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Original Contribution

Nonallergenic urushiol derivatives inhibit the oxidation of unilamellar vesicles and of rat plasma induced by various radical generators

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ABSTRACT

Urushiols consist of an *o*-dihydroxybenzene (catechol) structure and an alkyl chain of 15 or 17 carbons in the 3-position of a benzene ring and are allergens found in the family Anacardiaceae. We synthesized various veratrole (1,2-dimethoxybenzene)-type and catechol-type urushiol derivatives that contained alkyl chains of various carbon atom lengths, including –H, $-C_1H_3$, $-C_5H_{11}$, $-C_{10}H_{21}$, $-C_{15}H_{31}$, and $-C_{20}H_{41}$, and investigated their contact hypersensitivities and antioxidative activities. 3-Decylcatechol and 3-pentadecylcatechol displayed contact hypersensitivity, but the other compounds did not induce an allergic reaction, when the ears of rats were sensitized by treatment with the compounds every day for 20 days. Catechol-type urushiol derivatives (CTUDs) exerted very high radical-scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl radical and inhibited lipid peroxidation in a methyl linoleate solution induced by 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). However, veratrole-type urushiol derivatives did not scavenge or inhibit lipid peroxidation. CTUDs also acted as effective inhibitors of lipid peroxidation of the egg yolk phosphatidylcholine large unilamellar vesicle (PC LUV) liposome system induced by various radical generators such as AMVN, 2,2′-azobis(2-amidino-propane) dihydrochloride, and copper ions, although their efficiencies differed slightly. In addition, CTUDs suppressed formation of cholesteryl ester hydroperoxides in rat blood plasma induced with copper ions. CTUDs containing more than five carbon atoms in the alkyl chain showed excellent lipophilicity in a *n*-octanol/water partition experiment. These compounds also exhibited high affinities to the liposome membrane using the ultrafiltration method of the PC LUV liposome system. Therefore, CTUDs seem to act as efficient antioxidative compounds against membranous lipid peroxidation owing to their localization in the phospholipid bilayer. These results suggest that nonallergenic CTUDs act as antioxidants to protect against oxidative damage of cellular and subcellular membranes.

Urushiols are major components contained in the sap of the lacquer tree (*Rhus verniciflua* Stokes, Anacardiaceae) [1]. Urushiols consist of *o*-dihydroxybenzene (catechol) coupled with a saturated or unsaturated alkyl side chain of 15 or 17 carbons and are amphipathic compounds [1–3]. These compounds exert various biological effects such as antioxidant [4,5], antimicrobial [6,7], and anticancer [8] activities. However, urushiols cause skin redness, swelling, inflammation, and irritation on contact, which is known as urushiol-induced contact dermatitis [9–11]. Therefore, nonallergenic urushiol derivatives would be useful as a bioactive compound in the human body.

Excessive reactive oxygen species generated in the body cause oxidative damage. In particular, lipid peroxidation has been implicated in various human diseases, including atherosclerosis, cancer, and aging [11–16]. α-Tocopherol and flavonoids (quercetin, luteolin, catechins, etc.) are distributed widely in food, including cereals, vegetables, and fruits, and are excellent antioxidants in humans [17–20]. In addition, the catechol structure of various phenolic compounds including flavonoids is important as an active site for their antioxidative activity [21,22]. The lipophilicity and localization of antioxidants in biological systems are generally considered important to understanding the biological activities of these compounds on cellular and intracellular membranes. Natural urushiols possessing a catechol structure and an alkyl side chain of 15 or 17 carbons are amphipathic compounds; therefore, attention has been focused on the inhibitory effects of these compounds on biomembrane peroxidation. However, the urushiols have a fatal

Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); AsA, ascorbic acid; *n*-BuLi, *n*-butyllithium; CE-OOH, cholesteryl ester hydroperoxide; CTUD, catechol-type urushiol derivative; DPPH, 1,1-diphenyl-2-picrylhydrazyl; PC LUV, phosphatidylcholine large unilamellar vesicle; MeL-OOH, methyl linoleate hydroperoxide; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; α-Toc, α-tocopherol; VTUD, veratrol-type urushiol derivative

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defect, as they induce strongly allergic reactions on contact, although natural urushiols act as excellent antioxidants. Therefore, the functions of urushiol are desirable but unattainable.

In this study, we chemically synthesized veratrole- and catechol-type urushiol derivatives (compounds **1–12**) that contain alkyl side chains of different lengths. The urushiol derivatives (**1–12**) were subjected to a contact hypersensitivity evaluation using rats. We also measured radical-scavenging activity of the urushiol derivatives (**1–12**) using the 1,1-diphenyl-2-picrylhydrazyl (DPPH)[2] radical and methyl linoleate system. In addition, the inhibitory effects of the urushiol derivatives (**1–12**) against lipid peroxidation on phosphatidylcholine large unilamellar vesicles (PC LUV) and copper ion-induced rat blood plasma were investigated.

Materials and methods

General experimental procedures

Nuclear magnetic resonance (NMR) spectral data were measured with Varian ^{Unit}INOVA 300 and 500 (Varian, Walnut Creek, CA, USA) spectrometers using tetramethylsilane in $CDCl_3$ as the internal standard. Mass spectral data were obtained by electrospray ionization mass spectrometry (API 3200Q trap, Applied Biosystems, Foster City, CA, USA) under the following conditions: ion source temperature, 0 °C; electron voltage of positive and negative mode, 5000 and −4500 V, respectively. Column chromatography was performed with silica gel (Kieselgel 60, 70–230 mesh; Merck, Darmstadt, Germany) resin. The high-performance liquid chromatography (HPLC) analysis was performed on a Shimadzu LC-6AD with a SPD-M20A detector and silica gel (4.6 × 250 mm, TSK-gel, Silica-60, Tosoh Co., Tokyo, Japan), LiChroprep Lobar (RP-8, 40–63 μm, 25 × 310 mm, Merck), and Octyl-80Ts (4.6 × 150 mm, TSK-gel, Tosoh Co.) columns. Thin-layer chromatography was performed on silica gel 60 F_{254} (0.25 mm thickness, Merck).

Chemicals

Veratrole (1,2-dimethoxybenzene, **1**), 3-methylveratrole (**2**), 3-methylcatechol (**8**), 1-bromopentane, 1-bromodecane, 1-bromopentadecane, 1-bromoeicosane, butyllithium (1.6 M solution in *n*-hexane), boron tribromide (BBr_3; 1.0 M solution in *n*-hexane), 2,6-di-*tert*-butyl-4-methylphenol (BHT), 70% perchloric acid ($HClO_4$), diethylenetriaminepentaacetic acid (DTPA), and methyl linoleate were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Pyrocatechol (catechol, **7**) was purchased from Kanto Chemical Co. (Tokyo, Japan). *n*-Octanol was obtained from Samchun Pure Chemical Co. (Pyeongtaek, Korea). DPPH, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), ascorbic acid (AsA), and egg yolk 3-*sn*-phosphatidylcholine were obtained from Wako (Osaka, Japan). (±)-α-Tocopherol (α-Toc) was purchased from Fluka (Buchs SG, Lucerne, Switzerland). All other chemicals and solvents were of analytical grade.

Synthesis of veratrole-type urushiol derivatives

Synthesis of veratrole-type urushiol derivatives (VTUDs) was carried out according to the method of Niimura et al. [23] and Satoh et al. [24]. Exactly 3.82 ml veratrole (0.03 mol) was added to a solution (130 ml) of dry tetrahydrofuran (THF) and the mixture was stirred for 30 min at 0 °C. A solution of 28.2 ml *n*-BuLi (1.6 M solution in *n*-hexane, 0.045 mol) in 5 ml THF was slowly added to the mixture and then stirred successively for 1 h at 0 °C and for 1 h at room temperature. A solution (5.56 ml) of 1-bromoalkanes (0.045 mol), with different carbon chain lengths, was slowly poured into the solution and then refluxed for 5 h at 210 °C. The resulting mixture was added to a saturated NH_4Cl solution (150 ml, twice) and partitioned with ethyl acetate (EtOAc; 200 ml, twice). The organic layer was washed with brine (200 ml, twice) and added to anhydrous K_2CO_3. After filtration, the organic layer was evaporated in vacuo at 35 °C. The concentrates was purified by silica gel (100 g, 2.3 × 67 cm) column chromatography eluting with toluene to give the VTUDs. Four VTUDs (**3, 4, 5,** and **6**) were synthesized using 1-brompentane, 1-bromodecane, 1-bromopentadecane, and 1-bromoeicosane by the same procedure described above.

