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Antioxidant activities of a new lignan and a neolignan from *Saururus chinensis*

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Abstract—A new diarylbutane lignan, 2′-hydroxy dihydroguaiaretic acid (**4**), and a known 8-*O*-4′-type neolignan, machilin D (**5**), were isolated from the ethyl acetate extracts of the underground parts of *Saururus chinensis*. Compounds **4** and **5** exhibited low-density lipoprotein (LDL)-antioxidant activity in the thiobarbituric acid-reactive substances (TBARS) assay (**4**: IC_{50} = 3.3 μM and **5**: IC_{50} = 3.8 μM), the lag time of conjugated diene production, the relative electrophoretic mobility (REM) of ox-LDL, the apoB-100 fragmentation on copper-mediated LDL oxidation and the macrophage-mediated LDL oxidation, and radical DPPH scavenging activity.

Since it has been observed that oxidized low-density lipoproteins (ox-LDLs) play important role in early stage of atherosclerosis,[1] there has been increasing interest to intake antioxidant-rich foods, such as fruits, vegetables, and beverage plants.[2] *Saururus chinensis* Baill (Saururaceae) is a perennial herbaceous plant used in treatment of various diseases, such as edema, jaundice, gonorrhea, antipyretic, diuretic, and anti-inflammatory agents in Korean folk medicine.[3] So far, diarylbutane lignans and neolignans including 8-*O*-4′-types have been isolated mainly from root of *S. chinensis*, which were shown various biological activities such as cell adhesion inhibitory,[4] anti-inflammatory,[5] antiplasmodial,[6] murine neuroleptic,[7] and antifeedant activities.[8] Sadhu et al. reported that a neolignan, machilin C, showed an antioxidant activity as the DPPH radical scavenger.[9] Recently, we reported that a sesquineolignan, saucerneol B (**1**), and dineolignans, manassantin A (**2**) and manassantin B (**3**), were isolated by bioassay-guided fractionation of the methanolic extracts of the root of *S. chinensis* and showed specificity of inhibitory activity against hACAT-1 and -2 (Fig. 1).[10] In connection of our studies on the screening of cholesterol-lowering and antiatherosclerotic agents, we found that the ethyl acetate extracts of root of *S. chinensis* exhibited significant LDL-antioxidant activities (87% inhibition at 40 μg/mL). Subsequent bioactivity-guided fractionation of the ethyl acetate extracts led to a new diarylbutane lignan, 2′-hydroxy dihydroguaiaretic acid (**4**), and a known 8-*O*-4′-type neolignan, machilin D (**5**) (Fig. 1).[11] Compounds **4** and **5** did not inhibit hACAT-1 and -2 activities, whereas compounds **1**, **2**, and **3** did not inhibit LDL oxidation in TBARS assay (data not shown). In this study, we wish to describe the isolation, structure characterization, and biological activities of compounds **4** and **5**. Antioxidant activities of LDL were detected by various tools, such as TBARS assay, conjugated diene formation, relative electrophoretic mobility (REM), fragmentation of apoB-100 on copper-mediated LDL oxidation and macrophage-mediated LDL oxidation. Also, radical DPPH scavenging activities of compounds **4** and **5** were measured.

Compound **4** exhibited a molecular formula of $C_{20}H_{26}O_5$ from its positive HREIMS data (*m*/*z* $[M]^+$, 346.1780, calcd 346.1780 for $C_{20}H_{26}O_5$) and has the value of $[\alpha]_D^{25}$ − 13.8 (*c* 0.29, $CHCl_3$). The mass spectrum showed a base peak at *m*/*z* 346 $[M]^+$ (61.8%)

