Low Density Lipoprotein (LDL)-Antioxidant Flavonoids from Roots of *Sophora flavescens*

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Oxidation of low density lipoprotein (LDL) is strongly implicated as a key process in the onset of atherosclerosis. In this study, nine alkylated (C_{10}–C_5) flavonoids from *Sophora flavescens* were examined for their inhibitory effects on copper-induced LDL oxidation. Of the flavonoids tested, sophoraflavanone G (1), kurarinone (2), kurarinol (3), norkurarinol (4), and kuraridin (9) inhibited the generation of thiobarbituric acid reactive substances (TBARS) with IC_{50}s of 7.9, 14.5, 22.0, 26.9, and 17.5 μM, respectively. The most potent inhibitor, compound 1, also demonstrated significant activities in complementary *in vitro* investigations, such as lag time (130 min at 5 μM), relative electrophoretic mobility (REM) of ox-LDL (80% inhibition at 20 μM), and fragmentation of apoB-100 (inhibition of 71% at 20 μM). Analysis of the structures of these compounds reveals that a resorcinol moiety in the B-ring is strongly correlated with protection of LDL-oxidation.

Key words *Sophora flavescens*; low density lipoprotein oxidation; sophoraflavanone G; kurarinone; resorcinol

Oxidative stress caused by reactive oxygen species (ROS) threatens the integrity of various biomolecules including proteins, lipids and DNA. These degradation pathways have been implicated in a number of degenerative processes, including ageing, Alzheimer's disease and atherosclerosis. In the case of the latter, oxidative stress is believed to be involved through the formation of oxidized low density lipoprotein (ox-LDL). When LDL is oxidized by ROS, it is modified in several ways and the build-up of oxidized LDL within arterial walls promotes several steps in atherosclerosis,[1,2] including endothelial cell damage.[3] Moreover monocyte-derived macrophages recognize and absorb ox-LDL through scavenger receptors, which unlike Brown/Goldstein LDL receptors are unregulated. These results in massive accumulation of lipids and the proliferation of foam cells.[4] For these reasons, LDL-antioxidants are of great interest, with particular attention focused on therapeutic strategies to prevent atherosclerosis.

In order to find out the crucial component of naturally-derived remedies believed to serve for the prevention or treatment of atherosclerosis, many polyphenols with LDL-antioxidant activity have been isolated from various fruits, vegetables, and plants.[5] Recently, we also reported that lignan, neolignan, and xanthone isolated from *Myristica fragrans* and *Cudrania tricuspidata* inhibit LDL oxidation.[6,7] In our continued screening of cholesterol-lowering, antiatherosclorotic agents, we found that the methanolic extracts of roots of *S. flavescens* exhibited significant LDL-antioxidant activity.

Sophora flavescens, which is known as the Chinese drug 'kushen' has been used as a stamachic, antipyretic, analgesic, and insecticide.[8] Biological investigations have mainly focused on its antibacterial, cytotoxic, tyrosinase inhibitory, and glycosidase inhibitory properties.[9—12] Although the antioxidant benefits of flavonoids from *S. flavescens* have been reported in a few cases,[13,14] there is no report as to whether the compounds inhibit LDL oxidation and thus their utility toward atherosclerosis treatment is currently not known.

In this study, we isolated 9 compounds from the roots of *S. flavescens* and their structures were identified using spectroscopic methods. The isolated compounds were evaluated for their inhibitory activity against copper-induced LDL oxidation by four methods: thiobarbituric acid reactive substances (TBARS) assay,[15] measurement of the formation of conjugated dienes,[16] relative electrophoretic mobility (REM),[17] and fragmentation of apoB-100.[18]

MATERIALS AND METHODS

General All purifications were monitored by TLC using commercially available glass-backed plates. Column chromatography was carried out using 230—400 mesh silica gel. Melting points were measured on a Thomas Scientific capillary melting point apparatus (Electrothermal 9300, U.K.) and are uncorrected. IR spectra were recorded on a Bruker IFS66 (Bruker, Karlsruhe, Germany) IR Fourier transform spectrophotometer (KBr). ^{1}H- and ^{13}C-NMR as well as 2D-NMR data were obtained on a Bruker AM 500 (^{1}H-NMR at 500 MHz, ^{13}C-NMR at 125 MHz) spectrometer (Bruker, Karlsruhe, Germany) in either $CDCl_3$, acetone-d_6, and CD_3OD. Optical rotation values were measured by a Perkin-Elmer 343 polarimeter and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. Chromatographic separation was achieved using an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a quaternary HPLC pump, a degasser, and autosampler and UV detector (PDA). All the reagent grade chemicals were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Plant Material *Sophora flavescens* was collected in Hamyang (Korea) and identified by Prof. Myong Gi Chung. A voucher specimen (Park, K. H. 112) of this raw material is deposited at Herbarium of Gyeongsang National University

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(GNU).