Synthesis of catechol-type urushiol derivatives

VTUDs (**3**–**6**, 2.85 g, 13.68 mmol), with different side chain lengths, were dissolved respectively with 29.4 ml dry methylene chloride (CH_2Cl_2) at 0 °C. The sample solution was added to a solution (19.38 ml) of BBr_3 (1.0 M solution in hexane, 19.38 mmol) in dry CH_2Cl_2, and the mixture was stirred successively for 2 h at 0 °C and then for 12 h at room temperature. Each solution was partitioned with H_2O to give the CH_2Cl_2 layer. The aqueous layer was partitioned with CH_2Cl_2 (60 ml, twice). The combined organic layer was washed with brine (200 ml, twice) and added to anhydrous K_2CO_3. After filtration, the organic layer was evaporated in vacuo at 35 °C. The crude products were purified by silica gel (Kieselgel 60, Merck, 2.3 × 59 cm, benzene/EtOAc 6/1, v/v) column chromatography to give catechol-type urushiol derivatives (CTUDs). 3-Pentylcatechol (**9**), 3-decylcatechol (**10**), 3-pentadecylcatechol (**11**), and 3-eicosylcatechol (**12**) were synthesized using 3-pentylveratrole (**3**), 3-decylveratrole (**4**), 3-pentadecylveratrole (**5**), and 3-eicosylveratrole (**6**) by the same procedure described above.

Induction of contact hypersensitivity by the urushiol derivatives on rat ears

Sprague–Dawley rats (male, 6 weeks of age, 180–200 g) were obtained from Samtako Bio Korea (Osan, Korea). The rats were housed under controlled humidity (55 ± 5%), room temperature (25 ± 1 °C), and light cycle (12 h light/12 h dark). Food and water were available ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (CNU IACUC-YB-R-2012-26). All rats were acclimated for 1 week with a standard rodent diet (Harlan Rodent diet, 2018S) before experiments. An ethanol (EtOH) solution (3 μmol/50 μl) of one of the VTUDs or CTUDs (**1–12**) was applied to the rear (1 cm^2) of the left ear of the rats ($n = 6$) daily for 20 days. The erythema visualized on the rat ears treated with the VTUDs and CTUDs was reflective of contact hypersensitivity.

Determination of hematological biomarkers in the blood of rats treated with the urushiol derivatives

After the VTUD and CTUD treatments for 20 days, rats ($n = 6$) were anesthetized with diethyl ether, the abdominal wall was opened, and blood was collected from the abdominal aorta into glass tubes. The numbers of white blood cell, neutrophil, and eosinophil in the whole blood were determined by Veterinary Multi-species Hematology System (Hemavet 850, CDC Technologies Inc., Oxford, MI, USA). Blood serum was obtained by centrifugation (1500 *g*) at 4 °C for 20 min and stored at −70 °C until analysis. Serum IgE and histamine levels were measured using Rat IgE ELISA kit (Komabiotec, Seoul, Korea) and histamine level was measured using a histamine enzyme-linked immunosorbent assay kit (Oxford Biomedical Research, Rochester Hills, MI, USA) according to the manufacturer's instructions.

Determination of DPPH radical-scavenging activity

Antioxidant activity of urushiol derivatives was evaluated using the DPPH radical [25]. In brief, VTUDs (**1–6**) were prepared at final concentrations of 50, 100, and 250 μM in ethanol. CTUDs (**7–12**) were also prepared at final concentrations of 10, 50, 100, 150, 200, and 250 μM in ethanol. The ethanol solution (0.5 ml) of each compound at the various concentrations was added to the DPPH radical ethanol solution (2.0 ml, final concentration, 250 μM). The mixture was incubated at room temperature for 30 min in the dark. The free radical-scavenging activity of the urushiol derivatives was evaluated by decolorization at 517 nm, and activity was also determined as the percentage decrease in absorbance as shown by a blank test.

Measurement of peroxyl radical-scavenging activity in AMVN-induced methyl linoleate peroxidation

Peroxyl radical-scavenging activity of the urushiol derivatives (**1–12**) and α-Toc was evaluated by the inhibition of AMVN-induced methyl linoleate peroxidation [26,27]. Purified methyl linoleate (120 mM, final concentration) and each compound (40 μM, final concentration) were mixed with a solution (1.0 ml, final volume) of *n*-hexane/2-propanol/EtOH 8/3/0.1 (v/v). After a 37 °C preincubation for 10 min in the dark, a solution of AMVN (10 mM, final concentration) in *n*-hexane/2-propanol/EtOH 8/3/0.1 (v/v) was added to the mixture and incubated in the dark at 37 °C with continuous shaking. During the incubation, the samples were collected at intervals of 0, 20, 40, 60, 80, 100, 120, 140, and 160 min. A 10-μl aliquot of sample was subjected to HPLC using a silica gel column (4.6 × 250 mm, TSK-gel). The mobile phase was a mixture of *n*-hexane/2-propanol/EtOH 10/0.2/0.1 (v/v), and flow rate was 1.0 ml/min. Methyl linoleate hydroperoxide (MeL-OOH) was monitored at 235 nm (SPD 10A, Shimadzu Co., Kyoto, Japan). Calibration curves were constructed using external standard solutions (0.1–20 nmol in *n*-hexane) of MeL-OOH prepared from methyl linoleate by autoxidation and purification.

Calculation of the urushiol derivative kinetic parameters for AMVN-induced methyl linoleate peroxidation

The kinetic parameters of the antioxidative reaction of the CTUDs (**7–12**) and α-Toc against AMVN-induced methyl linoleate hydroperoxide were measured [28]. The oxidation rate during the induction period (R_{inh}), the oxidation rate after the induction period, and the rate of propagation (R_p) were obtained from the slope of the curve of methyl linoleate hydroperoxide formation. The apparent induction period (t_{inh}) was calculated from the intersecting point of the curves for R_{inh} and R'_p of the urushiol derivatives [29].

Quantitative analysis of the urushiol derivatives

The contents of the urushiol derivatives (**1–12**) and AsA and α-Toc in the filtrate after ultrafiltration of PC LUVs were measured by HPLC equipped with an Octyl-80Ts column (Tosoh Co.). The mobile phases for separating the urushiol derivatives (**1–12**) were solvent mixtures of MeOH/H_2O: **1,** MeOH/H_2O 40/60 (v/v); **2,** MeOH/H_2O 50/50 (v/v); **3,** MeOH/H_2O 74/26 (v/v); **4,** MeOH/H_2O 86/14 (v/v); **5,** MeOH/H_2O 92/8 (v/v); **6,** MeOH/H_2O 96/4 (v/v); **7,** MeOH/H_2O 14/86 (v/v); **8,** MeOH/H_2O 30/70 (v/v); **9,** MeOH/H_2O 63/27 (v/v); **10,** MeOH/H_2O 80/20 (v/v); **11,** MeOH/H_2O 92/8 (v/v); **12,** MeOH/H_2O 96/4 (v/v); α-Toc, MeOH/H_2O 93/7 (v/v). Flow rate was 1.0 ml/min, and the compounds were monitored at 240 nm (SPD-20A, Shimadzu). Standard solutions of urushiol derivatives in MeOH were prepared at concentrations of 0–150 nmol. Calibration curves were constructed by plotting the peak area vs the concentration of each compound. The content of urushiol derivatives was quantified in triplicate experiments.