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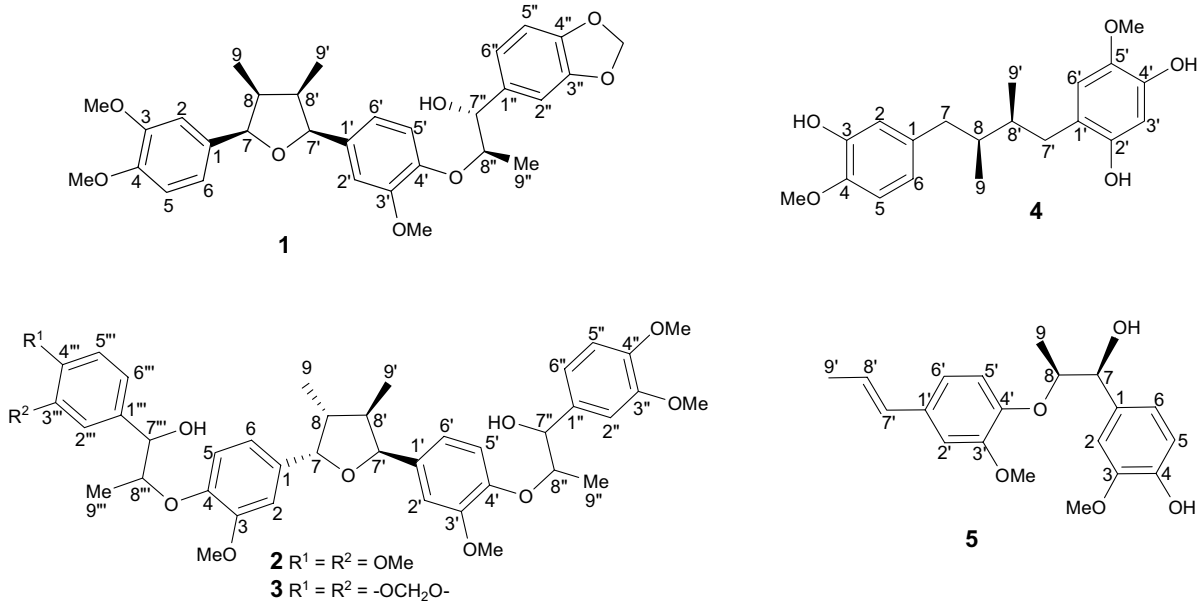

Figure 1. Chemical structures of **1**–**5** isolated from *S. chinensis*.

corresponding to compound **4** and the UV spectrum (MeOH) of **4** exhibited λ_{max} at 289 nm (ε 3500). The IR spectrum of **4** showed strong hydroxyl band at 3428 cm^{-1}. The ^1H NMR spectrum of **4** showed doublets at δ_H 0.82 and δ_H 0.84 corresponding to 6H that were attributed to two methyl groups (C-9 and C-9′) and multiplets at δ_H 1.75 corresponding 2H that were attributed to two methine groups (C-8 and C-8′). The signals at δ_H 3.74 (3H, s, C5′–OMe) and δ_H 3.79 (3H, s, C4–OMe) indicated the presence of two methoxy groups and showed HMBC correlations to two aromatic carbon signals at δ_H 56.4 (C-5′) and δ_H 57.2 (C-3). The positions and existence of hydroxyl groups were determined by HMBC correlations and D_2O exchangeable techniques, as shown in Table 1.

Compound **5** demonstrated a molecular formula of $C_{20}H_{24}O_5$ by HREIMS data (*m/z* $[M]^+$, 344.1628, calcd 344.1624 for $C_{20}H_{24}O_5$) and $[\alpha]_D^{25}$ − 50.0 (*c* 0.1, $CHCl_3$) (lit. $[\alpha]_D^{25}$ − 160.1 (*c* 0.7, $CHCl_3$), −88 (*c* 4.0, $CHCl_3$)).[12] The ^1H NMR spectrum of **5** revealed the presence of methyl protons at δ_H 1.09 (s, 3H, H-9), allylic methyl protons at δ_H 1.80 (s, 3H, H-9′), two methoxy protons at δ_H 3.81 (s, 3H), and δ_H 3.84 (s, 3H), the methine proton at δ_H 4.06 (m, 1H, H-8), a benzylic methine proton at δ_H 4.54 (d, 1H, H-7), and two vinyl protons at δ_H 6.09 (m, 1H, H-7′) and δ_H 6.28 (d, 1H, H-8′), respectively. Also, the structure of **5** was elucidated to be machilin D, comparing its spectroscopic data with reported data.[12]

Compounds **4** and **5** were isolated and evaluated in vitro for their potential to protect human LDL against Cu^{2+}-induced peroxidation.[13] The ability of compounds **4** and **5** to attenuate LDL oxidation was measured by measuring the amount of TBARS.[14] Effect of compound **4** on production of TBARS was examined by incubating human LDL (120 μg/mL) at the presence of 10 μM $CuSO_4$ as an oxidation initiator. Compounds **4** and **5** showed potent antioxidant activities with IC_{50} values of 3.3 μM and 3.8 μM, respectively. Then probucol was employed as a positive control (IC_{50} = 2.8 μM).