Extraction and Isolation Dried roots of *Sophora flavescens* (2.4 kg) were repeatedly extracted with methanol at room temperature. The combined extracts were filtered and concentrated to yield a dark brown residue, which was partitioned between water and chloroform. The organic layer was concentrated to give a brown residue (53 g). The chloroform extract was column chromatographed, eluted with a mixture of hexane/EtOAc, of increasing polarity (20 : 1→1 : 10). Seven pooled fractions (Fr. I—Fr. VII) were obtained after combining fractions with similar TLC profiles from this initial purification step. Repeated column chromatography of these pooled fractions on silica gel furnished 230 mg of compound **1**, 270 mg of compound **2**, 60 mg of compound **3**, 30 mg of compound **4**, 410 mg of compound **5**, 170 mg of compound **6**, 210 mg of compound **7**, 40 mg of compound **8**, and 97 mg of compound **9**.[12)]

Isolation of LDL Blood was obtained from healthy volunteers who had given their consent according to the 'Guidelines of Blood Donation Program for Research' of the Korean Red Cross Blood Center. EDTA was used as an anticoagulant (1.5 mg/ml of blood). After low speed centrifugation of the whole blood to obtain plasma and to prevent lipoprotein modification, 0.1% EDTA, 0.05% NaN_3, and 0.015% PMSF (phenylmethanesulfonyl fluoride) were added. LDL (1.019—1.063 g/ml) was isolated from the plasma by sequential density ultracentrifugation at 4 °C in a Beckman TL ultracentrifuse (Beckman Instruments, Mountain View, CA, U.S.A.) as described previously.[19)] After isolation, LDL was dialyzed overnight against three changes of phosphate buffer (pH 7.4), containing 150 mM NaCl, in the dark at 4 °C to remove EDTA. The LDL in phosphate-buffered saline (PBS) was stored at 4 °C and used within 4 weeks.

Cu^{2+}-Induced LDL Oxidation and TBARS Assay The TBARS assay of Buege and Aust[15)] was used with some modifications. Briefly, a solution of LDL (250 μl, 120 μg of protein) in PBS (10 mM, pH 7.4, 0.15 M NaCl) was supplemented with 10 mM $CuSO_4$. The oxidation was performed in a screw-capped 5 ml glass vial at 37 °C in the presence or absence of test compounds. After 4 h incubation, the reaction was terminated by addition of 1 ml of 20% trichloroacetic acid. Following precipitation, 1 ml of 0.67% thiobarbituric acid in 0.05 N NaOH was added and vortexed, and the final mixture was heated for 5 min at 95 °C, then cooled on ice, and centrifuged for 2 min at 1000×***g***. The optical density of the produced malondialdehyde (MDA) was measured at 532 nm.

Conjugated Diene Formation The formation of conjugated diene was measured by monitoring the absorbance at 234 nm using the modification method.[16)] Briefly, LDL (120 mg/ml), in PBS (pH 7.4) was incubated with 5 μM aqueous $CuSO_4$ in the presence or absence of test compounds (**1**—**9**), at 37 °C for 4 h; thereafter, the absorbance at 234 nm was measured every 10 min. The lag time was measured as the intercept between the baseline and the tangent of the absorbance curve during the propagation phase.

Relative Electrophoretic Mobility (REM) The electrophoretic mobility of native or oxidized LDL was detected by agarose gel electrophoresis (Ciba Corning Diagnostics, Palo Alto, CA, U.S.A.) using the method of Reid and Mitchinson.[17)] The LDL (120 μg/ml) in PBS (pH 7.4) was oxidized with 5 mM $CuSO_4$ for 12 h at 37 °C in the presence or absence of test compounds (**1**—**9**). Thereafter, the mixture was electrophoresed (85 V) on an agarose gel (0.7% agarose) in a buffer containing 40 mM Tris, 40 mM glacial acetic acid, and 1 mM EDTA for 1 h. After electrophoresis, lipoprotein bands were stained with coomassie blue; REM was defined as the ratio of the migrating distance of oxidized LDL to that of the control.