Preparation of egg yolk phosphatidylcholine (EYPC)

EYPC was prepared according the method of Terao et al. [30], with a slight modification. Briefly, commercial EYPC reagent was purified by LiChroprep Lobar (RP-8, 40–63 μm, 25 × 310 mm, Merck) column chromatography eluted with $CHCl_3$/MeOH/H_2O 1/10/0.5 (v/v). EYPC content in the EYPC-containing fraction was quantified at 830 nm by spectrophotometer using 2,4-diaminophenol dihydrochloride. The solvent was removed with a stream of nitrogen gas followed by evaporation under vacuum. The EYPC concentrate was stored at −40 °C until use.

Determination of the inhibitory effects of the urushiol derivatives against AMVN, AAPH, and copper-induced lipid peroxidation of PC LUV

The ethanol solutions of CTUDs (**7–12**, final concentration, 25 μM) and α-Toc (final concentration, 25 μM), a solution of the purified PC LUVs (final concentration 5 mM) in $CHCl_3$/MeOH (95/5, v/v), and a solution of AMVN (final concentration, 1 mM) in *n*-hexane were placed in a test tube. After the solvent was evaporated, the residue was dispersed in 1 ml of 0.01 M Tris–HCl buffer (pH 7.4) containing 0.5 mM DTPA. The PC LUV suspension was obtained by the same ultrasonication and membrane procedures (pore size 100 nm; 21 times). The PC LUV suspension was diluted with 1 ml of 0.01 M Tris–HCl buffer (pH 7.4) containing 0.5 mM DTPA and incubated at 37 °C for 270 min in the dark with continuous shaking. PC-OOH content in the reaction mixture was determined under the same HPLC conditions described below.

To determine the inhibitory effects of CTUDs against AAPH- and copper ion-induced oxidation in PC LUVs, a solution of AAPH (final concentration, 10 mM) and $Cu(NO_3)_2$ (final concentration, 0.05 mM) in 0.01 M Tris–HCl buffer (pH 7.4) containing 0.5 mM DTPA was added to the PC LUV suspension, which was preincubated at 37 °C for 5 min in the dark with continuous shaking after the ultrasonication and membrane procedures. Other conditions were the same as those for AMVN.

Quantitative analysis of PC-OOH during oxidation of the LUV solution induced by initiators

PC-OOH content was determined according to the method described by Shirai et al. [31]. Briefly, aliquots were subjected to ODS-HPLC using a TSK-gel Octyl-80Ts column (Tosoh). The mobile phase consisted of MeOH/H_2O 90/0 (v/v), and the flow rate was constant at 1.0 ml/min. The eluate was monitored by UV detection at 235 nm (Shimadzu SPD-10A). PC-OOH concentration was calculated from a standard curve of PC-OOH. Detailed procedures for preparing the PC-OOH standards have been published previously [30].

Measurement of the partition coefficient in the n-octanol/H_2O mixture

The partition coefficients of the urushiol derivatives (**1–12**), AsA, and α-Toc were determined using an *n*-octanol/H_2O system [32]. A 100-nmol aliquot of each compound in MeOH and the antioxidants (α-tocopherol and AsA, each 100 nmol) was placed in a test tube. After the solvent was removed with nitrogen gas, *n*-octanol (100 μl) and 50 mM Tris–HCl buffer (pH 7.4, 100 μl) were dispersed into the test tube. After the suspension was vigorously mixed for 30 s, it was centrifuged at 6000 rpm for

5 min at 4 °C to give the octanol and Tris–HCl buffer phases. The content of urushiol derivatives in the two phases was determined by ODS-HPLC analysis as described above.

Ultrafiltration of PC LUVs

The ethanol solution of CTUDs (**7–12,** final concentration, 25 μM) and α-Toc (final concentration, 25 μM) and a solution of the purified PC LUVs (final concentration, 5 mM) in $CHCl_3$/MeOH (95/5, v/v) were placed in a test tube, and the solvent was removed with nitrogen gas followed by evaporation in vacuo for 30 min. The residue was dispersed in 400 μl of 0.01 M Tris–HCl buffer (pH 7.4). The suspension was mixed using a vortex mixer for 30 s followed by sonication for 30 s. The suspension was passed through a polycarbonate membrane (pore size 100 nm) 21 times to give the LUV suspension [33]. The filtrate was centrifuged at 12,000 rpm for 40 min at 4 °C [34]. The supernatant was evaporated under a stream of nitrogen gas, and the concentrate was dissolved with ethanol (100 μl). The concentration of CTUDs in the LUV phase was determined by ODS-HPLC analysis as described above.

Determination of the inhibitory effects of CTUDs against copper ion-induced oxidation in rat blood plasma

The antioxidative activities of the CTUDs (**7–12**) were evaluated by measuring their inhibitory effects against cholesteryl ester hydroperoxide (CE-OOH) formation in copper ion-induced oxidation of rat blood plasma [26]. Sprague–Dawley rats (male, 6 weeks of age, 180–200 g) were obtained from Samtako Bio Korea. The rats were kept at 20 ± 2 °C under a 12-h light/dark cycle and fasted for 15 h before blood collection. After diethyl ether anesthesia, blood was collected from the abdominal aorta into heparinized tubes. Rat plasma was isolated by centrifugation (3000 *g*) at 4 °C for 20 min and stored at −40 °C. Blood plasma was diluted fourfold with phosphate-buffered saline (PBS; pH 7.4). The diluted plasma (650 μl) was added to 20 μl ethanol solution of the CTUDs (10 μM) and 100 μl of $CuSO_4$–PBS solution (final concentration, 100 μM). The mixture was incubated at 37 °C for 16 h with continuous shaking. The CE-OOH concentration was determined according to the method of Arai et al. [35]. Briefly, 100-μl aliquots were withdrawn from the incubating solutions and mixed with 3 ml MeOH containing 2.5 mM BHT. The mixture was sonicated for 1 min, and then partitioned with 3 ml *n*-hexane by vortexing vigorously for 1 min. The upper layer (*n*-hexane) was collected and extraction of the lower layer was repeated with 3 ml of *n*-hexane. The combined *n*-hexane phases were evaporated in a rotary evaporator at room temperature. The remaining lipids were dissolved in 100 μl MeOH/$CHCl_3$ (95/5, v/v), and aliquots were subjected to RP-HPLC using a TSK-gel Octyl-80TS column (Tosoh) to determine CE-OOH content. The effluent was monitored by UV detection at 235 nm (Shimadzu SPD-10A). MeOH/H_2O (97/3, v/v) served as the mobile phase, and the flow rate was constant at 1.0 ml/min. CE-OOH concentration was calculated from a standard curve of CE-OOH. Detailed procedures for preparing the CE-OOH standard have been published previously [35].

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) using the Statistical Package for Social Sciences (SPSS, IBM, Armonk, NY, USA) 19.0 package program. Statistical differences were measured by one-way analysis of variance followed by Duncan's multiple comparison test. A $p < 0.05$ was considered significant.

Results

Synthesis of VTUDs and CTUDs

VTUDs (**3–6**) were synthesized by lithiation of *n*-BuLi and alkylation of 1-bromoalkanes, which have different carbon atom lengths of C_5, C_{10}, C_{15}, and C_{20}. The crude VTUD products were purified by silica gel column chromatography (toluene). Yields of **3, 4, 5,** and **6** were 62.2, 82.4, 99.7, and 59.3%, respectively, and purities of all compounds were above 99%. The structures of **3, 4, 5,** and **6** were determined based on one-dimensional (1D) and two-dimensional (2D) NMR and mass spectroscopic (MS) data (Supplementary Data 1, Fig. 1).