Table 1. ^1H and ^{13}C NMR spectral data and HMBC correlations for compound **4** in $CDCl_3$[a]

Position	δ_H[b]	δ_C[c]	HMBC
1		134.3	
2	6.53 s	111.9	C-3, C-4, C-6, C-7
3		146.9	
4		144.1	
5	6.70 d (7.9)	114.5	C-1, C-3, C-4
6	6.56 d (7.9)	122.3	C-2, C-4, C-5
7	2.39 dd (7.8, 13.6), 2.52 dd (7.0, 13.8)	41.7	C-1, C-2, C-6, C-8, C-8′, C-9
8	1.75 m	38.5	C-7, C-8′, C-9
9	0.84 d (7.1)	14.6	C-7, C-8, C-8′
1′		118.9	
2′		148.4	
3′	6.44 s	114.1	C-1′, C-2′, C-4′, C-5′, C-7′
4′		144.8	
5′		141.4	
6′	6.41 s	103.5	C-1′, C-2′, C-4′, C-5′
7′	2.31 dd (8.0, 13.9), 2.55 dd (6.8, 13.9)	35.5	C-1′, C-2′, C-3′, C-8′, C-8, C-9′
8′	1.75 m	37.0	C-7′, C-8, C-9′
9′	0.82 d (7.1)	14.7	C-7′, C-8′, C-8
–OCH_3	3.79 s	57.2	C-3
–OCH_3	3.74 s	56.4	C-5′
–OH	5.52 s (D_2O exchangeable)		
–OH	5.54 s (D_2O exchangeable)		
–OH	4.53 br s (D_2O exchangeable)		

[a] Chemical shift are shown in the δ scale with *J* values (Hz) in parentheses.
[b] 500 MHz in $CDCl_3$ at 25 °C.
[c] 125 MHz in $CDCl_3$ at 25 °C.

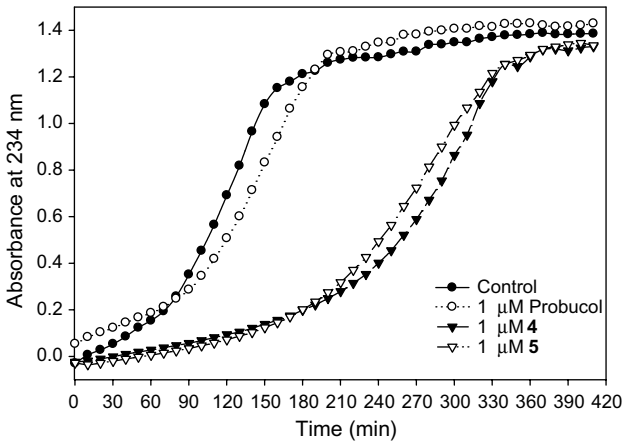

Figure 2. Effects of **4** and **5** on Cu^{2+}-mediated LDL oxidation. LDL (100 μg/mL) in PBS (pH 7.4) was incubated with 5 μM $CuSO_4$ at 37 °C in the presence or absence of antioxidants, **4** and **5**. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 7 h. Probucol was used as a reference antioxidant.

