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Anti-Oxidative Effects of Pyrogollol Lipid Derivatives on the Oxidation of Unilamellar Vesicles and Rat Blood Plasma

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ABSTRACT

Amphipathic pyrogallol lipid derivatives (PD) containing different carbon atomic length alkyl chains, including –H, $-C_5H_{11}$, $-C_{10}H_{21}$, $-C_{15}H_{31}$, and $-C_{20}H_{41}$ in the C-4 position of the benzene ring were synthesized. 4-Decylpyrogallol displayed contact hypersensitivity, but other compounds did not induce an allergic reaction when rat ears were sensitized with the compounds daily for 10 days. PDs containing more than five carbon atoms in the alkyl chain showed high lipophilicity in *n*-octanol/water partition experiments and high rate of incorporation to liposome membranes. In addition, PDs showed higher radical-scavenging activity on 1,1-diphenyl-2-picrylhydrazyl than α-tocopherol and 3-pentylcatechol. PDs efficiently inhibited lipid peroxidation of egg yolk phosphatidyl choline large unilamellar vesicle liposomes induced by lipid- and aqueous-soluble radical generators. Moreover, PDs attenuated the formation of cholesteryl ester hydroperoxides in rat blood plasma induced by copper ions. These results suggest that non-allergenic PDs may act as potent antioxidants against the oxidative damage of cellular and subcellular membranes.

INTRODUCTION

Urushiols are major components in the sap of the lacquer tree (*Rhus verniciflua* Stokes, Anacardiaceae), which has been used as food and a medicinal herb for preventing stomach diseases, inflammatory diseases, and various cancers in Korea [1–4], but they cause contact hypersensitivity [5,6]. Urushiols consist of a catechol structure and an alkyl side chain of 15 or 17 carbons in the C-3 position of the benzene ring, and their diverse biological effects such as antioxidant [7,8], antimicrobial [9,10] and anticancer [11] activities have been reported. In addition, urushiols exert various biological effects in cellular and subcellular membranes because of their amphipathic properties and high rate of incorporation for membranes [12]. However, urushiols are the main allergens that induce contact hypersensitivity, even though these compounds have excellent bioactivities [8].

In a previous study, we chemically synthesized urushiol derivatives that possessed different of alkyl side chain lengths [12]. Some of the synthesized urushiol derivatives did not induce contact hypersensitivity, whereas 3-decylcatechol and 3-pentadecylcatechol, which consist of $-C_{10}H_{21}$ and $-C_{15}H_{31}$ alkyl side chains, caused serious allergic reactions. In addition, the non-allergenic urushiol derivatives, 3-pentylcatechol and 3-eicosylcatechol, showed high lipophilicity and the rate of incorporation to liposome membranes, and they efficiently scavenged free radicals and inhibited lipid peroxidation of liposome membranes and in rat blood plasma.

Phenolic compounds, such as phenolic acids and flavonoids, are distributed widely in food materials, including cereals,

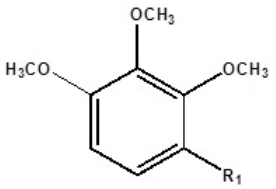
vegetables, and fruits, and have high antioxidative activities [13,14]. The number and position of their phenolic hydroxyl groups are associated with their antioxidative activities, and the catechol and pyrogallol structures are important active sites [15,16]. In particular, the pyrogallol structure more efficiently scavenges free radicals and chelates metal ions than the catechol structure [17,18]. Additionally, similar to the structure of urushiol, phenolic lipids, such as anacardic acid, cardanol, resorcinol, and gingkolic acid are found in plants [19]. These compounds show antioxidative, anticancer, and antimicrobial activities, because of their high lipophilicity and the rate of incorporation for membranes [19,20]. Therefore, we hypothesized that the pyrogallol derivatives, which possess different alkyl side chain lengths, may more efficiently inhibit oxidative damage to cellular and intracellular membranes in comparison with urushiol derivatives, which have a catechol structure.

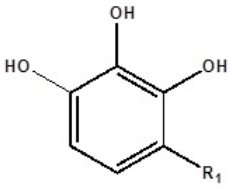
In the present study, we chemically synthesized pyrogallol lipid derivatives (PDs), which contained alkyl side chains of different lengths in the C-4 position of the benzene ring. The contact hypersensitivity of the PDs 1–10 was evaluated in rats. We also measured the radical-scavenging activity of the PDs 1–10 using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and tested their inhibitory effects on lipid peroxidation of phosphatidylcholine large unilamellar vesicles (PhC LUV) and in rat blood plasma.

RESULTS

PD Synthesis

The trimethylated PDs 2–5 were synthesized by lithiation of butyllithium (*n*-BuLi) and alkylation of 1-bromoalkanes, which have different carbon lengths, such as C_5, C_{10}, C_{15}, and C_{20} [12].The crude trimethylated PD products were purified by silica gel column chromatography (toluene). The determined structures were 2 (1,2,3-tri-*O*-methyl-4-pentylpyrogallol), 3 (1,2,3-tri-*O*-methyl-4-decylpyrogallol), 4 (1,2,3-tri-*O*-methyl-4-petadecylpyrogallol), and 5 (1,2,3-tri-*O*-methyl-4-eicosylpyrogallol), using NMR and MS analyses **(Figure 1)**. PDs 7–10 were also synthesized from trimethylated PDs 2–5 by demethylation with boron tribromide (BBr_3) [12]. The crude PD products were purified on silica gel column chromatography using a mobile phase of *n*-hexane/ether =1:1.5 (v/v). The structures were determined to be 7 (3-pentylpyrogallol), 8 (3-decylpyrogallol), 9 (3-pentadecylpyrogallol), and 10 (3-eicosylpyrogallol) by NMR and MS spectroscopic data **(Figure 1)**.





	R_1	
1,2,3-Tri-*O*-methylpyrogallol (**1**)	H_1	Pyrogallol (**6**)
1,2,3-Tri-*O*-methyl-4-pentylpyrogallol (**2**)	C_5H_{11}	4-Pentylpyrogallol (**7**)
1,2,3-Tri-*O*-methyl-4-decylpyrogallol (**3**)	$C_{10}H_{21}$	4-Decylpyrogallol (**8**)
1,2,3-Tri-*O*-methyl-4-pentadecylpyrogallol (**4**)	$C_{15}H_{31}$	4-Pentadecylpyrogallol (**9**)
1,2,3-Tri-*O*-methyl-4-eicosylpyrogallol (**5**)	$C_{20}H_{41}$	4-Eicosylpyrogallol (**10**)

Figure 1. Structure of the synthetic pyrogallol derivatives 1–10.

PD Contact Hypersensitivity on Rat Ears

Rats were sensitized on the rear (1 cm^2) of their left ear with a EtOH solution (3 μmol) of PDs 2–5 and 7–10 every day for 10 days [12]. As shown in **Figure 2**, treatment with 8, which consisted of $-C_{10}H_{21}$ in the alkyl side chain of the C-4 position of the pyrogallol structure, caused severe erythema and swelling on rat ear skin. In contrast, treatment of other PDs 7,9 and 10 did not cause erythema or swelling of rat ears. In addition, none of the trimethylated PDs 2–5 induces contact hypersensitivity regardless of alkyl side chain length.