Electrophoresis of Fragmented ApoB-100 The degree of fragmentation of apoB-100 was evaluated using electrophoresis as described previously.[18)] After the oxidation in the presence or absence of antioxidants, samples were denatured with 3% SDS, 10% glycerol, and 5% 2-mercaptoethanol at 95 °C for 10 min. SDS-polyacrylamide gel electrophoresis (SDS PAGE, 3—5% gradient) was performed to detect the apoB-100 fragmentation. The gel was subjected to electrophoresis at 48 V for 150 min. After the electrophoresis, the gel was dried and stained with coomassie brilliant blue R250 and subjected to densitometric scanning by a Bio Rad® Model GS-800 with Bio Rad® Quantity One-4.4.0 software.

HPLC Apparatus and Chromatographic Conditions The pulverized roots of *S. flavescens* (1.0 g) were extracted with 40 ml MeOH using sonication at 60 °C. The extracts used for HPLC analysis were passed through 0.45-μm filters (Millipore, MSI, Westboro, MA, U.S.A.). HPLC separation of isolated polyphenols was performed on a Agilent 1100 series instrument equipped with PDA using a C_{18} column [LichiroCART 125-4 HPLC-Cartridge (Lichrphore 100 RP-18e, φ 5 μm, Merck)]. The mobile phase for HPLC consisted of solvent A, 0.1% acetic acid in water, and solvent B, 0.1% acetic acid in acetonitrile. The solvent gradient was as follows (relative to solvent A): 0 min, 30% B; 25 min, 60% B; 35 min, 100% B. The flow rate was 1.0 ml/min and the injection was volume 10 μl. The eluent was detected at 286 nm and all HPLC analyses were performed at 30 °C.

Data Analysis All values are expressed as mean standard deviation (S.D.) of two independent experiments performed in duplicate. Statistical analysis was performed using a *t*-test. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The methanol (95%) extract of *Sophora flavescens* at 40 μg/ml caused 80% inhibition of LDL-oxidation (TBARS assay). Subsequent bioactivity-guided fractionation of the MeOH extracts led to the isolation of nine compounds: seven flavanones (**1**—**7**), one flavanol (**8**), and one chalcone (**9**). The structures of compounds **1**—**9** were identified as sophoraflavanone G (**1**), kurarinone (**2**), kurarinol (**3**), norkurarinol (**4**), kushenol A (**5**), (2*S*)-2′-methoxykurarinone (**6**), isoxanthohumol (**7**), 3,7,4′-trihydroxy-5-methoxy-8-prenylflavanone (**8**), and kuraridin (**9**) as shown in Table 1 on the basis of spectroscopic analyses. The physical and spectroscopic data of **1**—**9** agree with the previously reported data.[12,20—24)] Herein, we report some important physical data including ^{13}C-NMR spectroscopic data.

Sophoraflavanone G (**1**): Amorphous white powder; mp 178—180 °C; EI-MS *m*/*z* (relative intensity): 424 (M^+, 11.5%), 301 (79.7), 283 (100), 219 (14), 165 (79.1); [α]

−71.4° (c=2.0, MeOH); UV λ_{max} nm: 292, 332 (MeOH); ^{13}C-NMR (125 MHz, CD_3OD) δ: 76.2 (d, C-2), 43.7 (t, C-3), 199.4 (s, C-4), 163.6 (s, C-5), 96.7 (d, C-6), 166.9 (s, C-7), 109.1 (s, C-8), 103.7 (s, C-4a), 163.0 (s, C-8a), 118.8 (s, C-1′), 157.1 (s, C-2′), 108.1 (d, C-3′), 159.9 (s, C-4′), 103.8 (d, C-5′), 129.1 (d, C-6′), 28.4 (t, C-1″), 48.7 (d, C-2″), 32.8 (t, C-3″), 125.2 (d, C-4″), 132.4 (s, C-5″), 18.3 (q, C-6″), 26.3 (q, C-7″), 150.1 (s, C-8″), 19.6 (q, C-9″), 111.6 (t, C-10″).[20,21]

Kurarinone (**2**): Colorless powder; mp 117—119 °C; EI-MS m/z (relative intensity): 438 (M^+, 1.9%), 436 (2.6), 418 (16.7), 299 (100), 297 (29), 179 (27.7), 153 (33.6); [α] −35.2° (c=2.0, MeOH); UV λ_{max} nm: 286, 371 (MeOH); ^{13}C-NMR (125 MHz, CD_3OD) δ: 75.9 (d, C-2), 46.0 (t, C-3), 194.3 (s, C-4), 162.3 (s, C-5), 93.7 (d, C-6), 165.3 (s, C-7), 110.0 (s, C-8), 106.2 (s, C-4a), 165.1 (s, C-8a), 118.9 (s, C-1′), 157.7 (s, C-2′), 108.1 (d, C-3′), 159.9 (s, C-4′), 103.8 (d, C-5′), 128.9 (d, C-6′), 28.6 (t, C-1″), 48.6 (d, C-2″), 32.8 (t, C-3″), 125.2 (d, C-4″), 132.4 (s, C-5″), 18.2 (q, C-6″), 26.3 (q, C-7″), 150.2 (s, C-8″), 19.6 (q, C-9″), 111.6 (t, C-10″), 56.3 (C5–OCH_3).[22]