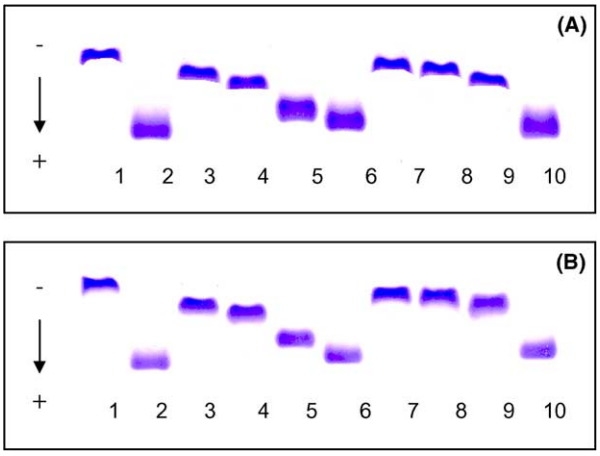

Figure 3. Effects of **4** (A) and **5** (B) on the Cu^{2+}-mediated oxidation and electrophoretic mobility of LDL. LDL (240 μg/mL in PBS) was incubated for 12 h at 37 °C with 5 μM $CuSO_4$. After incubation, approximately 2.0 μg of LDL protein was loaded onto 0.7% agarose gel for electrophoresis. The gel was stained with Coomassie brilliant blue R-250. (A) Lane 1: native LDL (absence of $CuSO_4$), lane 2: ox-LDL, lane 3: **4** (20 μM), lane 4: **4** (10 μM), lane 5: **4** (5 μM), lane 6: **4** (2 μM), lane 7: BHT(20 μM), lane 8: BHT (10 μM), lane 9: BHT (5 μM), lane 10: BHT (2 μM); (B) lane 1: native LDL (absence of $CuSO_4$), lane 2: ox-LDL, lane 3: **5** (20 μM), lane 4: **5** (10 μM), lane 5: **5** (5 μM), lane 6: **5** (2 μM), lane 7: BHT(20 μM), lane 8: BHT (10 μM), lane 9: BHT (5 μM), lane 10: BHT (2 μM).

Oxidation of the LDL was determined by measuring the conjugated diene formation at 234 nm[15] and typical effects of compounds **4** and **5** were shown in Figure 2. The formation of the conjugated diene during LDL oxidation represents the early peroxidation of the LDL. The LDL (120 μg protein/mL) in PBS buffer (pH 7.4, 10 mM) was incubated with 5 μM $CuSO_4$ alone to have a lag time of 85 min. When treated each 1.0 μM of **4** and **5**, the lag phase of LDL oxidation was retarded to 228 and 198 min, respectively, whereas, probucol extended lag time to 100 min. As a result, compounds **4** and **5** inhibited much more the formation of conjugated diene formation during Cu^{2+}-induced LDL oxidation than did probucol.

The effects of **4** and **5** on Cu^{2+}-mediated oxidation of LDL were determined by the relative electrophoretic mobility (REM), another parameter that is affected by LDL oxidation (Fig. 3).[16] The LDL was incubated with 5 μM $CuSO_4$ alone to induce the oxidation of LDL for 12 h (lane 2). The mobility of LDL in **4** and **5** was reduced dose dependently. When treated 20, 10, 5, and 2 μM of **4** (Fig. 3A, lanes 3–6), LDL oxidation was protected in 81%, 65%, 30%, and 19%, respectively, compared to that of oxidized LDL. Whereas the use of 20, 10, 5, and 2 μM of **5** (Fig. 3B, lanes 3–6) showed inhibition of LDL oxidation in 79%, 71%, 36%, and 14%, respectively, compared to that of oxidized LDL. Then, butylated hydroxytoluene (BHT), a positive control, inhibited the oxidation of LDL in 90–96% (at 20 μM), 86–90% (at 10 M), 77–79% (at 5 μM), and 14–15% (at 2 μM) as shown in Figure 3.

Oxidative inhibition of compounds **4** and **5** was evaluated by fragmentation of the apoB-100 through the electrophoretic analysis on 4% polyacrylamide gel in the presence of sodium dodecylsulfate (SDS-PAGE), because apoB-100 is a major component of the LDL.[17] As shown in Table 2, the densitometric values related to the areas of the peaks of the apoB-100 were expressed as absorbance units per millimeter for compounds **4** and **5** at 20 and 10 μM.[18] When the LDL (120 μg/mL in PBS) was incubated with $CuSO_4$ alone, band of the apoB-100 completely disappeared, whereas, in the presence of each 20 and 10 μM of **4** and **5**, the percentage of remaining apoB-100 against intact apoB-100 of native LDL were 76%, 64% for **4** and 67%, 58% for **5**, respectively. The percentage of remaining apoB-100 band in the presence of 20 and 10 μM of probucol was 66% and 43%, respectively.

Table 2. Antioxidant effects of **4** and **5** on the Cu^{2+}-mediated oxidation and apoB-100 fragmentation in LDL[a]

Compounds (μM)	Area[b] (AU/mm)
Native LDL	10.09
Ox-LDL	0
4 (20)	7.66
4 (10)	6.45
5 (20)	6.78
5 (10)	5.81
Probucol (20)	6.70
Probucol (10)	4.32

[a] LDL (120 μg/mL in PBS) was incubated for 4 h at 37 °C with 5 μM $CuSO_4$ in the absence or presence of 20 and 10 μM of **4**, **5**, and probucol. After incubation, approximately 2.0 μg of LDL protein was applied to SDS-PAGE (7.5%). After the electrophoresis, the gel was stained with Coomassie Brilliant blue R250 and subjected to densitometric scanning by Bio Rad Model GS-800 with Bio Rad Quantity One-4.4.0 software.