Effects of PDs on Hypersensitivity-related Blood Factors

Contact hypersensitivity-related biomarkers in rat blood after treatment with two trimethylated PDs 3 and 4 and four PDs 7–10 daily for 10 days were determined. Compound 8 showed significantly higher white blood cell number than that in the control ($p < 0.05$) **(Figure 3A).** The numbers of white blood cell were slightly different among the rats treated with other compounds 3,4,7,9, and 10, although these values were not significantly different from the control. The white blood cell numbers in rats treated with 7 and 9 were slightly higher than that of the control group. The numbers of white blood cells in rats treated with 3, 4, and 10 were relatively lower than that of the control. These white blood cell number patterns were similar to the eosinophil **(Figure 3B)** and neutrophil **(Figure 3C)** numbers as well as serum histamine levels **(Figure 3D)**. In addition, serum IgE levels in rats treated with 8 were significantly higher than those of other compounds 3,4,7,9, and 10 and the control ($p<0.05$) **(Figure 3E)**. The serum IgE levels in rats treated with 3,4,7,9, and 10 were similar to the levels of the control, with no significant differences. The patterns for hypersensitivity-related biomarkers analyzed in this study were consistent with the results of the contact hypersensitivity experiment **(Figure 2)**.

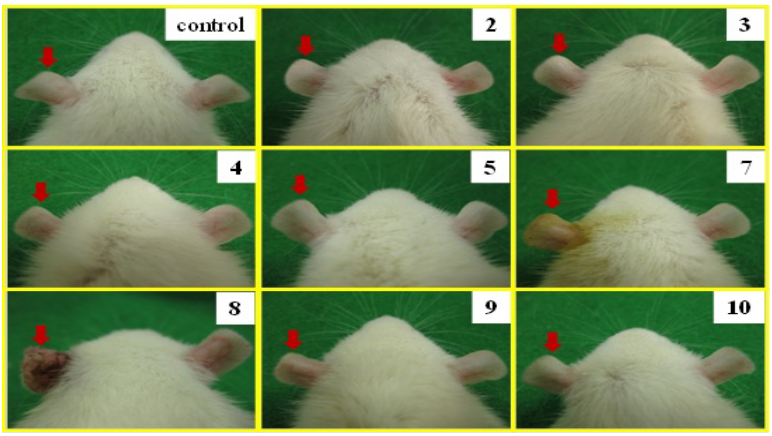


Figure 2. Effects of the synthetic pyrogallol derivatives on contact hypersensitivity induced after treating rat ears for 10 days. Six rats in one group were treated with each urushiol derivative. The synthetic pyrogallol derivatives 2–5 and 7–10 were not induced contact hypersensitivity on the rat ears like control group.

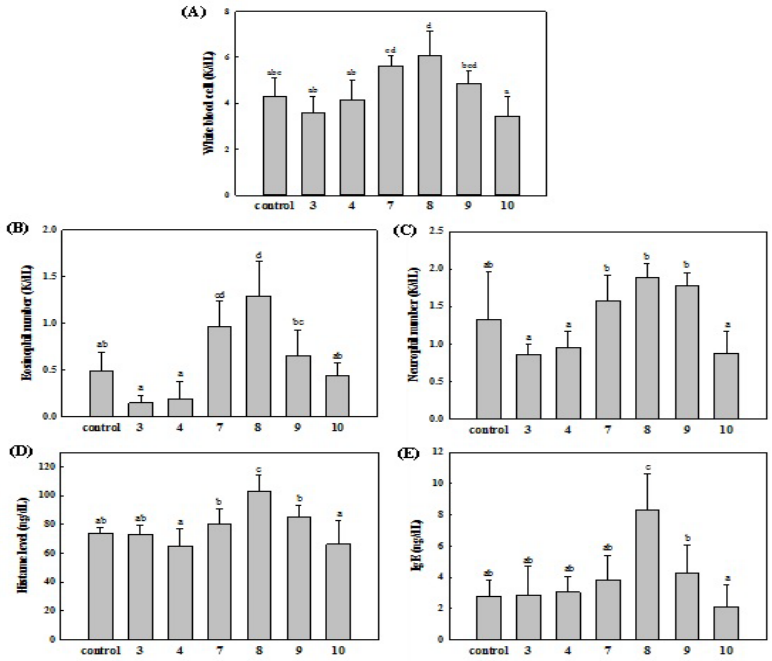


Figure 3. Numbers of white (A) blood cells, (B) neutrophils, (C) eosinophils, (D) IgE, and (E) histamine content in the blood after treatment with synthetic pyrogallol derivatives 3, 4, and 6–10 on rat ears for 20 days. Each value is mean ± standard deviation (n = 6). The different letters indicated a significant difference ($p < 0.05$) by the Tukey-Kramer test.

PD Behavior in n-Octanol/water Partition System

The pyrogallol derivatives 1–10, 4-pentylcatechol (PC), ascorbic acid (AsA), and α-tocppherol (α-Toc) were partitioned in a *n*-octanol/water system, and their contents were determined in two phases by ODS-HPLC analysis [21]. As shown in **Figure 4**, most of the PDs 1–5 and 7–10, except for 6, were detected in the *n*-octanol phase, although their partition coefficients were different. These patterns were similar to the α-Toc and PC results. Therefore, the PDs 6–10 seemed to have lipophilic properties similar to α-Toc and PC. However, the PDs 6–10 showed relatively lower contents in the *n*-octanol phase than the trimethylated PDs 1–5, α-Toc, and PC. Thus, the PDs 6–10 had slightly lower lipophilic properties than other compounds 1–6 and PC. The contents of the PDs in the *n*-octanol phase increased with extension of alkyl side chain length.

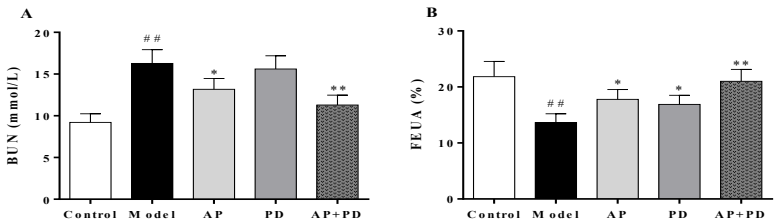


Figure 4. Concentration of synthetic pyrogallol derivatives 1–10 in each fraction after partitioning in n-octanol/water. α-Tocopherol (α-Toc), L-ascorbic acid (AsA), and 3-pentylcatechol (PC) were used as controls. Each value is mean ± standard deviation (n = 4).

PD Localization with a PhC LUV Ultrafiltration System

The affinities of the PDs 6–10 to phospholipid membranes were examined with ultrafiltration using a PhC LUV system [22]. Small amounts of most PDs 6–10 were detected in the filtrate, suggesting that these compounds have strong affinities for PhC LUV. The contents of PDs in the filtrate decreased with increases in alkyl side chain length. Compounds 9 and 10, which possess over 15-carbon atoms in the alkyl side chain of the pyrogallol structure, showed higher rate of incorporation for PhC LUV than α-Toc and PC. The incorporation rate of compound 8, which possesses 10-carbon atoms in the alkyl side chain of the pyrogallol structure, was similar to PC, which is composed of five carbon atoms in the alkyl side chain of catechol **(Figure 5)**. Compounds 6 and 7 also showed considerable incorporation for PhC LUV, although their affinities were slightly lower than those of other PDs 8–10, PC, and α-Toc.

