5 μM; Curve e, 75 μM; Curve f, 150 μM

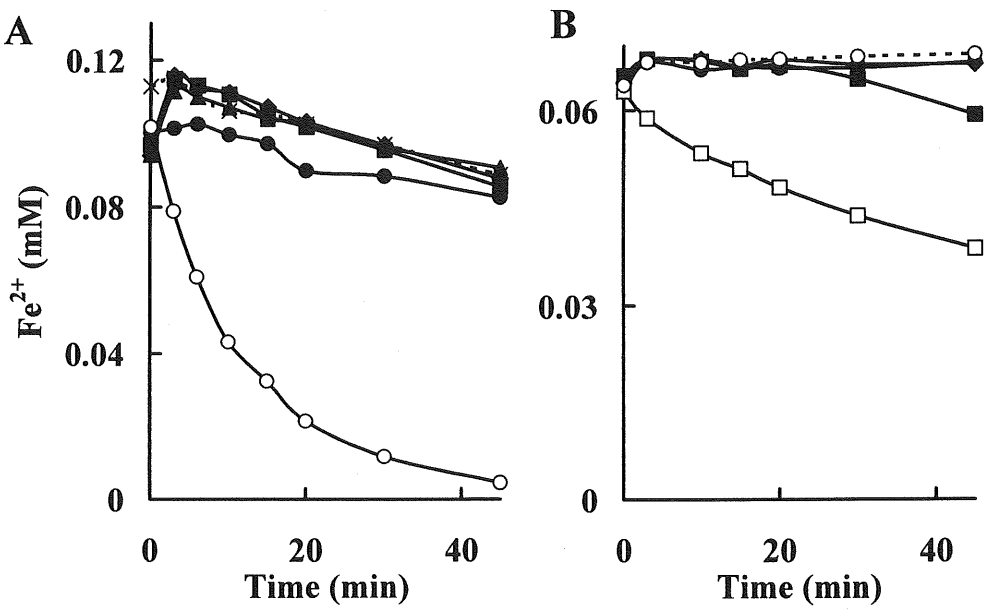

Fig. 4 Effects of eugenol and isoeugenol on the autooxidation of ferrous ion in the absence and presence of isocitrate. Iron autooxidation was followed by determining the ferrous ion concentration according to the bathophenanthroline disulfonate method[9]. Reaction mixture of 2 ml containing 0.1 mM eugenol compounds or other additives in 10 mM Tris-HCl buffer (pH 7.1) was incubated with 0.1 (A) or 0.06 mM $FeSO_4$ (B), in the absence (A) and presence of 0.2 mM isocitrate (B). Aliquots of 0.2 mL were mixed with 0.1 mL of 1 mM bathophenanthroline disulfonate at appropriate intervals, and the absorbance at 540 nm was measured. A. Effects of eugenol and isoeugenol on the Fe^{2+} autooxidation in the absence of isocitrate. ●, no addition; ■, 0.1 mM eugenol; ◆, 0.1 mM isoeugenol; ▲, 0.1 mM capsaicin; ×, butylhydroxyanisole; ○, 0.1 mM quercetin. B. Effects of eugenol and isoeugenol on the Fe^{2+} autooxidation in the presence of isocitrate. ●, no addition; □, 0.2 mM isocitrate added; ■, 0.1 mM eugenol with 0.2 mM isocitrate; ◆, 0.1 mM isoeugenol with 0.2 mM isocitrate; ○, 0.1 mM isocitrate with 0.1 mM ascorbate.

trolox and capsaicin (Fig. 5).