CTUDs (**9–12**) were synthesized from the VTUDs (**3–6**) by demethylation with BBr_3. The crude CTUD products were purified by silica gel column chromatography using a mobile phase of benzene/EtOAc 6/1 (v/v). The yields of **9, 10, 11,** and **12** were 78.3, 93.0, 80.3, and 90.5%, respectively, and purities of all compounds were above 99%. The structures of **9, 10, 11,** and **12** were determined, based on 1D and 2D NMR and MS data (Supplementary Data 2, Fig. 1).

*Contact hypersensitivity of urushiol derivatives (**1–12**) on rat ears*

Rats were sensitized on the rear (1 cm^2) of their left ear with an EtOH solution (3 μmol/50 μl) of VTUDs and CTUDs (**1–12**) every day for 20 days. The clinical features are shown in Fig. 2. Severe erythema and swelling were observed on the ear skin of rats treated with **10** and **11,** which are catechol-type compounds consisting of $-C_{10}H_{21}$ and $-C_{15}H_{31}$ in the alkyl side chains, respectively. In contrast, erythema and swelling symptoms were not observed on ears of rats treated with the other CTUDs (**7–9, 12**), which contain shorter or longer side chains than those of **10** and **11.** In addition, VTUDs (**1–6**) with an *o*-dimethoxyl-substituted catechol moiety did not induce any contact hypersensitivity regardless of side-chain length.

Effects of CTUDs on hypersensitivity-related factors in blood

Contact hypersensitivity-related biomarkers in blood of rats treated with a veratrol-type compound, 3-pentadecylveratrole (**5**), and four CTUDs (**9–12**) daily for 20 days were determined. The numbers of white blood cells in rats treated with **5** and **9–12** were similar to that of the control with no different significance (Fig. 3A). Treatment with compounds **10** and **11** increased the numbers of neutrophils compared to that of the control ($p < 0.05$) (Fig. 3B). The numbers of neutrophils in rats treated with **5, 9,** and **12** were lower than that of the control ($p < 0.05$). These neutrophil number patterns were similar to the eosinophil number results, although the numbers did not differ significantly (Fig. 3C). In addition, serum IgE (Fig. 3D) and histamine (Fig. 3E) levels in rats treated with **10** and **11** were higher than those of other compounds (**5, 9, 12**) and the control ($p < 0.05$). The serum IgE levels of rats treated with **5, 9,** and **12** were similar to that of the control with no significant difference. The serum histamine levels of rats treated with **5** and **9** were significantly lower than those treated with **10** and **11** but were not different from the control, although the serum histamine levels of **5** and **9** were slightly higher than that of the control group. The patterns for the numbers of neutrophils and eosinophils and the levels of serum IgE and histamine agreed with the results of the contact hypersensitivity experiment.

DPPH radical-scavenging activity of the urushiol derivatives

The radical-scavenging activities of VTUDs and CTUDs (**1–12**) and α-Toc as a positive control were evaluated using DPPH. As shown in

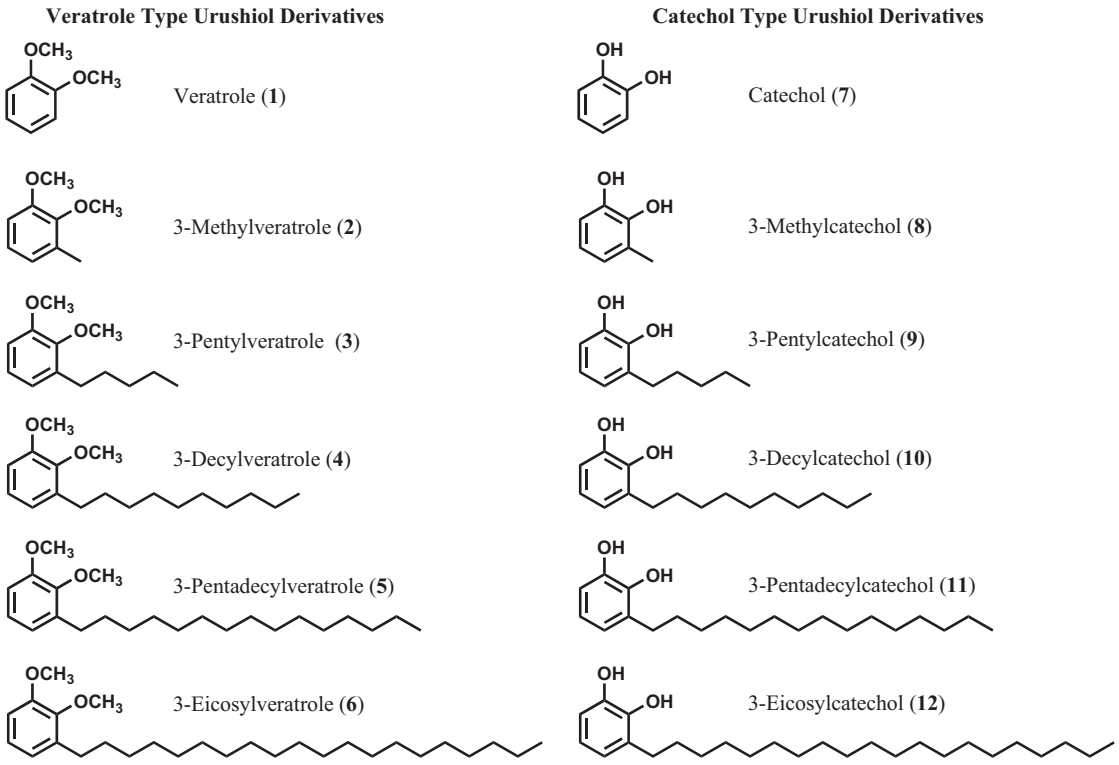

Fig. 1. Structures of synthetic veratrole-type and catechol-type urushiol derivatives (**1–12**).

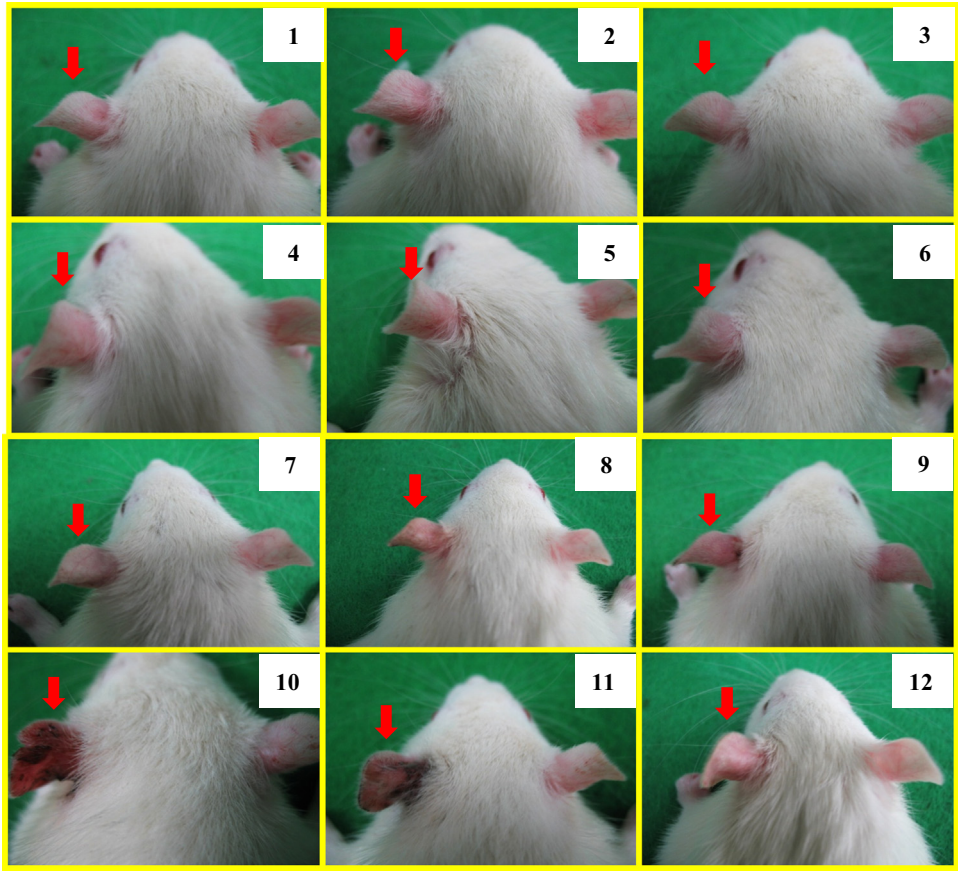

Fig. 2. Contact hypersensitivity effects of the urushiol derivatives (**1–12**) induced after treatment of rat ears for 20 days. Each group consisted of six rats that were treated with one of the urushiol derivatives.