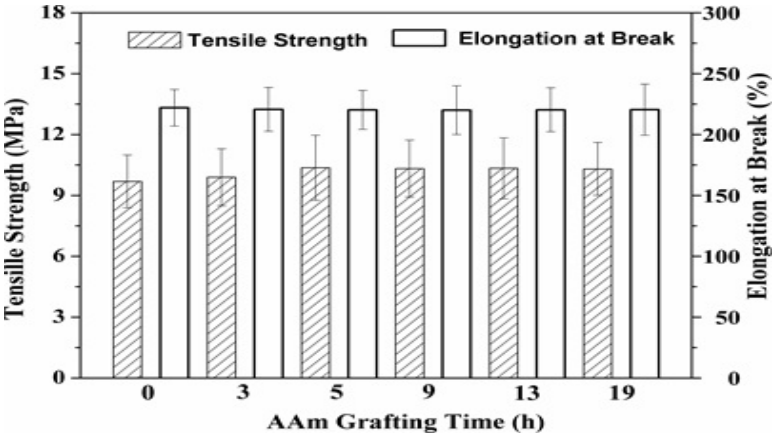


Figure 5. Concentration of synthetic pyrogallol derivatives 6–10 and 3-pentylcatechol in the filtrate after ultrafiltration of the phosphatidyl choline large unilamellar vesicle (PhC LUV) suspension. The concentration of pyrogallol derivatives in the LUV suspension was 25 μM. Each value is mean ± standard deviation (n = 3). The different letters indicated a significant difference ($p < 0.05$) by the Tukey-Kramer test.

DPPH Radical-scavenging Activity of PD

The radical-scavenging activities of pyrogallol derivatives 1–10, PC, and α-Toc as a positive control were evaluated using DPPH. As shown in **Figure 6**, the trimethylated PDs 1–5 did not scavenge DPPH radicals regardless of concentration, much like dimethoxylated urushiol derivatives [12]. In contrast, PDs 6–10 showed predominantly higher DPPH radical-scavenging activities, and their activities were very similar regardless of alkyl side chain length.

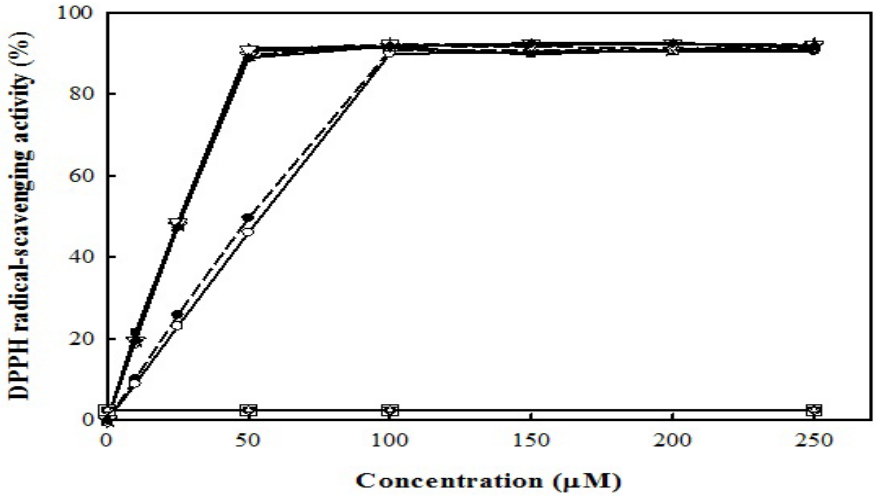


Figure 6. DPPH radical-scavenging activities of synthetic pyrogallol derivatives 1–10 in each fraction after partitioning in n-octanol/water. ●, α-Tocopherol (α-Toc); ○, 3-pentylcatechol (PC); □, 1 (1,2,3-tri-O-methylpyrogallol); ☐, 2 (1,2,3-tri-O-methyl-4-pentylpyrogallol); ☐, 3 (1,2,3-tri-O-methyl-4-decylpyrogallol); ▼, 4 (1,2,3-tri-O-methyl-4-pentadecylpyrogallol); ☐, 5 (1,2,3-tri-O-methyl-4-ecosylpyrogallol); ■, 6 (pyrogallol); ☐, 7 (3-pentylpyrogallol); ▲, 8 (3-decylpyrogallol); ☐, 9 (3-pentadecylpyrogallol); and ☐, 10 (3-ecosylpyrogallol). Each value is the mean ± standard deviation (n = 4).

Inhibition Effects of the PDs on AMVN- and AAPH-induced Lipid Peroxidation in the PhC-LUV System

As shown in **Figure 7A**, Phosphatidyl choline hydroperoxide (PhC-OOH) formation was induced when PhC LUV was exposed to 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN), which is a lipid-soluble radical generator. The PDs 6–10 efficiently inhibited PhC-

OOH formation during lipid peroxidation of PhC LUV induced by AMVN. These compounds exhibited significantly higher inhibitory effects than α-Toc, which is a representative lipophilic antioxidative vitamin. The inhibitory effects of 7–10, which contained more than five carbons in the alkyl side chain, were very similar regardless of the alkyl chain length and were significantly (p<0.05) higher than that of 6, which contained no carbons in its alkyl side chain. In addition, compounds 7–10, which contained three vicinal free hydroxyl groups in the benzene structure, more efficiently inhibited the PhC-OOH formation of lipid peroxidation of PhC LUV in comparison with PC, which consists of a catechol structure and a five-carbon alkyl chain, as a non-allergenic urushiol derivative. In addition, the antioxidative activity of 6 was very similar to that of PC.

We also evaluated the inhibitory effects of PDs 6–10 on PhC-OOH formation during lipid peroxidation of PhC LUV induced by 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH), which is a water-soluble radical generator. All PDs 6–10 exerted high inhibitory effects on PhC-OOH production of PhC LUV generated in the aqueous phase **(Figure 7B)**. The inhibitory effects of 6–10 were very similar regardless of alkyl side chain length. In addition, the inhibitory effects of these compounds were very similar to PC, which consists of a catechol structure and a five-carbon alkyl chain, and were predominantly greater than α-Toc, which was a positive control.