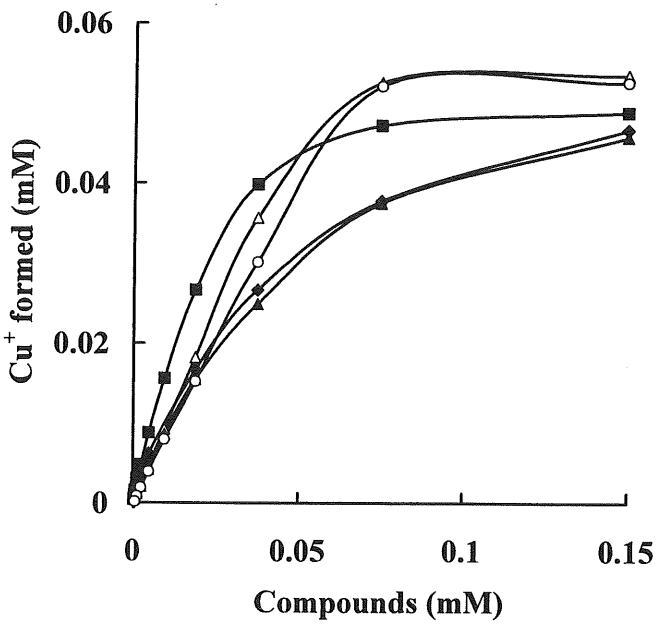

Fig. 5 Effects of eugenol and isoeugenol on the reduction of copper ion. Reaction mixture of 0.3 ml contained 10 mM Tris-HCl (pH 7.1), 0.05 mM $CuSO_4$, various concentrations of eugenol, isoeugenol capsaicin, trolox or ascorbate, and 0.5 mM neocuproine. The mixture was incubated at room temperature, and the absorbance at 450 nm was recorded. ■, eugenol; ◆, isoeugenol; ▲, capsaicin; ○, ascorbate; △, trolox.

Discussion

Reactive oxygen species including superoxide anion radical, hydrogen peroxide and hydroxyl radical are related to various pathological conditions such as ischemia-perfusion injuries, aging and carcinogenesis[11-13]. Superoxide anion is readily produced by one-electron reduction of oxygen *in vivo*, and is dismuted into dioxygen and hydrogen peroxide by enzymatic and nonenzymatic mechanisms[12]. Hydrogen peroxide is further converted to a more reactive hydroxyl radical by the Fenton reaction[14, 15], which requires reduced iron or copper[13]. Reactive oxygen species, in particular the hydroxyl radical, the reactivity of which is highly toxic, attack most molecules found *in vivo*, and several antioxidant mechanisms toward reactive oxygen species are prerequisite[16]. Antioxidant effects can be classified into (a) direct action scavenging reactive oxygen species, and (b) the inhibition of the formation of reactive oxygen species[16].

The present study analyzed the antioxidant properties of eugenol and isoeugenol on the basis of the protective effect on iron-mediated lipid peroxidation. Antioxidant action of eugenol compounds has been explained by its methoxyphenolic structure[17]. However, the protective effect of isoeugenol on the lipid peroxidation of microsomes was 10-fold more potent than that of eugenol (see Fig. 1), although the radical-scavenging activity of isoeugenol was only slightly different from that of eugenol. The initiation phase of lipid peroxidation depends on the perferryl ion[18, 19] and/or ferrous-dioxygen-ferric (Fe[II]-oxygen-Fe[III]) chelate complex[20]. The potent inhibitory effect of isoeugenol on lipid peroxidation may be explained by inhibiting the formation of these iron/oxygen complexes. Effective inhibition by isoeugenol of the isocitrate-mediated enhancement of Fe^{2+} autooxidation results in decreased formation of perferryl ion and ferrous-dioxygen-ferric complex by keeping the iron at a reduced state. The conjugated diene

structure of isoeugenol is suggested to be responsible for binding and reducing iron. However, the weak inhibitory effect of eugenol on lipid peroxidation may be due to only a limited inhibition of the formation of perferryl ion and Fe[II]-oxygen-Fe[III] complex by weak protection of isocitrate-dependent Fe^{2+} autooxidation.

On the other hand, eugenol and isoeugenol inhibited the oxidation of LDL to the same extent. Although the molecular mechanisms underlying LDL oxidation are not completely understood, reduction of transition metals, in particular copper ion, has been claimed to play a key role mediating the oxidative modification of LDL[21]. No difference in copper-reducing activity between isoeugenol and eugenol may explain the same-order protective effect on LDL oxidation.

Eugenol, known as a common antioxidant, is widely used as a flavoring agent in cosmetic and food products[22], and is also utilized as an antiseptic drug in dentistry[2, 3]. Structure-activity relationship studies on eugenol compounds revealed that the side-chain structure in addition to the phenolic ring had an important role in antioxidant function. Isoeugenol may be more useful for application to food additives and some therapeutics.

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