Kurarinol (**3**): Amorphous yellow powder; mp 166—169 °C; [α] −40° (c=1.0, MeOH); UV λ_{max} nm: 287, 367 (MeOH); ^{13}C-NMR (125 MHz, CD_3OD) δ: 75.9 (d, C-2), 46.0 (t, C-3), 194.4 (s, C-4), 106.2 (s, C-4a), 162.3 (s, C-5), 93.8 (d, C-6), 165.3 (s, C-7), 109.9 (s, C-8), 165.1 (s, C-8a), 118.8 (s, C-1′), 157.1 (s, C-2′), 128.9 (d, C-3′), 159.9 (s, C-4′), 103.8 (d, C-5′), 108.1 (d, C-6′), 29.1 (t, C-1″), 49.3 (d, C-2″), 28.5 (t, C-3″), 43.2 (t, C-4″), 71.9 (s, C-5″), 29.3 (q, C-6″), 29.6 (q, C-7″), 150.0 (s, C-8″), 15.9 (q, C-9″), 112.0 (t, C-10″), 56.4 (C5–OCH_3).[12]

Norkurarinol (**4**): Colorless powder; mp 102—104 °C; EI-MS m/z (relative intensity): 442 (M^+, 1.9%), 301 (76.8), 283 (100), 165 (67.9); [α] −75.8° (c=0.5, MeOH); ^{13}C-NMR (125 MHz, acetone-d_6) δ: 75.8 (d, C-2), 43.0 (t, C-3), 198.6 (s, C-4), 163.4 (s, C-5), 96.7 (d, C-6), 165.8 (s, C-7), 108.3 (s, C-8), 118.3 (s, C-4a), 162.5 (s, C-8a), 108.4 (s, C-1′), 156.7 (s, C-2′), 104.0 (d, C-3′), 159.8 (s, C-4′), 103.7 (d, C-5′), 129.1 (d, C-6′), 43.0 (t, C-1″), 48.6 (d, C-2″), 28.6 (t, C-3″), 28.1 (t, C-4″), 70.9 (s, C-5″), 30.1 (q, C-6″), 29.9 (q, C-7″), 149.6 (s, C-8″), 19.1 (q, C-9″), 111.8 (t, C-10″).[23]

Kushenol A (**5**): Amorphous white powder; mp 175—178 °C; EI-MS m/z (relative intensity): 408 (M^+, 9.9%), 285 (100), 267 (68.9), 165 (63.7); [α] −106.5° (c=2.0, MeOH); UV λ_{max} nm: 224, 291 (MeOH); ^{13}C-NMR (125 MHz, CD_3OD) δ: 76.3 (d, C-2), 43.6 (t, C-3), 199.0 (s, C-4), 163.7 (s, C-5), 96.9 (d, C-6), 167.0 (s, C-7), 109.1 (s, C-8), 103.7 (s, C-4a), 163.8 (s, C-8a), 127.6 (s, C-1′), 155.6 (s, C-2′), 116.7 (d, C-3′), 130.6 (d, C-4′), 121.0 (d, C-5′), 127.6 (d, C-6′), 28.4 (t, C-1″), 48.7 (d, C-2″), 32.7 (t, C-3″), 125.1 (d, C-4″), 132.5 (s, C-5″), 18.2 (q, C-6″), 26.2 (q, C-7″), 150.1 (s, C-8″), 19.7 (q, C-9″), 111.6 (t, C-10″).[12]