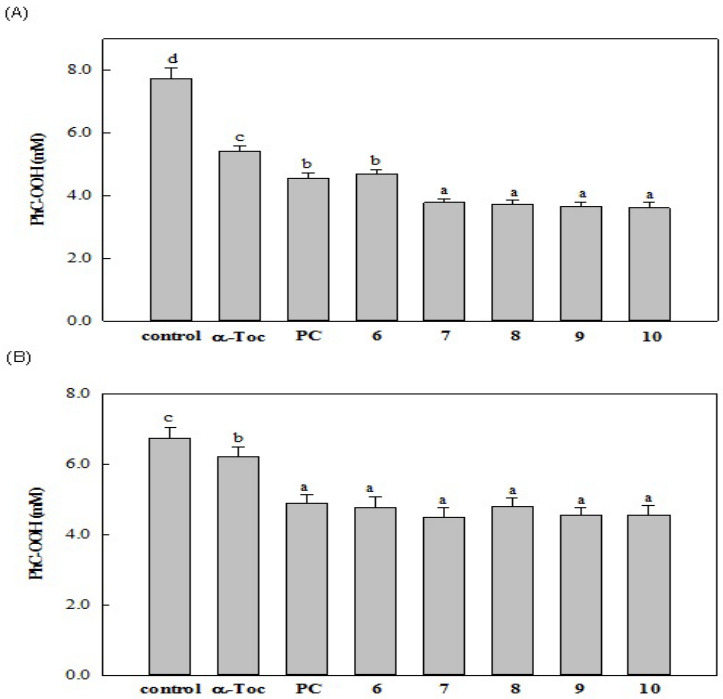


Figure 7. Inhibitory effects of synthetic pyrogallol derivatives 6–10 on (A) AMVN- and (B) AAPH-induced lipid peroxidation of phosphatidyl choline large unilamellar vesicle (PC LUV). α-Tocopherol (α-Toc) and 3-pentylcatechol (PC) were used as controls. The PC LUV solution was mixed with solutions of AMVN, AAPH, and copper ions followed by adding the catechol type urushiol derivatives (5 μM). The reaction mixture was incubated at 37 °C with continuous shaking. Each value is mean ± standard deviation (n = 3). The different letters indicated a significant difference ($p < 0.05$) by the Tukey-Kramer test.

PD Inhibitory Effects on the Copper Ion-induced Lipid Peroxidation of Rat Blood Plasma

When rat blood plasma was treated with copper ions, cholesteryl ester hydroperoxide (CE-OOH) content as a lipid peroxidation product increased gradually during the incubation. All of the PDs 6–10 and PC, which served as a positive control, efficiently inhibited CE-OOH formation during copper ion-induced lipid peroxidation of rat plasma, although their inhibitory effects were different **(Figure 8)**. Compound 7, with five-carbon atoms in its alkyl side chain of the pyrogallol structure, showed significantly higher inhibitory effects than those of the other PDs 6 and 8–10 and PC. After a 14 h incubation, the inhibitory effects on CE-OOH formation decreased in the order of 7>9>8>6 ≥ PC>10 **(Figure 8)**. These results indicate that PDs 6–10 exerted an increased inhibitory effect on CE-OOH formation during the lipid peroxidation of rat plasma induced by copper ions.

DISCUSSION

Our previous study demonstrated that a catechol structure and the alkyl side chain lengths of urushiol derivatives are associated with contact hypersensitivity and with the antioxidative activity of liposome membranes and rat blood plasma [12]. We hypothesized that the PDs, which consist of gallol and alkyl side chain structures, may be more attractive and potential antioxidative compounds on cellular and subcellular membranes than urushiol derivatives. Therefore, we synthesized PDs 1–10 containing different carbon lengths in the alkyl side chains **(Figure 1)** and their contact hypersensitivities and antioxidative activities were determined in this study.

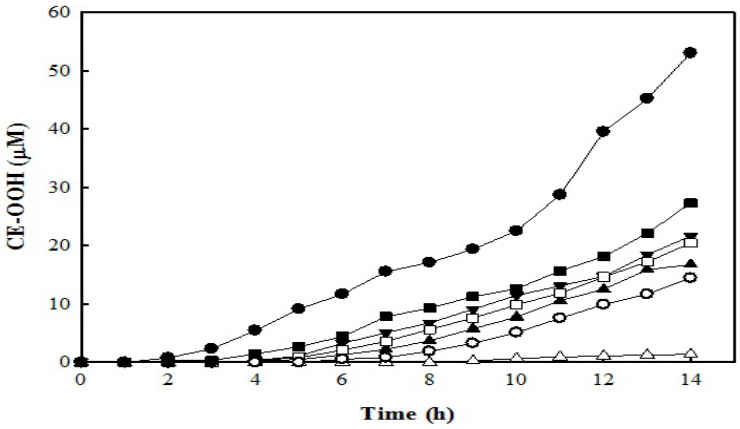


Figure 8. Inhibitory effects of the pyrogallol derivatives 6–10 on copper ion-induced lipid peroxidation of rat blood plasma. ●, control; □, 6 (pyrogallol); △, 7 (4-pentylpyrogallol); ▲, 8 (4-decylpyrogallol); ○, 9 (4-pentadecylpyrogallol); and ■, 10 (4-ecosylpyrogallol). 4-pentylcatechol (▼, PC) was used as a control. A diluted blood plasma solution was added to each catechol-type urushiol derivative (5 μM) followed by CuSO4 (100 μM) to induce CE-OOH formation. The reaction mixture was incubated at 37 °C with continuous shaking. Data are representative of two experiments.

The synthesized PDs 1–10 were treated at high amounts (3 *μ*mol) on the left ear of rats daily for 10 days. The morphological results indicated that only 8 (3-decylpyrogallol), which contains 10-carbon atoms in the alkyl side chain, caused serious contact hypersensitivity **(Figure 2)**. The morphological characteristics of 8 were similar to those of 3-decylcatechol and 3-pentadecylcatechol, which are urushiol derivatives that induced an allergic reaction in our previous study [12]. However, other PDs 1–7, 9, and 10 did not cause contact hypersensitivity. The contact hypersensitivity of the synthetic PDs derivatives **(Figure 2)** was closely correlated with the results of the blood biomarkers, including neutrophils, eosinophils, serum IgE, and histamine **(Figure 3)**. These observations suggest that the number and position of phenolic hydroxyl groups and alkyl side chain lengths are associated with the induction of contact hypersensitivity similar to the urushiol derivatives.

The localization of an antioxidative compound on a biomembrane is a vital factor to protect against oxidative damage on a membrane. We assumed that localization of the PDs 6–10 in biomembranes affected their antioxidative activities. In particular, the antioxidative mechanism of compounds in a biomembrane is frequently dependent upon a balance between the lipophilic and hydrophilic portions of the antioxidants with membranes. α-Toc, which possesses a chromanol group and phytyl side chain, is that protect against oxidative damage on a membrane. Several studies have reported that phytyl side chain length of α-Toc, a representative lipid antioxidant, is important factor in incorporation and localization of α-Toc in the membrane [23,24]. A water-soluble α-Toc derivative, which contains glycosyl group instead of phytyl side chain of α-Toc, enhanced antioxidative activity in membrane surface [25]. The hydroxyl groups of an antioxidative compound decrease lipophilicity. Therefore, the PDs seemed to have lower lipophilicity than catechol derivatives. In addition, low lipophilicity of the PDs may affect incorporation rate and localization of the PDs in membrane. This assumption is supported by results showing that 7 had relatively lower lipophilicity than PC, which was measured in a *n*-octanol/water partition experiment, although both compounds possess the same five-carbon alkyl side chain. In addition, PD lipophilicity was proportional to the lengths of carbon atoms in their alkyl side chains. We also found that PD lipophilicity in compounds with more than 10 carbon atoms in their alkyl side chains showed relatively higher lipophilicity than that of PC. The sum of the PDs 6–10 in the *n*-octanol and water phases did not reach 100%, suggesting that the missing portion might be located in the interface between *n*-octanol and water, much like the catechol-type urushiol derivatives [12]. These trends were similar to the incorporation rate of PDs 6–10 observed in the PhC LUV liposome membrane experiments (Figure 5). Therefore, these results suggest that the hydroxyl group of the pyrogallol structure may contribute to lower lipophilicity and incorporation rate to membranes.