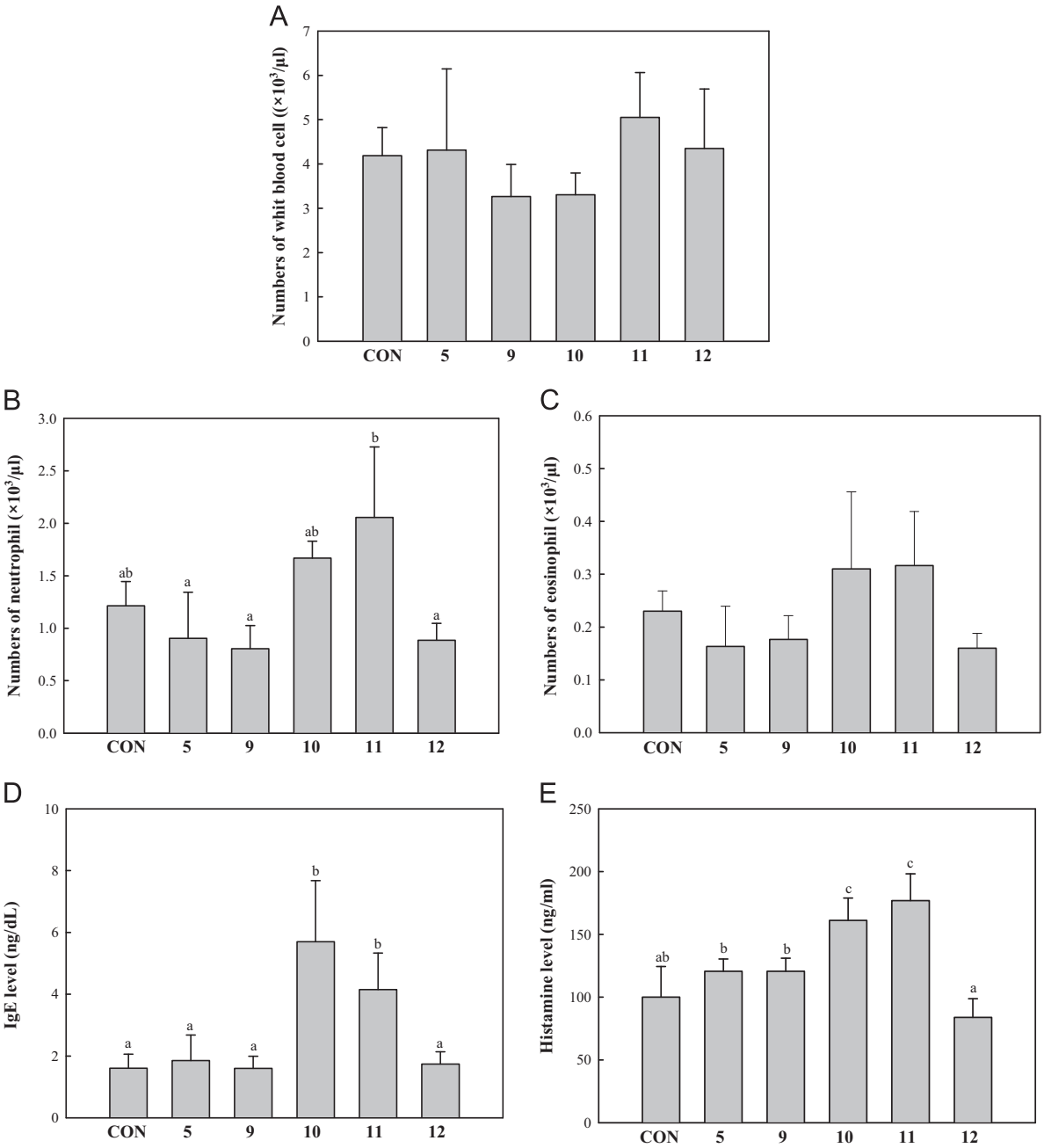

Fig. 3. Numbers of (A) white blood cells, (B) neutrophils, and (C) eosinophils and (D) IgE and (E) histamine content in the blood obtained after treatment with urushiol derivatives (**5, 9–12**) of rat ears for 20 days. Each value is the mean ± SD ($n = 6$). CON: control.

Supplementary Fig. S1, CTUDs (**7–12**) showed predominantly higher DPPH radical-scavenging activity than VTUDs (**1–6**), which did not exhibit DPPH radical-scavenging activity regardless of concentration. In addition, the DPPH radical-scavenging activities of CTUDs (**7–12**) were very similar regardless of carbon atom side-chain length. Moreover, the number of DPPH radical molecules scavenged by one molecule of CTUDs was calculated by assuming that one molecule of α-Toc scavenges two molecules of DPPH. One molecule of each CTUD trapped two molecules of free radicals corresponding to the number scavenged by α-Toc.

Peroxyl radical-scavenging activity of urushiol derivatives against AMVN-induced methyl linoleate peroxidation

The peroxyl radical-scavenging activities of urushiol derivatives were evaluated by measuring inhibition against methyl linoleate peroxidation induced by AMVN, a lipophilic radical generator, in a solution of *n*-hexane/2-propanol/EtOH 8/3/0.1, v/v). VTUDs (**1–6**) at a concentration of 40 μM did not inhibit AMVN-induced oxidation of methyl linoleate (Supplementary Fig. S2A) and showed a pro-oxidant effect compared with the control. In contrast, the CTUDs (**7–12**) strongly inhibited AMVN-induced oxidation of methyl linoleate (Supplementary Fig. S2B). Interestingly, the CTUDs (**7–12**) showed slightly higher peroxyl radical-scavenging activity in comparison to α-Toc and their peroxyl radical-scavenging activities were very similar regardless of the carbon atom side-chain length.

The kinetic parameters of CTUDs (**7–12**) were calculated by the method of Niki et al. [28]. All CTUDs showed a slightly lower induction period ($t_{inh} = 3.73–3.97$) and a slightly higher oxidation rate during the induction period ($R_{inh} = 0.13–0.16$) compared to α-Toc ($t_{inh} = 3.66$, $R_{inh} = 0.17$) (Table 1). The stoichiometric factors

Table 1
Kinetic parameters for the antioxidative reaction of urushiol derivatives against AMVN-initiated oxidation of methyl linoleate.

Catechol-type urushiol derivative	R_p	R'_p	R_{inh}	t_{inh}	k_{inh}/k_p	R_i	Kinetic chain length	Oxidizability	n
α-Toc	1.03	0.98	0.17	3.66	1.61	2.20	4.68	6.94	2.0
Catechol (**7**)	1.03	0.98	0.16	3.73	1.67	2.20	4.68	6.94	2.1
3-Methylcatechol (**8**)	1.03	0.81	0.13	3.96	1.94	2.20	4.68	6.94	2.2
3-Pentylcatechol (**9**)	1.03	0.85	0.16	3.84	1.62	2.20	4.68	6.94	2.1
3-Decylcatechol (**10**)	1.03	0.80	0.13	3.97	1.93	2.20	4.68	6.94	2.2
3-Pentadecylcatechol (**11**)	1.03	0.81	0.16	3.85	1.62	2.20	4.68	6.94	2.1
3-Eicosylcatechol (**12**)	1.03	0.81	0.16	3.83	1.63	2.20	4.68	6.94	2.1

R_p, 10^{-7} M s^{-1}; R'_p, 10^{-7} M s^{-1}; R_{inh}, 10^{-7} M s^{-1}; t_{inh}, 10^3 s; k_{inh}/k_p, 10^3; R_i, 10^{-8} M s^{-1}; kinetic chain length, R_p/R_i; oxidizability, $10^{-3}(M\ s)^{-1/2}$.