[b] Areas of the peaks of the apoB-100 expressed as absorbance units per millimeter.

Also, we were interested in antioxidant activities of compounds **4** and **5** on macrophage-mediated oxidation of

LDL. Differentiation of THP-1 cells to macrophage was induced by treatment of phorbol 12-myristrate 13-acetate (PMA) for 3 days.[19] In order to compare activities of Cu^{2+}-induced LDL oxidation with macrophage-mediated LDL oxidation, the LDL was incubated with 4 μM $CuSO_4$ alone to give the value of the ox-LDL (95.5 ± 9.5 MDA nmol/mg LDL protein), whereas the content of ox-LDL by incubation of LDL and 4 μM $CuSO_4$ in the presence of the macrophage was 468.7 ± 22.7 MDA nmol/mg LDL protein. The value of ox-LDL by macrophage-mediated LDL oxidation has proven to be much higher than that by Cu^{2+}-induced LDL oxidation. Therefore, antioxidant activities of compounds **4** and **5** were tested by macrophage-mediated LDL oxidation at 0.1 μM. These results are summarized in Table 3. In the concentration of each 0.1 μM of **4** and **5**, the content of ox-LDL was 358.9 ± 22.6 and 118.3 ± 0.2 MDA nmol/mg LDL protein, respectively. In the presence of 1.0 μM of probucol, a positive control, the content of ox-LDL was 412.6 ± 25.0 MDA nmol/mg LDL protein. As a result, antioxidant activities of compounds **4** and **5** were much higher than that of probucol in macrophage-mediated LDL oxidation (Table 3). Compounds **4** and **5** showed very similar antioxidant activities in the TBARS assay, the lag time of conjugated diene production, REM of ox-LDL, and the apoB-100 fragmentation on copper-mediated LDL oxidation, however, **5** exhibited more potent antioxidant activity in macrophage-mediated LDL oxidation. Even though the roles of **4** and **5** within the macrophage are not yet known, it may be influenced at lipophilicity of antioxidants that are through cell membrane. Also, in order to confirm whether antioxidant activity is influenced by cytotoxicity of **4** and **5** in macrophage-mediated LDL oxidation, cell viabilities for compounds **4** and **5** were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[20] Then, the viable cells were determined by amount of conversion of MTT to formazan (a blue colored product) by mitochondrial enzyme succinate-dehydrogenase existing at THP-1 macrophage-like cells. As shown in Figure 4, the viabilities of THP-1 macrophage-like cells were preserved more than 90% in the presence of 100 μM of **4** or **5**. Therefore, these results indicated that compounds **4** and **5** did not influence for cell toxicity to show antioxidant activity in macrophage-mediated LDL oxidation.

Table 3. Effects of **4** and **5** on macrophage-mediated LDL oxidation

Incubation conditions[a]	MDA nmol/mg LDL protein[b]
LDL+Cu^{2+}	95.5 ± 9.5*
LDL+cell+Cu^{2+} (control)	468.7 ± 22.7
LDL+cell+Cu^{2+}+0.1 μM **4**	358.9 ± 22.6*
LDL+cell+Cu^{2+}+0.1 μM **5**	118.3 ± 0.2*
LDL+cell+Cu^{2+}+1 μM probucol	412.6 ± 25.0

* $P < 0.01$ versus control.

[a] LDL (120 μg/mL) was incubated for 24 h at 37 °C in serum-free RPMI 1640 medium with 2 μM of Cu^{2+} in 12-well plate containing macrophages, in the absence (control) or presence of each 0.1 μM of compounds **4** and **5**. Probucol was used as a positive control.

[b] The extent of LDL oxidation was determined directly in the harvested medium using the TBARS assay. Data are shown as means ± SD ($n = 3$).