Several studies have suggested that the number and position of phenolic hydroxyl groups is important as an active site for radical-scavenging activity [15,16]. In particular, the pyrogallol structure has been well studied and shows higher free radical-scavenging activity than that of the catechol structure. In the results of DPPH radical experiments **(Figure 6)**, the PDs 6–10 with the pyrogallol structure more efficiently scavenged DPPH radicals than PC, which possesses a catechol structure. The PDs 6–10 showed high free radical-scavenging activities regardless of the alkyl side chain lengths, suggesting that the alkyl side chain length does not contribute to free radical scavenging **(Figure 6)** in a homogeneous solution system. However, the trimethylated PDs 1–5, which have a tri-methylated pyrogallol structure, did not scavenge DPPH radicals. Therefore, these results confirm that the pyrogallol structure in PDs plays an important role in free radical scavenging. Moreover, the number of molecules of DPPH radical scavenged by one molecule of pyrogallol or urushiol derivative was calculated by assuming that one molecule of α-Toc scavenges two molecules of DPPH radical [26,27]. One molecule of the catechol-type urushiol derivative PC trapped two molecules of free radicals, which are similar to α-Toc. However, one molecule of the PDs 6–10 used in the present study was significantly higher than those of α-Toc and catechol-type urushiol derivatives [12]. Therefore, each PD of one molecule probably scavenges approximately five radical molecules.

The PC LUV model system has been frequently used as a biomembrane model to evaluate the inhibitory effects of antioxidants against lipid peroxidation on cellular membranes [28]. A lipid-soluble AMVN generates peroxyl radical in the interior of liposomal membranes [29]. whereas a water-soluble AAPH generates peroxyl radical in the exterior and surface of liposomal membranes [30]. AMVN and AAPH were respectively added in PhC LUV suspension and the amount of PC-OOH was measured at 30 min intervals for 360 min. The lag time of PC-OOH induced by radical generators was about 60 min and thereafter linearly increased during incubation of 360 min. PD inhibited PC-OOH formation in PhC LUV induced by radical generators compared to control without the addition of antioxidants, although their inhibitory activities were different. After 270 min incubation, the inhibitory activities of pyrogallol derivatives against PC-OOH formation were significantly different when compared with α-Toc and/or PC as positive control. These results indicate that PDs 7–10 containing the pyrogallol structure effectively suppress PhC-OOH formation in AMVN- and AAPH-induced membranous PhC LUV systems, although their effects differed slightly depending on alkyl side chain length. This trend was similar to the results **(Figure 7)** for the antioxidative activities of urushiol derivatives, which possess a catechol structure, as reported by our previous study [12]. Interestingly, our data showed that PDs 7–10 with over a 10-carbon alkyl side chain more effectively inhibited lipid peroxidation induced by AMVN in the interior of LUV liposome membranes than α-Toc and PC, which are composed of a catechol structure and a five-carbon atom alkyl side chain **(Figure 7A)**. In addition, all PDs strongly inhibited lipid peroxidation of LUV liposome membranes induced by AAPH regardless of alkyl side chain length, and their inhibitory activities were significantly more effective than that of α-Toc ($p<0.05$); however, the PD activities did not differ significantly from PC activities. These results suggest that PDs 6–10 more effectively scavenged radicals induced in both the aqueous and lipid phases of LUV liposome membrane systems than that of α-Toc. Therefore, these results suggest that the hydroxyl group of the pyrogallol structure may be distributed on the surface as well as in the interior of the PhC LUV membrane. Furthermore, the PDs had relatively lower amount incorporated to PhC LUV membrane than the urushiol derivatives, when compared between compounds possessing identical alkyl side chain length. Therefore, PDs may more effectively scavenge radicals distributed at the interface of membranes.

Several studies have indicated that antioxidants suppress the oxidation of components, such as lipoproteins in blood plasma, and prevent the development of cardiovascular diseases, such as atherosclerosis [30–33]. CE-OOH is one of blood plasma lipid peroxidation biomarkers because CE-OOH produced from oxidation is very stable and is present in healthy human plasma at a concentration of about 3 Nm [34,35]. We have reported that all catechol-type urushiol derivatives that have different alkyl side chain lengths strongly inhibit the formation of CE-OOH during lipid peroxidation of blood plasma induced by copper ions. In the present study, we also found that all PDs 6–10 with the pyrogallol structure had high inhibitory effects on CE-OOH formation in this system. This result indicates that PDs 6–10 efficiently chelated copper ions and/or scavenged copper ion-induced radicals in blood plasma. However, our previous and present observations indicate that the antioxidative activities of both PDs and catechol-type urushiol derivatives did not correlate with alkyl side chain length during rat blood plasma oxidation (Figure 8). Compound 7 and PC possess a five-carbon atom alkyl side chain but contain three vicinal and two hydroxyl groups in the benzene structure, respectively. However, compound 7 more overwhelmingly inhibited CE-OOH formation in blood plasma than that of PC. Moreover, compound 7 showed higher inhibitory effects on lipid peroxidation than that of other PDs 6 and 8–10. Compounds 8 and 9, which have 10- and 15-carbon atom alkyl side chains, showed relatively higher inhibitory effects on lipid peroxidation than PC. This result suggests that PDs possessing 5- and 15-carbon atoms in their alkyl side chains may efficiently suppress copper ion-induced lipid peroxidation in blood plasma.

In this study, we demonstrated that PDs 6–10 possessing different alkyl side chain lengths effectively scavenged radicals generated in both aqueous and lipid phases. However, 4-decylpyrogallol 8, which has 10-carbon atoms in the alkyl chain, caused serious contact hypersensitivity, much like the urushiols. In contrast, other PDs 6, 7, 9, and 10 did not cause an allergic reaction on contact. Therefore, the number and position of the phenolic hydroxyl groups and the length of the carbon atoms in the alkyl chains of the PDs were clarified as important factors in allergy induction. Moreover, the non-allergenic PDs 7, 9, and 10 may be excellent antioxidative compounds in biomembrane systems because they showed high incorporation for phospholipid membranes and effectively scavenged radicals induced at the interface as well as in the interior of the PhC LUV membranous system. Therefore, the non-allergenic PDs are considered potent antioxidants that prevent biomembrane oxidation. Now, the adsorption and metabolism of the non-allergenic PDs 7, 9, and 10 and their *in vivo* antioxidative effects on membranous peroxidation are being evaluated.