(n) of the CTUDs were 2.1–2.2, which were slightly higher compared with that (n = 2.0) of α-Toc. The efficiencies (k_{inh}/k_p) of peroxyl radical scavenging (the ratio of t_{inh}/rate of chain propagation (k_p)) for CTUDs (**7–12**) were 1620–1940, which was very similar to that of α-Toc (k_{inh}/k_p = 1610). The kinetic parameters of the CTUDs (**7–12**) were not influenced by carbon atom side-chain length. Consequently, it was concluded that the lipid peroxyl radical-scavenging capacity was the same and/or slightly higher in magnitude in comparison to that of α-Toc.

Inhibitory effects of CTUDs against AMVN-induced lipid peroxidation of PC LUVs

The antioxidative activities of the CTUDs (**7–12**) and α-Toc as a positive control were evaluated by measuring the inhibition of PC-OOH formation on lipid peroxidation of PC LUVs that were exposed to AMVN, a lipid-soluble radical generator. As shown in Fig. 4A, all CTUDs more efficiently inhibited PC-OOH formation than the control in this reaction system. The inhibitory effects of **9–12,** which contained more than five carbons in the alkyl side chain, were very similar regardless of carbon length of the side chains and were higher than those of **7** and **8.** In addition, **9–12** showed slightly higher inhibitory effects in comparison with α-Toc, which was used as a positive control and as a representative lipophilic antioxidative vitamin. However, the antioxidative activities of **7** and **8** were weaker compared to that of α-Toc.

Inhibitory effects of CTUDs against AAPH-induced lipid peroxidation of PC LUVs

We also evaluated the inhibitory effects of CTUDs (**7–12**) against PC-OOH production from PC-LUVs induced by AAPH, a water-soluble radical generator. All CTUDs (**7–12**) exerted a greater inhibitory effect than the control against lipid peroxidation of PC LUVs generated in the aqueous phase (Fig. 4B) as well as in the lipid phase (AMVN) (Fig. 4A). The inhibitory effects of **9**–**12** were very similar regardless of the carbon length of their side chains. In addition, compounds **7** and **8** also showed greater inhibitory effects than α-Toc, different from AMVN-induced oxidation (Fig. 4A), but their inhibitory effects were weaker than those of **9–12** in this oxidation system.

Inhibitory effects of CTUDs against copper ion-induced lipid peroxidation of PC LUVs

The antioxidative activities of CTUDs (**7–12**) and α-Toc against peroxidation of PC LUVs induced by copper ions showed a very similar pattern to that of AAPH-induced PC LUV oxidation. That is, all CTUDs had greater inhibitory effects than α-Toc. In addition, compounds **9–12** exhibited more efficient antioxidative activities than **7** and **8.** Moreover, the activities of **9–12** were very similar regardless of side-chain length (Fig. 4C).

Behavior of urushiol derivatives in an n-octanol/water partition system

The urushiol derivatives (**1–12**), AsA, and α-Toc were respectively partitioned in an *n*-octanol/water system to determine their relative lipophilicities. Their contents were quantified in two phases by ODS-HPLC analysis. As shown in Fig. 5, most of the AsA was detected in the water phase, whereas most of the α-Toc was detected in the *n*-octanol phase. Similar to α-Toc, the urushiol derivatives (**1–12**) were also mostly detected in the *n*-octanol phase, although they showed some differences in partition coefficients. The VTUD (**1–6**) contents in the *n*-octanol phase were not statistically significant regardless of the side-chain carbon atom length. However, the CTUD (**7**–**12**) contents in the *n*-octanol phase, except for **12,** which had an alkyl side chain of $C_{20}H_{41}$, increased proportionally with the increase in length of their side chains. In addition, compounds **7–9** showed relatively lower contents in the *n*-octanol phase than other CTUDs (**10–12**). α-Toc and all of the urushiol derivatives (**1–12**) were not detected or were detected in very small amounts in the water phase. Therefore, all urushiol derivatives (**1–12**) used in this study seemed to have lipophilic properties similar to α-Toc rather than hydrophilic properties, although **7**–**9** have slightly less lipophilic properties than others (**1–6, 10–12**).

Localization of the urushiol derivatives using PC-LUV system ultrafiltration

PC LUVs were prepared with each CTUD and α-Toc as a control to determine the affinity of the CTUDs (**7–12**) to phospholipid membranes. Each PC LUV suspension was filtered through an ultrafiltration membrane (pore size, 10 nm) and the contents of each compound in the filtrate were measured. That is, if a compound presented as a small amount in the filtrate, it suggests that the compound has a very high affinity for phospholipid membranes. CTUDs (**7–12**) and α-Toc were detected in the filtrate in small amounts, indicating that the CTUDs possess a high affinity for PC LUVs, similar to α-Toc (Fig. 6). In addition, the affinity of PC LUVs for the CTUDs showed proportional increases with the length of the alkyl side chain. That is, compounds **11** and **12** with >15 carbon atoms in the alkyl side chain had the highest affinity for PC LUVs. The filtrate contents of **9** and **10,** with 5 and 10 carbon atoms in the alkyl side chain, were lower than those of **11** and **12.** Compounds **7** and **8** also showed considerable affinities for PC LUVs, although their affinities were slightly lower than those of other CTUDs (**9–12**) and α-Toc. These results indicate that CTUDs (**7–12**) possess considerable affinity for PC LUVs.

Inhibitory effects of CTUDs against copper ion-induced lipid peroxidation of rat blood plasma

The antioxidative activities of the CTUDs (**7–12**) were also determined using the copper ion-induced oxidation system of

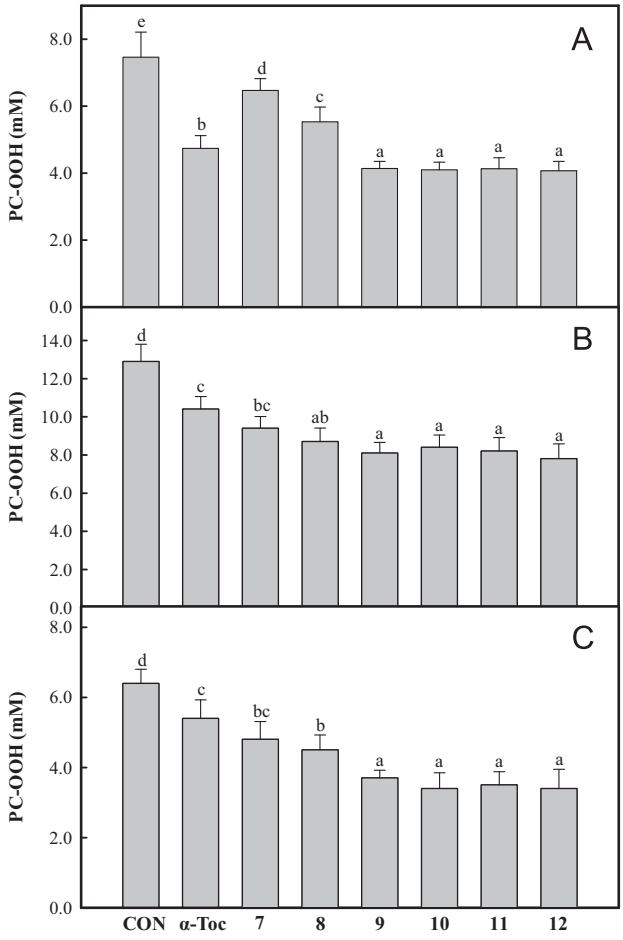

Fig. 4. Inhibitory effects of catechol-type urushiol derivatives (**7–12**) (A) against AMVN-, (B) AAPH-, and (C) copper ion-induced lipid peroxidation of PC LUVs. The PC LUV solution was mixed with solutions of AMVN, AAPH, and copper ions followed by addition of the catechol-type urushiol derivatives (5 μM). The reaction mixture was incubated at 37 °C with continuous shaking. Each value is the mean ± SD ($n = 3$). CON: control.