(2*S*)-2′-Methoxykurarinone (**6**): Pale yellow powder; mp 112—115 °C; EI-MS m/z (relative intensity): 452 (M^+, 6.3%), 329 (32.5), 179 (100); [α] −35.4° (c=2.0, MeOH); UV λ_{max} nm: 277, 288 (MeOH); ^{13}C-NMR (125 MHz, CD_3OD) δ: 75.9 (d, C-2), 46.0 (t, C-3), 194.3 (s, C-4), 106.2 (s, C-4a), 165.3 (s, C-5), 93.7 (d, C-6), 165.3 (s, C-7), 110.0 (s, C-8), 165.1 (s, C-8a), 118.9 (s, C-1′), 157.1 (s, C-2′), 108.1 (d, C-3′), 159.9 (s, C-4′), 103.8 (d, C-5′), 128.9 (d, C-6′), 28.6 (t, C-″), 48.6 (d, C-2″), 32.8 (t, C-3″), 125.2 (d, C-4″), 132.4 (s, C-5″), 18.2 (q, C-6″), 26.3 (q, C-7″), 150.2 (s, C-8″), 19.6 (q, C-9″), 111.6 (t, C-10″), 56.3 (OCH_3), 56.2 (OCH_3).[12,22]

Isoxanthohumol (**7**): Amorphous white powder; mp 196—208 °C; EI-MS m/z (relative intensity): 354 (M^+, 100%), 311 (49.9), 299 (19.5), 217 (47.2), 191 (43.8), 179 (91.5); [α] −19.6° (c=2.0, MeOH); UV λ_{max} nm: 286, 368 (MeOH); ^{13}C-NMR (125 MHz, CD_3OD) δ: 80.4 (d, C-2), 46.6 (t, C-3), 193.3 (s, C-4), 106.3 (s, C-4a), 162.2 (s, C-5), 93.9 (d, C-6), 164.7 (s, C-7), 110.4 (s, C-8), 164.2 (s, C-8a), 132.1 (s, C-1′), 129.1 (s, C-2′), 116.7 (d, C-3′), 159.2 (s, C-4′), 116.5 (d, C-5′), 129.3 (d, C-6′), 23.1 (t, C-1″), 124.3 (d, C-2″), 132.0 (s, C-3″), 18.3 (q, C-4″), 26.4 (q, C-5″), 56.4 (C5–OCH_3).[12]

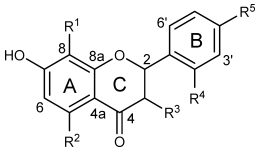
3,7,4′-Trihydroxy-5-methoxy-8-prenylflavanone (**8**): Colorless powder; mp 194—198 °C; EI-MS m/z (relative intensity): 370 (M^+, 3.2%), 354 (65.9), 311 (44), 219 (39.5), 179 (100); [α] −4.2° (c=0.5, MeOH); ^{13}C-NMR (125 MHz, CD_3OD) δ: 74.0 (d, C-2), 84.2 (d, C-3), 191.7 (s, C-4), 161.5 (s, C-5), 94.2 (d, C-6), 163.5 (s, C-7), 109.6 (s, C-8), 103.8 (s, C-4a), 163.1 (s, C-8a), 131.7 (s, C-1′), 130.5 (d, C-2′), 116.2 (d, C-3′), 159.0 (s, C-4′), 116.2 (d, C-5′), 130.5 (d, C-6′), 22.7 (t, C-1″), 123.9 (d, C-2″), 130.1 (s, C-3″), 18.2 (q, C-4″), 26.2 (q, C-5″), 56.4 (C5-OCH_3).[24]

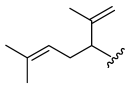
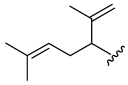
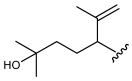
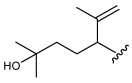
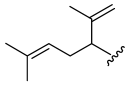
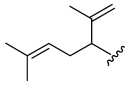
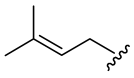
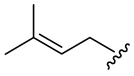
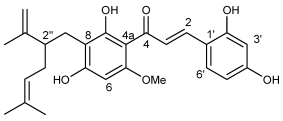
Kuraridin (**9**): Amorphous yellow powder; mp 114—116 °C; EI-MS m/z (relative intensity): 438 (M^+, 2.7%), 299 (26.3), 297 (13.3), 179 (24.9), 153 (100); [α] −1.0° (c=0.5, MeOH); ^{13}C-NMR (125 MHz, CD_3OD) δ: 140.2 (d, C-2), 125.9 (d, C-3), 195.2 (s, C-4), 162.9 (s, C-5), 92.0 (d, C-6), 164.4 (s, C-7), 109.3 (s, C-8), 107.0 (s, C-4a), 109.3 (s, C-8a), 160.7 (s, C-1′), 116.7 (s, C-2′), 109.4 (d, C-3′), 162.8 (s, C-4′), 104.1 (d, C-5′), 132.0 (d, C-6′), 28.6 (t, C-1″), 48.2 (d, C-2″), 32.8 (t, C-3″), 125.5 (d, C-4″), 132.2 (s, C-5″), 18.3 (q, C-6″), 26.3 (q, C-7″), 150.3 (s, C-8″), 111.5 (t, C-9″), 19.5 (q, C-10″), 56.5 (C5–OCH_3).[12]