EXPERIMENTAL SECTION

NMR spectral data were obtained with a Varian UnityINOVA (Varian, Walnut Creek, CA) spectrometer using tetramethylsilane in $CDCl_3$ and acetone-d_6 as the internal standard. Mass spectral data were obtained with electrospray ionization mass spectrometry (API 3200Q trap, Applied Biosystems, Foster City, CA) under the following conditions: ion source temperature, 0°C; and electron voltage of the positive and negative modes, 5000 V and −4500 V, respectively. Column chromatography was performed with silica gel (70–230 mesh) (Merck, Darmstadt, Germany) resin. HPLC analysis was performed on a Shimadzu LC-6AD with a SPD-M20A detector and silica gel (4.6×250 mm) (Tosoh, Tokyo, Japan), LiChroprep Lobar (40–63 μm, 25×310 mm) (Merck), and Octyl-80Ts (4.6×150 mm) (Tosoh) columns. Thin-layer chromatography was performed on a silica gel 60 F_{254} (0.25 mm thickness) (Merck).

Compound 1 (1,2,3-trimethylbenzene), 1-bromopentane, 1-bromodecane, 1-bromopentadecane, 1-bromoeicosane, *n*-BuLi (1.6 M in *n*-hexane), boron tribromide (BBr_3, 1.0 M in *n*-hexane), 2,6-di-*tert*-butyl-4-methyl-phenol (BHT), 70% perchloric acid

($HClO_4$), and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Compound 6 (pyrogallol) was purchased from Kanto Chemical Co. (Tokyo, Japan). PC was synthesized in our laboratory [12]. *n*-Octanol was obtained from the Samchun Pure Chemical Co. (Pyeongtaek, Korea). DPPH, AMVN, AAPH, AsA, and egg yolk 3-*sn*-phosphatidyl choline were obtained from Wako (Osaka, Japan). α-Toc was purchased from Fluka (Buchs SG, Lucerne, Switzerland). All other chemicals and solvents were analytical grade.

Synthesis of Tri-O-methyl-4-alkylpyrogallol Derivatives

1,2,3-Tri-*O*-methyl-4-alkylpyrogallol derivatives were synthesized according to a urushiol derivative synthesis method [12]. A 3.82 mL aliquot of 1,2,3-tri-*O*-methyl-4-alkylpyrogallol (24 mmol) was added to a solution (50 mL) of dry tetrahydrofuran (THF), and the mixture was stirred for 30 min at 0°C. A solution of 72 ml *n*-BuLi (1.6 M in *n*-hexane, 72 mmol) in 5 mL THF was slowly added to the mixture and then stirred successively for 1 h at 0°C and for 3 h at room temperature. A solution (5.94 mL) of 1-bromoalkanes (48 mmol) with different carbon chain lengths was slowly poured into the solution and then refluxed for 20 h at 210°C. The resulting mixture was added to a saturated NH_4Cl solution (50 mL, three times) and partitioned with ethyl acetate (EtOAc, 50 mL, three times). The organic layer was washed with a brine solution (50 mL, three times) and evaporated *in vacuo* at 35°C. The concentrates were purified on silica gel (140 g, 2.3×67 cm) column chromatography eluting with *n*-hexane/EtOAc=18:1 (v/v) to provide the trimethylated PDs. Four trimethylated PDs 2–5 were synthesized using 1-brompentane, 1-bromodecane, 1-bromopentadecane, and 1-bromoeicosane using the procedure described above. The crude trimethylated PD products were purified by silica gel column chromatography (toluene). The yields were 62.9, 59.5%, 60.1, and 60.5% for 2, 3, 4, and 5, respectively. The purities of all compounds were >99%.

1,2,3-Tri-O-methyl-4-pentylpyrogallol 2

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.83 (1H, d, *J* = 8.5 Hz, H-5), 6.61 (1H, d, *J* = 8.5 Hz, H-6), 3.85 (3H, s, OCH_3 of C-1), 3.88 (6H, s, OCH_3 of C-2 and C-3), 2.55 (2H, t, *J* = 8.0 Hz, H-1'), 1.57 (2H, m, H-2'), 1.34 (4H, m, H-3', H-4'), 0.91 (3H, t, *J* = 7.0 Hz, H-5'); ^{13}C NMR (125 MHz, $CDCl_3$) δ 142.3 (C-1), 151.8 (C-2), 151.7 (C-3), 129.0 (C-4), 123.7 (C-5), 107.1 (C-6), 56.0 (3H, s, OCH_3 of C-1), 60.9 (OCH_3 of C-2) , 60.7 (OCH_3 of C-3), 29.6 (C-1'), 30.6 (C-2'), 31.8 (C-3'), 22.6 (C-4'), 14.0 (C-5'); ESI-MS (positive) *m/z* 239.2 $[M + H]^+$, 261.2 $[M + Na]^+$.

1,2,3-Tri-O-methyl-4-pentylpyrogallol 3

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.83 (1H, d, *J* = 8.3 Hz, H-5), 6.61 (1H, t, *J* = 8.3 Hz, H-6), 3.85 (3H, s, OCH_3 of C-1), 3.85 (6H, s, OCH_3 of C-2 and C-3), 2.54 (2H, t, *J* = 7.8 Hz, H-1'), 1.55 (2H, m, H-2'), 1.30 (14H, m, H-3'–H-9'), 0.89 (3H, t, *J* = 7.0 Hz, H-10'); ^{13}C NMR (125 MHz, $CDCl_3$) δ 142.2 (C-1), 151.8 (C-2), 151.7 (C-3), 129.0 (C-4), 123.7 (C-5), 107.1(C-6), 60.0 (OCH_3 of C-1), 60.9 (OCH_3 of C-2), 60.9 (OCH_3 of C-3), 31.0 (C-2'), 29.8–29.3 (C-1', C-3'–C-7'). 31.9 (C-8'), 22.7 (C-9'), 14.1 (C-10'); ESI-MS (positive) *m/z* 309.3 $[M + H]^+$.

1,2,3-Tri-O-methyl-4-decylpyrogallol 4

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.82 (1H, d, *J* = 8.5 Hz, H-5), 6.60 (1H, d, *J* = 8.5 Hz, H-6), 3.84 (3H, s, OCH_3 of C-1), 3.87 (6H, s, OCH_3 of C-2 and C-3), 2.53 (2H, t, *J* = 7.8 Hz, H-1'), 1.54 (2H, m, H-2'), 1.29 (24H, m, H-3'–H-14'), 0.88 (3H, t, *J* = 7.0 Hz, H-15'); ^{13}C NMR (125 MHz, $CDCl_3$) δ 142.3 (C-1), 151.9 (C-2), 151.7 (C-3), 129.0 (C-4), 123.7 (C-5), 107.1 (C-6), 56.0 (OCH_3 of C-1), 60.9 (OCH_3 of C-2), 60.7 (OCH_3 of C-3), 29.7–29.4 (C-1', C-3'–C-12'), 31.0 (C-2'), 31.9 (C-13'), 22.7 (C-14'), 14.1 (C-15'); ESIMS (positive) *m/z* 379.3 $[M + H]^+$, 401.3 $[M + Na]^+$.