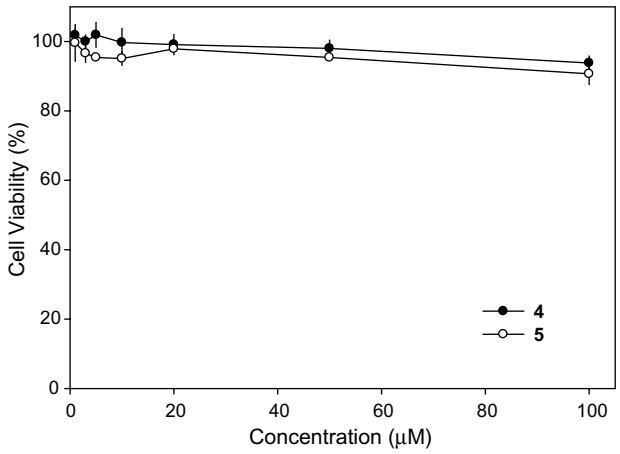

Figure 4. Cell viability as a percentage of control for THP-1 macrophage-like cells. Cells (1×10^5/well) were treated with **4** and **5** (1–100 μM) or without compound for 20 h and the cell viabilities were assessed by MTT assay. Data are shown as means ± SD ($n = 3$).

As an another parameter determining antioxidant activities, compounds **4** and **5** were evaluated for their radical 1,1-diphenyl-2-picrylhydrasyl (DPPH) scavenging activity.[21] The radical DPPH scavenging activity of compounds **4** and **5** were measured as decolorizing activity following the trapping of the unpaired electron of DPPH (Fig. 5).[22] After 20 min, 69% and 50% DPPH radicals, respectively, were remained at the presence of each 100 μM of compounds **4** and **5**, whereas only 18% DPPH radicals were remained at 100 μM of probucol. Therefore, compounds **4** and **5** have proven to have mild radical DPPH scavenging capacity compared to probucol. Also, we tested copper-chelating capacity of **4** and **5**, however, each maximum absorbance at 289 and 262 nm did not change even after 18 h incubation (data not shown). Therefore, the LDL-antioxidant activities of compounds **4** and **5** did not due to copper-chelating capacity.

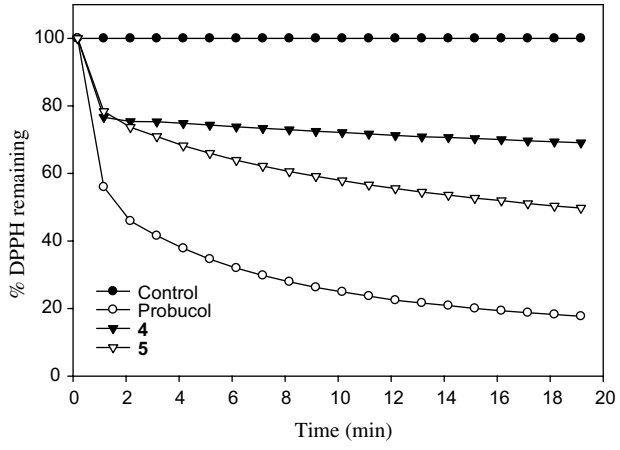

Figure 5. Effects of **4** and **5** on radical DPPH scavenging. Compounds **4** and **5** (100 μM) were incubated with 100 μM of DPPH in methanol at room temperature for 20 min. The absorbance at 517 nm of each compound solution was measured. The antiradical activity was expressed by the remaining DPPH percentage.

In conclusion, a new diarylbutane lignan, 2′-hydroxy dihydroguaiaretic acid (**4**), and a known 8-*O*-4′-type neolignan, machilin D (**5**) were isolated by bioassay-guided fractionation of ethyl acetate extracts from root of *S. chinensis*. Compounds **4** and **5** exhibited LDL-antioxidant activities in the TBARS assay, conjugated diene formation, REM of ox-LDL, fragmentation of apoB-100, macrophage-mediated LDL oxidation, and radical DPPH scavenging activities.