Initially, compounds **1**—**9** were measured in order to compare their LDL-antioxidant activities using the TBARS assay, which is a good general method to measure the amount of oxidized lipid present in a medium. The results are summarized in Table 1. Among of them, compound **1** bearing 5,7-dihydroxy and 2′,4′-dihydroxy groups showed the highest LDL-antioxidant activity (IC_{50}=7.9 μM). On the other hand compound **6**, the direct analogue of **1** with hydroxyl functions at positions 5 and 2′ capped with methyl groups, was significantly less active. Compound **5** which possess the same functionality in the A-ring as compound **1**, but has a lone hydroxyl group in the B-ring, displayed very low activity. Interestingly, C5-*O*-methylated derivative **2**, exhibited a two folds lower activity than compound **1**. Compounds **7** and **8** which possess the same structure as compound **2** in the A-ring, but contain only a 5′-hydroxy group in the B-ring were practically inactive against LDL-oxidation. Perhaps most poignantly, inactive flavonoids (**5**—**8**) differed from the most effective inhibitors screened, flavanones **1** and **2**, only in the fact that they lacked a resorcinol moiety within the B ring.

In terms of structure–activity relationship, these results clearly implicate the resorcinol moiety within the B-ring as the key functional element: it was present in all active flavonoids (**1**—**4**) and absent from the inactive one. To investigate this we also assayed chalcone **9**. This compound, despite being structurally very different from the flavones studied above, nonetheless contains a resorcinol-type appendage and thus should show activity. Indeed, **9** was found to have an

Table 1. Inhibitory Effects of Isolated Compounds **1**—**9** on Cu^{2+}-Mediated LDL Oxidation by Measurement of TBARS Assay



Compound	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM) values[a]
1		OH	H	OH	OH	7.9
2		OMe	H	OH	OH	14.5
3		OMe	H	OH	OH	22.0
4		OH	H	OH	OH	26.9
5		OH	H	OH	H	46.9±1.6[b]
6		OMe	H	OMe	OH	16.2±0.3[b]
7		OMe	H	H	OH	6.3±0.9[b]
8		OMe	OH	H	OH	14.2±3.8[b]
9		—	—	—	—	17.5

a) All compounds were examined in triplicate; IC_{50} values of compounds represent the concentration that caused 50% inhibition of LDL antioxidant activity. *b*) Percentage at 40 μM.

IC_{50} of 17.5 μM. The preeminence of the resorcinol moiety can be accounted for at least in part by the fact that it is highly electron-rich and thus may serve as a sacrificial oxidant. On the other hand, the 5-hydroxy group within the A-ring is not mandatory for high activity. For instance, compounds **1** and **2** only differ by a factor of **2** despite the latter being C5-*O*-methylated. The alkyl group on the A-ring (R^1) was also shown to confer most potency when it was more hydrophobic as shown by compound **1** *vs.* compound **4**. On the basis of these results, compounds **1** and **2** were selected for further studies as they displayed the most potent LDL-antioxidant activity.

The formation of conjugated dienes constituents the first step of LDL oxidation. Monitoring diene formation (234 nm) as a function of time can elucidate the relative resistance of LDL to oxidation. As shown in Fig. 1, LDL (120 μg/ml) incubated with 5 μM of $CuSO_4$ had a lag time of 58 min (control). At an inhibitor concentration of 5 μM, the lag time was extended to 130 min in the presence of **1** and 115 min in the presence of **2**. These data correlate with the activities found in the TBARS assay.

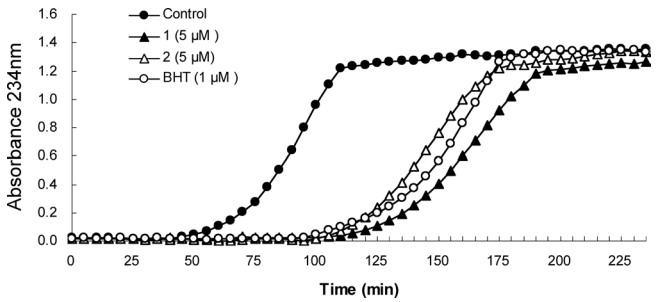


Fig. 1. Effects of Compounds **1** and **2** on the Generation of Conjugated Diene

Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 4 h. Butylated hydroxytoluene (BHT) was used as a reference antioxidant. Values shown are a representative of 2 experiments.