1,2,3-Tri-O-methyl-4-eicosylpyrogallol 5

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.82 (1H, d, *J* = 8.3 Hz, H-5), 6.60 (1H, d, *J* = 8.3 Hz, H-5), 3.84 (3H, s, OCH_3 of C-1), 3.87 (6H, s, OCH_3 of C-2 and C-3), 2.53 (2H, t, *J* = 7.8 Hz, H-1'), 1.54 (2H, m, H-2'), 1.31 (34H, m, H-3'–H-19'), 0.88 (3H, t, *J* = 7.0 Hz, H-20'); ^{13}C NMR (125 MHz, $CDCl_3$) δ 142.3 (C-1), 151.9 (C-2), 151.7 (C-3), 129.0 (C-4), 123.7 (C-5), 107.1 (C-6), 56.0 (OCH_3 of C-1), 60. 9 (OCH_3 of C-2), 60.7 (OCH_3 of C-3), 29.7–29.4 (C-1', C-3'–C-17'), 31.0 (C-2'), 31.9 (C-18'), 22.7 (C-19'), 14.1 (C-20'); ESI-MS (positive) *m/z* 449.4 $[M + H]^+$, 471.4 $[M + Na]^+$.

Synthesis of PDs

PDs were synthesized according to a synthetic urushiol derivative method described previously [12]. Briefly, the trimethylated PDs 2–5 (5.7 g, 22.5 mmol) were dissolved in 60 mL dry methylene chloride (CH_2Cl_2) at 0°C. This sample solution was added to a solution (45 mL) of 1.0 M BBr_3 hexane solution (45 mmol) in dry CH_2Cl_2, and the mixture was stirred for 2 h at 0°C and then for 20 h at room temperature. Each solution was partitioned with H_2O to produce the CH_2Cl_2 layer. The aqueous layer was partitioned with CH_2Cl_2 (50 mL, twice). The combined organic layer was washed with a brine solution (100 ml, twice). The organic layer was evaporated *in vacuo* at 35°C. The crude products were purified on silica gel (2.9×56 cm, *n*-hexane/ether =1:1.5, v/v) column chromatography to produce the PDs. PDs 7,8,9 and 10 were synthesized from 1,2,3-tri-*O*-methyl-4-alkylpyrogallol derivatives 2,3,4 and 5, respectively, using the procedure described above. PDs 7–10 were also synthesized from trimethylated PDs 2–5 by demethylation with BBr_3 [12]. The yields were 95.9, 86.5, 81.1 and 94.5% for 7, 8, 9 and 10, respectively, and the purities of all compounds were >99%.

4-Pentylpyrogallol 7

^{1}H NMR (500 MHz, acetone-d_6) δ 7.84–7.02 (3H, s, OH of C-1, C-2, and C-3), 6.42 (1H, d, *J* = 8.5 Hz, H-5), 6.30 (1H, d, *J* = 8.5 Hz, H-6), 2.52 (2H, t, *J* = 7.8 Hz, H-1'), 1.56 (2H, m, H-2'), 1.32 (4H, m, H-3' and H-4'), 0.88 (3H, t, *J* = 7.0 Hz, H-5'); ^{13}C NMR (125 MHz, acetone-d_6) δ 133.5 (C-1), 144.8 (C-2), 144.4 (C-3), 121.7 (C-4), 120.6 (C-5), 107.3 (C-6), 30.5 (C-1'), 30.8 (C-2'), 32.5 (C-3'), 23.4 (C-4'), 14.5 (C-5'); ESI-MS (negative) *m/z* 195.1 [M – H]$^-$.

4-Decylpyrogallol 8

^{1}H NMR (500 MHz, acetone-d_6) δ 7.34 (3H, s, OH of C-1, C-2, and C-3), 6.43 (1H, d, *J* = 8.5 Hz, H-5), 6.30 (1H, d, *J* = 8.5 Hz,), 2.52 (2H, t, *J* = 7.8 Hz, H-1'), 1.55 (2H, m, H-2'), 1.31 (14H, m, H-3'–H-9'), 0.88 (3H, t, *J* = 7.0 Hz, H-10'); ^{13}C NMR (125 MHz, acetone-d_6) δ 132.6 (C-1), 143.9 (C-2), 143.5 (C-3), 120.8 (C-4), 119.7 (C-5), 106.4 (C-6), 29.6-29.4 (C-1', C-3'–C-9'), 30.2 (C-2'), 31.8 (C-8'), 22.5 (C-9'), 13.5 (C-10'); ESI-MS (negative) *m/z* 265.2 [M – H]$^-$.

4-Pentadecylpyrogallol 9

^{1}H NMR (500 MHz, acetone-d_6) δ 7.82–7.01 (3H, s, OH of C-1, C-2, and C-3), 6.43 (1H, d, *J* = 8.0 Hz, H-5), 6.30 (1H, d, *J* = 8.0 Hz, H-6), 2.52 (2H, t, *J* = 7.8 Hz, H-1), 1.56 (2H, m, H-2'), 1.31 (24H, m, H-3'–H-14'), 0.88 (3H, t, *J* = 7.0 Hz, H-15'); ^{13}C NMR (125 MHz, acetone-d_6) δ 133.5 (C-1), 144.8 (C-2), 144.4 (C-3), 121.7 (C-4), 120.6 (C-5), 107.3 (C-6), 30.5–30.4 (C-1', C-3'–C-12'), 31.2 (C-2'), 32.7 (C-13'), 23.4 (C-14'), 14.4 (C-15'); ESI-MS (negative) *m/z* 335.2 [M – H]$^-$.

4-Eicosylpyrogallol 10

^{1}H NMR (125 MHz, acetone-d_6) δ 7.85–7.03 (3H, s, OH of C-1, C-2, and C-3), 6.42 (1H, d, *J* = 8.0 Hz, H-5), 6.30 (1H, d, *J* = 8.0 Hz, H-6), 2.52 (2H, t, *J* = 7.5 Hz, H-1'), 1.56 (2H, m, H-2'), 1.31 (34H, m, H-3'–H-19'), 0.88 (3H, t, *J* = 7.3 Hz, H-20'); ^{13}C NMR (125 MHz, acetone-d_6) δ 133.5 (C-1), 144.8 (C-2), 144.5 (C-3), 121.7 (C-4), 120.6 (C-5), 107.3 (C-6), 30.5–30.4 (C-1', C-3'–C-19'), 31.2 (C-2'), 32.7 (C-18'), 23.4 (C-19'), 14.4 (C-20); ESI-MS (negative) *m/z* 405.3 [M – H]$^-$.

Contact Hypersensitivity Assay for the PDs on Rat Ears

Sprague–Dawley rats (male, 6-weeks-old, 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 a 12 h light/dark cycle. Food and water were available *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (no. CNU IACUC-YB-R-2012-26). All rats were acclimated for 1 week with a standard rodent diet (Harlan Rodent diet, 2018S) prior to experiments. The EtOH solution (3 μmol 50 μL^{-1}) of PDs 1–10 was applied to the rear (1 cm^2) of the left rat ear (*n*=6) daily for 20 days. The erythema visualized on the rat ears treated with the PDs was reflective of contact hypersensitivity.