Acknowledgements

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11. Procedure for extraction and isolation. The dried root of *S. chinensis* (1.0 kg), which was collected in Guchang, Korea, was extracted three times each with EtOAc (4 L × 3) for 10 days. The EtOAc extracts (40 g) were subjected to silica gel column chromatography (230–400 mesh, Merck) with a gradient elution of *n*-hexane/EtOAc (9:1, 1.0 L; 9:5, 1.0 L; 1:1, 1.0 L; 5:9, 1.0 L; 1:9, 1.0 L) to afford five fractions. The active fraction 3 (3 g) was purified by column chromatography on SiO_2 with a gradient elution of $CHCl_3$/MeOH (100:1, 0.6 L; 98:2, 0.6 L; 97:3, 0.6 L; 96:4, 0.6 L, 96:5, 0.6 L) to obtain five fractions. The active fraction 4 (270 mg) was performed by column chromatography on SiO_2 with gradient elution of $CHCl_3$/acetone (100:1, 0.1 L; 9:1, 0.1 L; 8:2, 0.1 L; 7:3, 0.1 L, v/v) to give five fractions. The active fraction 3 (59 mg) was purified by reverse-phase column chromatography (ODS-A, RP-18; 70–230 mesh; YMC-gel), eluting successively with a gradient of aqueous MeOH (90%) to produce compound **4** (18 mg) as a brownish oil. Then, the purity of **4** was 95%, which was determined by reverse-phase HPLC under the following conditions: column, Hydrosphere-C_{18} (i.d. 4.6 × 250 mm, YMC Co. Ltd); mobile phase, MeOH/H_2O = 85:15; wavelength, 280 nm; flow rate, 1 mL/min, with t_R = 2.1 min. In the second fractionation step, the active fraction **2** (420 mg, eluted with $CHCl_3$/EtOAc 100:1, 0.1 L; 98:2, 0.1 L; 96:4, 0.1 L, 94:6, 0.1 L, 92:8, 0.1 L, 90:10, 0.1 L, v/v) was purified by column chromatography on SiO_2 to give compound **5** (48 mg) as a colorless oil.
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19. Cell culture and oxidative modification of LDL: Human monocytic THP-1 cells (ATCC) were cultured in RPMI 1640 medium (Gibco/BRL) with phenol red containing 10% fetal bovine serum (Gibco/BRL), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C under 5% CO_2 in air. Cells in RPMI 1640 medium with serum and antibiotics were plated in 12-well plates (1×10^6 cell per well in 1 mL). Differentiation of THP-1 cells to macrophage was induced by treatment of PMA (150 ng/mL, Sigma) for 3 days.[23] THP-1 macrophages were washed three times with serum-free RPMI 1640 media. To examine the effect of the compounds on macrophage-mediated LDL oxidation, cells were incubated with LDL (120 μg/mL) in the culture medium with or without compounds, supplemented with 2 μM $CuSO_4$ for 24 h at 37 °C. The extent of LDL oxidation was determined directly in the harvested medium using the TBARS assay.
20. Denizot, F.; Lang, R. *J. Immunol. Methods* **1986**, *89*, 271. Measurement of MTT conversion assay: THP-1 cells were plated at a density of 1×10^5 cell per well onto a 96-well plate. Differentiation of THP-1 cells to macrophage was performed described upper.[17] The THP-1 macrophage-like cells were incubated in the absence (control) and presence of compounds **4** and **5** (1–100 μM) in RPMI 1640 medium for 20 h at 37 °C in humidified CO_2 incubator. After incubation, 10 μL of 5 mg/mL (50 μg) of MTT was added to above 100 μL of cell and incubated 4 h at the same condition. Then, the MTT is converted into water-insoluble crystals of formazan, which was dissolved by the addition of 100 L of 10% SDS containing 0.01 M HCl. After 12 h, the absorbance was measured at 595 nm using a Microplate reader (Bio-Rad, Model 680).
21. (a) Graham, A.; Wood, J. L.; O'Leary, V. J.; Stone, D. *Free Rad. Res.* **1994**, *21*, 295; (b) Lesnik, P.; Dachet, C.; Petit, L.; Moreau, M.; Griglio, S.; Brudi, P.; Chapman, M. J. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 979; (c) Lin, K.-Y.; Chen, Y.-L.; Shih, C.-C.; Pan, J.-P.; Chan, W.-H.; Chiang, A.-N. *J. Cell. Biochem.* **2002**, *86*, 258.

22. Blois, M. S. *Nature* **1958**, *181*, 1199.
23. Zhou, K.; Yu, L. *J. Agric. Food Chem.* **2004**, *52*, 1112. Radical DPPH scavenging activity: Radical DPPH scavenging activity was studied by methanol solution of compounds **4**, **5**, and probucol, a positive control, at 100 μM. Fresh made DPPH radical solution (2 mL, final concn 100 μM) was added into each 1 mL of **4**, **5**, and probucol. The absorbance of DPPH radical remaining was measured at 517 nm against a blank of pure methanol including only DPPH radical for 20 min using the UV–vis spectrophotometer at room temperature. Radical DPPH scavenging capacity was calculated from the difference in absorbance with **4**, **5**, and probucol and expressed as percent DPPH radical remaining, according to the following equation: DPPH remaining=100 ×(absorbance of sample/absorbance of control).