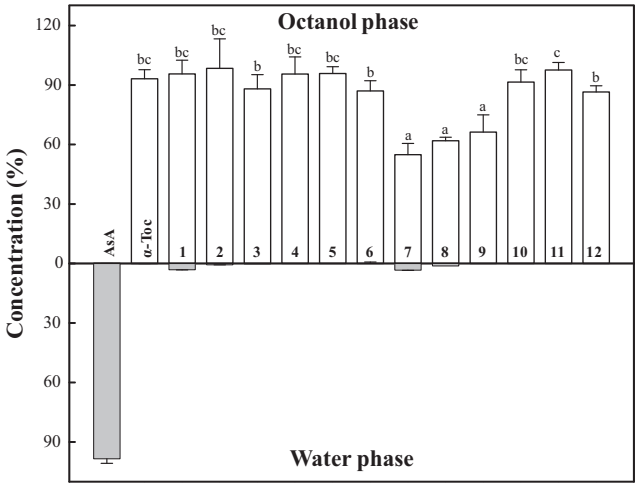

Fig. 5. Concentration of urushiol derivatives (**1–12**) in each fraction after partitioning in *n*-octanol/water. Each value is the mean ± SD ($n = 4$).

rat blood plasma. CTUDs more effectively inhibited CE-OOH formation during lipid peroxidation of rat plasma than the control group (Fig. 7). In addition, CTUDs (**7–12**) showed a significantly higher inhibitory effect on CE-OOH formation than the control

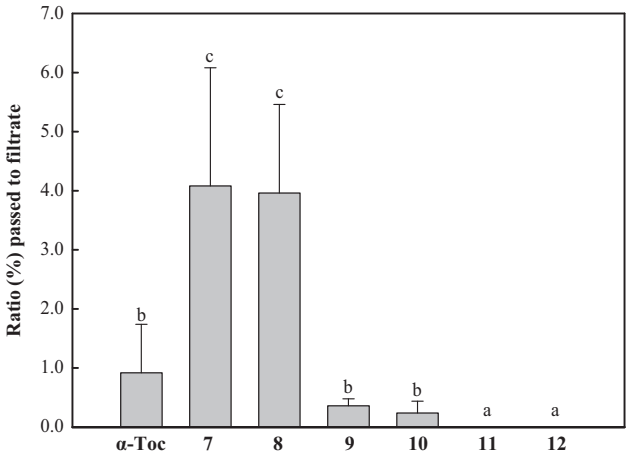

Fig. 6. Concentration of catechol-type urushiol derivatives (**7–12**) in the filtrate after ultrafiltration of the PC LUV suspension. The concentration of catechol-type urushiol derivatives in the LUV suspension was 25 μM. Each value is the mean ± SD ($n = 3$).

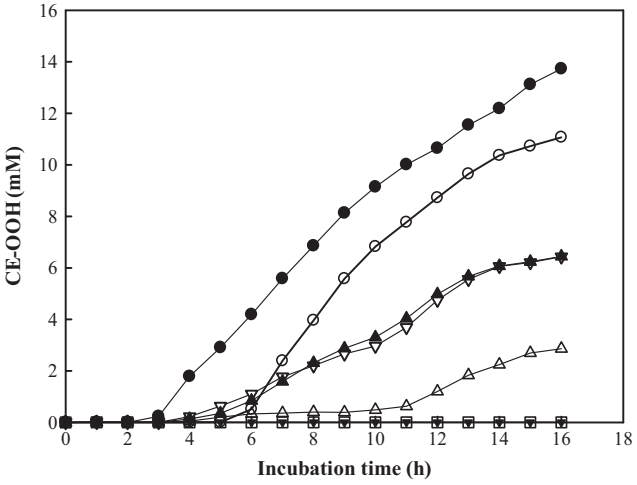

Fig. 7. Inhibitory effects of the catechol-type urushiol derivatives (**7–12**) against copper ion-induced lipid peroxidation in rat blood plasma. •, control; ▽, catechol (**7**); ▲, 3-methylcatechol (**8**); ○, 3-pentylcatechol (**9**); ▼, 3-decylcatechol (**10**); □, 3-pentadecylcatechol (**11**); △, 3-ecosylcatechol (**12**). A diluted blood plasma solution was added to each catechol-type urushiol derivative (5 μM) followed by the addition of $CuSO_4$ (100 μM) to induce CE-OOH formation. The reaction mixture was incubated at 37 °C with continuous shaking. Data are representative of two experiments.

during the incubation. The inhibitory effects decreased in order of **10** and **11** > **12** > **8** ≥ **7** > **9** (Fig. 7). These results confirm that CTUDs (**7–12**) exert a high inhibitory effect against CE-OOH formation during lipid peroxidation of rat plasma induced by copper ions, which is responsible for metal ion-initiated oxidation.

Discussion

We hypothesized that urushiols could be an attractive and potential antioxidative candidate on cellular and subcellular membranes owing to their amphipathic property. However, urushiol causes skin problems such as contact dermatitis [9–11]. Natural urushiol-induced contact dermatitis is dependent on the carbon atom length and the degree of unsaturation of the alkyl chain [1–3]. However, studies on the formation mechanism of urushiol-induced contact dermatitis are not available. We hypothesized that nonallergenic urushiol derivatives would act as excellent antioxidants that would inhibit lipid peroxidation in membrane systems. Therefore, VTUDs and CTUDs (**1–12**)

containing various carbon atom lengths of the alkyl side chains were synthesized to produce nonallergenic urushiol derivatives (Fig. 1). Contact hypersensitivities and antioxidative activities of the synthesized urushiol derivatives (**1–12**) were evaluated with 3-pentadecylcatechol (**11**), which is a natural urushiol, to understand the structural relationships between their hypersensitivities, antioxidative activities, and side-chain lengths. In our preliminary study, a contact hypersensitivity test using **11** as a model natural urushiol compound was performed with sensitization of 0.01–1.0 μmol dosages on the left ears of rats ($n = 6$) daily for 20 days (data not shown). The concentration that approximated the threshold inducing contact hypersensitivity by **11** was about 0.55 μmol (176 μg). Therefore, the synthesized urushiol derivatives (**1–12**) were treated in high amounts (3 μmol) to the left ear of rats daily for 20 days, which was about a 5.5-fold increased dosage of the concentration that approximated the threshold. The morphological results are shown in Fig. 2. 3-Decylcatechol (**10**), which contains 10 carbon atoms in the alkyl side chain, caused more serious contact hypersensitivity than **11,** which is a natural urushiol. However, interestingly, the VTUDs (**1–6**) and other CTUDs (**7, 8, 12**) did not cause contact hypersensitivity. In addition, the results for the numbers of neutrophils and eosinophils and the levels of serum IgE and histamine (Fig. 3) were closely correlated with the contact hypersensitivities of the synthetic urushiol derivatives (**5, 9–12**) (Fig. 2). These results strongly indicate that the catechol structure and the length of the side chain in urushiol are important factors for inducing contact hypersensitivity. Therefore, the nonallergic urushiol derivatives may be excellent antioxidants in biological systems.