Hematological Biomarkers in Rats Treated with PDs

After 10 days of PD treatment, the 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 cells, neutrophils, and eosinophils in whole blood were determined with the Veterinary Multi-species Hematology System (CDC Technologies Inc., Oxford, MI). Blood serum was obtained by centrifugation (1,500 g) at 4°C for 20 min and stored at –70°C until use. Serum IgE and histamine levels were measured using a Rat IgE Enzyme-Linked Immunosorbent Assay (ELISA) kit (Komabiotec, Seoul, Korea) and a histamine ELISA kit (Oxford Biomedical Research Inc., Oxford, MI) according to the manufacturer's instructions.

Measurement of the Partition Coefficient in the n-Octanol/H_2O Mixture

The partition coefficients of PDs 6–10, PC, AsA, and α-Toc were determined using a *n*-octanol/H_2O system [21]. A 100 nmol aliquot of each compound in MeOH and the antioxidants (α-Toc and AsA, each 100 nmol) were placed in a test tube. After removing the solvent 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 6,000 rpm for 5 min at 4°C to produce the *n*-octanol and Tris-HCl buffer phases. The content of each compound in the two phases was determined by the ODS-HPLC analysis described below.

PhC LUV Ultrafiltration

The ethanol solution of PDs 6–10, PC, and α-Toc (final concentration, 25 μM) and a solution of the purified PhC LUV (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 *in vacuo* evaporation 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 [36]. The filtrate was centrifuged at 12,000 rpm for 40 min at 4°C. The supernatant was concentrated under a stream of nitrogen gas, and the concentrate was dissolved with ethanol (100 μL).

PD Quantitative Analysis

The contents of PDs 1–10, AsA, and α-Toc in the filtrate after PhC LUV ultrafiltration were measured by HPLC equipped

with an Octyl-80Ts column (Tosoh). The mobile phases for separating the PDs 1–10 were solvent mixtures of MeOH/H_2O: 1, MeOH/H_2O=40:60 (v/v); 2, MeOH/H_2O=70:30 (v/v); 3, MeOH/H_2O=83:17 (v/v); 4, MeOH/H_2O=88:12 (v/v); 5, MeOH/H_2O=94:6 (v/v); 6, MeOH/H_2O=0:100 (v/v); 7, MeOH/H_2O=52:48 (v/v); 8, MeOH/H_2O=73:27 (v/v); 9, MeOH/H_2O=83:17 (v/v); 10, MeOH/H_2O=88:12 (v/v); PC, MeOH/H_2O=62:38 (v/v); α-Toc, MeOH/H_2O=93:7 (v/v); and AsA, MeOH/H_2O=0:100 (v/v). The flow rate was 1.0 mL min^{-1}, and the compounds were monitored at 240 nm (Shimadzu). Standard PD solutions 1–10 in MeOH were prepared at concentrations of 5–200 nmol. Calibration curves were constructed by plotting the peak areas vs. the concentration of each compound. The PD content was quantified in triplicate experiments

Determination of DPPH Radical-scavenging Activity

The free radical-scavenging activities of PDs 1–10, α-Toc, and PC were evaluated using the DPPH radical [37]. Briefly, the compounds were prepared at final concentrations of 10, 50, 100, 150, 200, and 250 μM in ethanol. The MeOH solution (0.5 mL) of each compound at the different 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 each compound was evaluated by decolorization at 517 nm, and activity was also determined as the percentage decrease in absorbance calculated from a blank test.

Preparation of Egg Yolk Phosphatidylcholine (EYPhC)

EYPhC was prepared as described previously by Terao et al. [38], with slight modifications. Briefly, a commercial EYPhC reagent was purified by LiChroprep Lobar column chromatography eluted with $CHCl_3$/MeOH/H_2O=1:10:0.5 (v/v/v). The EYPhC content in the EYPhC-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 EYPhC concentrate was stored at −40°C until use.

Determination of the Inhibitory Effects of PDs against AMVN- and AAPH-induced Lipid Peroxidation of PhC LUV

The inhibitory effects of PDs against AMVN- and AAPH-induced lipid peroxidation in PhC LUV system were determined according to a method described previously [12]. The ethanol solutions of PDs 6–10, PC, and α-Toc, a solution of the purified PhC LUV in $CHCl_3$/MeOH (95:5, v/v), or a solution of AMVN in *n*-hexane were placed in a test tube. After evaporating the solvent, the residue was dispersed in 1 mL of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.5 mM DTPA. The PhC LUV suspension was obtained using the same ultrasonication and membrane procedures (pore size=100 nm, 21 times). The PhC 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. The final concentrations of antioxidants, PhC LUV, and radical generators (AMVN and APPH) were 25,5 and 1 mM, respectively. PhC-OOH content in the reaction mixture was determined using the HPLC conditions described below.

To determine the inhibitory effects of the pyrogallol derivatives against APPH-induced oxidation in PhC LUV, a solution of AAPH (final concentration, 10 mM) in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.5 mM DTPA was added to the PC LUV suspension that was pre-incubated at 37°C for 5 min in the dark with continuous shaking after ultrasonication and membrane procedures. Other conditions were the same as those for AMVN.

PhC-OOH content was determined according to a method described previously by Shirai et al. [22]. Briefly, aliquots were subjected to ODS-HPLC using a TSK-gel Octyl-80Ts column (Tosoh). The mobile phase consisted of MeOH/H_2O=90:10 (v/v), and flow rate was constant at 1.0 mL min^{-1}. PhC-OOH was monitored by UV detection at 235 nm. PhC-OOH concentration was calculated from a PhC-OOH standard curve. Detailed procedures for preparing the PhC-OOH standards have been described previously [38].

Determination of the PD Inhibitory Effects on Copper Ion-induced Oxidation in Rat Blood Plasma

The antioxidative activities of PD 6–10 and PC were evaluated by measuring their inhibitory effects against the formation of CE-OOH during copper ion-induced oxidation of rat blood plasma [25]. Sprague–Dawley rats (male, 6-weeks-of-age, 180–200 g) were obtained from Samtako Bio Korea. The rats were maintained at 20 ± 2°C under a 12-h light/dark cycle and fasted for 15 h prior to blood collection. After diethyl ether anesthesia, blood was collected from the abdominal aorta into heparinized tubes. Rat plasma was isolated by centrifugation (3,000 *g*) at 4°C for 20 min and stored at −40°C. Blood plasma was diluted four-fold with PBS (pH 7.4). The diluted plasma (650 μL) was added to a 20 μL ethanol solution of PDs (10 μM) and 100 μL of $CuSO_4$ PBS solution (final concentration, 100 μM). The mixture was incubated at 37°C for 14 h with continuous shaking. The CE-OOH concentration was determined as described previously [39]. 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 vigorous vortexing 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 to determine CE-OOH content. The effluent was monitored by UV detection at 235 nm. MeOH/H_2O (97:3, v/v) served as the mobile phase, and flow rate was constant at 1.0 mL min^{-1}. CE-OOH concentration was calculated from a CE-OOH standard curve. Detailed

procedures for CE-OOH standard preparation have been published previously [39].

Statistical Analysis

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

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