We progressed to examine the effect of **1** and **2** on Cu^{2+}-mediated LDL oxidation using a REM assay. As shown in Fig. 2, incubation of the enzyme with $CuSO_4$ for 12 h re-

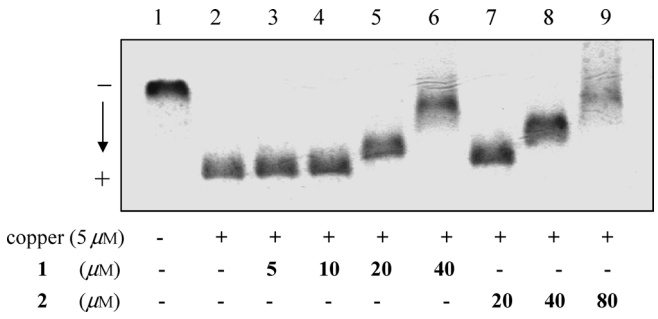


Fig. 2. Effect of Compounds **1** and **2** on Relative Electrophoretic Mobility of LDL

Lane 1, native LDL (absence of $CuSO_4$); lane 2, ox-LDL; lane 3, **1** (5 μM); lane 4, **1** (10 μM); lane 5, **1** (20 μM); lane 6, **1** (40 μM); lane 7, **2** (20 μM); lane 8, **2** (40 μM); lane 9, **2** (80 μM).

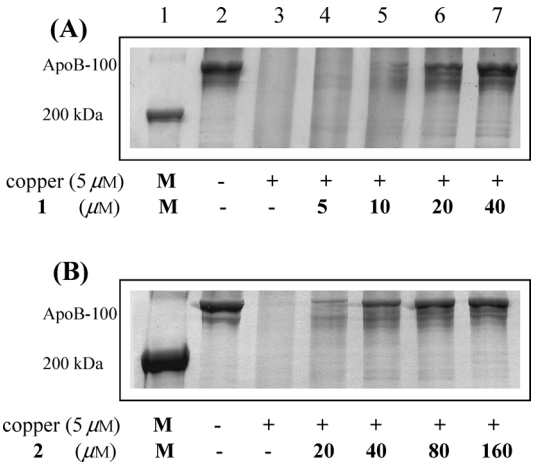


Fig. 3. Antioxidant Effects of Compounds **1** and **2** on the ApoB-100 Fragmentation

(A) Lane 1, molecular weight marker; lane 2, native LDL (absence of $CuSO_4$); lane 3, ox-LDL; lane 4, **1** (5 μM); lane 5, **1** (10 μM); lane 6, **1** (20 μM); lane 7, **1** (40 μM); M (marker); (B) lane 1, molecular weight marker; lane 2, native LDL (absence of $CuSO_4$); lane 3, ox-LDL; lane 4, **2** (20 μM); lane 5, **2** (40 μM); lane 6, **2** (80 μM); lane 7, **2** (160 μM); M (marker).

sulted in significant oxidation of the enzyme. As characterized by a significant change in the protein's REM. We were able to attenuate this effect considerably (up to 80%) by the addition of compound **1**. This was shown to be dose-dependent across an 8-fold concentration range. In the case of compound **2**, results were similar. However, in line with its lower potency, a larger concentration was required to engender a similar effect.

The inhibition of the oxidative process of compounds **1** and **2**, and probucol were evaluated also by the fragmentation of lipoprotein apoB-100 through analysis *via* sodium dodecylsulfate polyacrylamide gel (4%) electrophoresis (SDS-PAGE). When LDL (120 μg/ml in 10 mM PBS, pH 7.4) was incubated in the absence of $CuSO_4$, a band corresponding to the native protein was clearly visible. However, this band completely disappeared when LDL (120 μg/ml in PBS) was incubated with 5 μM of $CuSO_4$. We were able to retard this degradation process by adding inhibitor **1**. Again this was shown to be dose dependent, with >20 μM eliciting a significant effect. Compound **2** also showed similar but less pronounced activity. Table 2 shows the densitometric analysis of this data: in the presence of 20 and 40 μM of **1**, the percentage of remaining apoB-100 relative to intact apoB-100 was 71% and 109%, respectively. Thus, compound **1** has potent activity in the protection of apoB-100 fragmentation against copper-mediated oxidation of LDL.