Several studies have reported that the catechol (*o*-dihydroxylbenzene) structure is an important factor as an active site for the radical-scavenging activity of phenolic compounds [22]. In this study, VTUDs (**1–6**), which have a methoxyl-substituted catechol moiety, did not scavenge DPPH radicals, which are stable (Supplementary Fig. S1). However, CTUDs (**7–12**) exerted high radical-scavenging activity, and their activities were very similar regardless of carbon atom length of their alkyl side chains. Therefore, these results confirm that the catechol structure is essential for the radical-scavenging activity of urushiol derivatives (**7–12**).

α-Toc is an excellent lipophilic antioxidant that scavenges chain-propagating peroxyl radicals [36]. In this study, CTUDs (**7–12**) strongly suppressed MeL-OOH formation in the methyl linoleate solution induced by AMVN, a lipid-soluble radical generator (Supplementary Fig. S1B). In the kinetic parameter results, the t_{inh} and R_{inh} values of CTUDs (**7–12**) were very closely similar, and their k_{inh}/k_p values were slightly higher compared to those of α-Toc (Table 1). Several studies have suggested that α-Toc scavenges two peroxyl radical molecules [37,38]. In this study, the CTUD stoichiometric factor (n) was slightly higher than that of α-Toc (Table 1). Therefore, CTUDs probably scavenge more than two peroxyl radical molecules.

The PC LUV model system has been frequently used as a biomembrane model to evaluate the antioxidative activity of antioxidants on cellular and subcellular membranes [29,30]. Three different kinds of radical generators, including a lipid-soluble peroxyl radical generator (AMVN), a water-soluble peroxyl radical generator (AAPH), and transition copper ions were used in this biomembrane model. Radical generation by AMVN is initiated in the interior of liposomal membranes [38,39], whereas peroxyl radical generation by AAPH and transition copper ions added in the aqueous phase is initiated in the exterior and surface of liposomal membranes [40]. The results shown in Fig. 4 indicate that CTUDs (**7–12**) effectively suppress PC-OOH formation on AMVN-, AAPH-, and copper ion-induced membranous PC LUV systems, although their effects were slightly different depending on carbon atom length of their alkyl side chains. In particular, all CTUDs in the AAPH- and copper ion-induced oxidation model systems, except **7,** showed higher antioxidative activities than α-Toc (Fig. 4B and C), which was different from AMVN-induced oxidation (Fig. 4A). These results suggest that CTUDs (**7–12**) more effectively scavenged radicals and chelated metal ions induced in the aqueous phase of LUV liposome membrane systems than α-Toc. It may also be attributed to the difference in location of the CTUDs in the PC LUV liposome membrane model system.

In general, the location of the antioxidants should be accounted for to understand the effectiveness of the antioxidants in heterogeneous membrane systems. Several studies have reported that the efficiency of an antioxidant is influenced by its affinity for a membrane [41,42]. Therefore, the localization of an antioxidative compound on a biomembrane may be a particularly important factor to protect against oxidative damage on a membrane. Characteristics such as lipophilicity and hydrophilicity of compounds affect their localization on a biomembrane. Therefore, lipophilicity and affinity of antioxidants have been frequently evaluated using *n*-octanol/water partition and phospholipid membrane systems [32,33,43,44]. The results of the *n*-octanol/water partition experiment suggest that the CTUDs (**7–12**) possess high lipophilic properties (Fig. 5). In particular, lipophilicity increased depending on the carbon atom length of the alkyl side chains of the CTUDs. The lipophilicities of **10–12**, which contain >10 carbon atoms in the alkyl side chain, were similar to that of α-Toc. However, other CTUDs (**7–9**), containing less than 5 carbon atoms in the alkyl side chain, showed significantly lower lipophilicity than α-Toc. The sum of **7–9** in the *n*-octanol and water phases did not reach 100%, suggesting that the portion lacking might be located in the interface between *n*-octanol and water. Therefore, it may be related to the result that **7** and **8** showed patterns different from those of the other CTUDs (**7–12)** in comparison to antioxidative activities using the PC LUV liposome membrane model system.

It was assessed that CTUDs (**9–12**) containing more than five carbon atoms in their alkyl side chains possessed a higher affinity for PC LUV liposomes than α-Toc (Fig. 6). However, compounds (**7** and **8**) with no or one carbon atom in the alkyl side chain showed a relatively lower affinity than the other CTUDs (**9–12**), as well as α-Toc. In addition, the affinity of the CTUDs (**7–12**) to PC LUVs was proportional to the number of carbon atoms in their alkyl side chains. Although the difference in affinity to PC LUVs among the CTUDs was not prominent, small differences might negatively affect the antioxidative activity of **7** and **8** in the PC LUV liposome membrane model system and differences in antioxidative activity might be attributed to localization caused by a difference in carbon atom length in the alkyl side chains of the CTUDs on the PC LUV liposome membrane. In the PC LUV liposome membrane experiments (Fig. 4), CTUDs **9–12** showed high affinity for PC LUVs and strongly suppressed PC-OOH formation on the PC LUV membranous systems regardless of the radical initiator. Therefore, these results suggest that CTUDs (**7–12**) have high radical-scavenging and metal ion-chelating activities in both the aqueous and the lipid phases.

Several lines of evidence suggest that oxidative modification in blood plasma is involved in the development of cardiovascular disease [45]. Therefore, antioxidants in blood plasma may play a role in protecting against oxidation of components such as lipoproteins and delay or prevent the development of cardiovascular diseases such as atherosclerosis [46]. Because CE-OOH produced from oxidation is very stable and is present in healthy human plasma at a concentration of about 3 nM [47], CE-OOH has been selected as a blood plasma lipid peroxidation index [48]. Therefore, the antioxidant activity of CTUDs (**7–12**) in the copper ion-induced-blood plasma oxidation system was examined by measuring CE-OOH content. All CTUDs (**7–12**) exerted high inhibitory activity

against CE-OOH formation in this system, although structural relationships between the compounds and the antioxidative activity were not observed (Fig. 7). Surprisingly, **10** and **11,** which are allergenic urushiol derivatives, showed overwhelmingly higher activities in comparison to **9** and **12,** which are nonallergenic. However, the compounds may be different in practical use because of their allergenic characteristics. Interestingly, the concentration of α-Toc in the low-density lipoprotein fraction increases when incubated with additional α-Toc [49], and α-Toc taken with food is present in the lipoprotein of blood plasma [50]. It has not yet been investigated whether CTUDs are distributed in lipoproteins present in the blood plasma, similar to α-Toc. However, it is likely that the reaction patterns of each CTUD and lipoproteins containing cholesteryl esters are different because the inhibitory pattern against CE-OOH formation was different according to the CTUD. Therefore, the difference may be attributed to differences in carbon atom length of the alkyl side chains of the CTUDs.

In this study, we demonstrated that CTUDs (**7–12**) with various carbon atom lengths of the alkyl chain effectively scavenged radicals generated in both aqueous and lipid phases and chelated transition metal ions. However, of the synthesized CTUDs, **10** and **11,** which have 10 and 15 carbon atoms in the alkyl chain, caused serious contact hypersensitivity. In contrast, VTUDs (**1–6**) and other CTUDs (**7–9, 12**) did not cause an allergenic reaction on contact. Therefore, it was clarified that catechol structure is needed for the compounds to be allergenic and that the number of the carbon atoms in the alkyl chains of CTUDs is an important factor inducing allergy. The nonallergenic urushiol derivatives, **9** and **12,** which contain 5 and 20 carbon atoms in the alkyl chain, may act as excellent antioxidative compounds in biomembrane systems because they showed high affinity for phospholipid membranes and scavenged radicals induced in the exterior as well as the interior of the PC LUV membranous system. Therefore, the nonallergenic urushiol derivatives **9** and **12** acted as potential radical scavengers as well as metal-chelating agents in blood plasma. Moreover, the urushiol derivatives used in this study may provide very useful information for the development of antioxidants that prevent oxidation of biomembranes. Therefore, we have started to evaluate the antioxidative effects of the nonallergenic urushiol derivatives (**9** and **12**) against membranous peroxidation in vivo, and a metabolic study in an in vivo system is also in progress.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.03.041.

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