Table 2. Oxidative Modification on SDS-PAGE of ApoB-100

Compound (μM)	Protection (%)[a)]
Native LDL	100
Ox-LDL	0
1 (20)	71
1 (40)	109
2 (40)	56
2 (80)	80
2 (160)	89

a) Densitometric analysis of the apoB-100 expressed as relative protection % on the basis of native LDL.

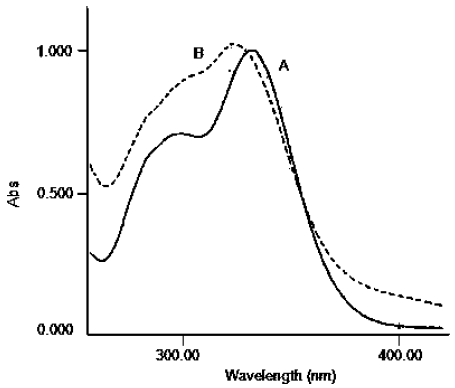


Fig. 4. UV–vis Spectrum of Compound **1** (50 μM), (A) without and (B) with $CuSO_4$ (100 μM)

LDL-oxidation was caused by a catalytic amount of copper(II) ions in solution. We accordingly speculated that the antioxidant effect could be attributed to the resorcinol moiety within the inhibitors chelating the copper. Consistent with this hypothesis, when compound **1** (50 μM) and copper(II) (100 μM) were mixed together, a 20 nm blue shift (338→320 nm) was observed at UV–vis spectrum of the inhibitor (Fig. 4). This indicates that the copper ion interacts with the inhibitor.

The quantity of active component contained within the plant source in one further important factor with which to evaluate neutraceuticals. Thus, the relative proportion of each of the isolated compounds was confirmed by HPLC analysis (C_{18}-column) (Fig. 5). The chromatographic profile implies that the most active LDL-antioxidants (**1**, **2**) detailed within this study are the predominant secondary metabolites of *Sophora flavescens*.

In summary, the bioassay-guided fractionation from methanol extract of *S. flavescens* yielded nine compounds, and among them, five [(**1**—**4** (flavonoids), **9** (chalcone)] showed LDL-antioxidant activities in TBARS assay with IC_{50}s of 7.9, 14.5, 22.0, 26.9, and 17.5 μM, respectively. From this data we conclude that a resorcinol moiety in the B-ring is pivotal for LDL-antioxidant activity. The most active species, compounds **1** and **2**, also exhibited potent dose-dependent activities in complementary *in vitro* investigation, such as lag time in diene formation, REM of ox-LDL, and fragmentation of apoB-100 on copper-mediated LDL oxidation. With re-

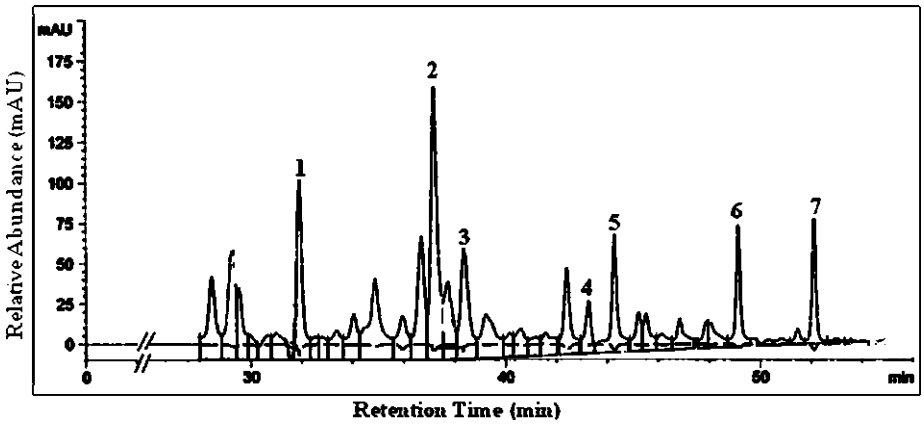


Fig. 5. Representative HPLC Chromatogram of Polyphenols in *S. flavescens*

1: isoxanthohumol (**7**) (retention time: 31.89 min), 2: kurarinone (**2**) (37.17 min), 3: kurarinol (**3**) (38.36 min), 4: (2*S*)-2′-methoxykurarinone (**6**) (43.24 min), 5: sophoraflavanone G (**1**) (44.26 min), 6: kuraridin (**9**) (49.12 min), 7: kushenol A (**5**) (52.10 min).

gard to structure–activity relationship, it has been demonstrated that resorcinol moiety of B-ring is a critical functionality for LDL-antioxidant.

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