Inhibitory Effect of Coumarins from *Weigela subsessilis* on Low Density Lipoprotein Oxidation

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Oxidation of low density lipoprotein (LDL) is thought to be a major factor in the pathophysiology of atherosclerosis. In the present study, we found that coumarins isolated from *Weigela subsessilis* (Caprifoliaceae) inhibited LDL oxidation mediated by either catalytic copper ions (Cu^{2+}) or free radicals generated with the azo compound 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH). Of the coumarins tested, scopoletin (1) and cleomiscosin A (2) increased the lag time of conjugated diene formation and inhibited the generation of thiobarbituric acid reactive substances (TBARS) in a dose-dependent manner. In addition, it was found that compounds 1 and 2 had the capacity to protect the fragmentation of apolipoprotein B-100 (apoB-100). These results suggest that *W. subsessilis* and its active coumarins, 1 and 2, may have a role to play in preventing the LDL oxidation involved in atherogenesis.

Key words *Weigela subsessilis*; coumarin; scopoletin; cleomiscosin A; LDL oxidation; apolipoprotein B-100

Oxidative modification of low density lipoprotein (LDL) in artery wall is now widely regarded to play an important role in the development of atherosclerosis.[1—4] The oxidation of LDL gives rise to atherogenic changes including the formation of oxidized lipids which act as chemotactic and mitogenic agents and the modification of the charge on the apolipoprotein B (apo-B) moiety of LDL creating a ligand for the scavenger receptors on macrophages.[1—4] With increasing evidence that LDL is oxidized *in vivo*, and that oxidized LDL is involved in atherogenesis, it is thought that antioxidants which could slow or inhibit the oxidative process may be an important therapeutic strategy to prevent and possibly to treat athrosclerosis.[1—4]

In our continuing study to search for antioxidants from plants, a MeOH extract of the leaves and stems of *Weigela subsessilis* exhibited antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide radical formation with IC_{50} values of 27.0 ± 0.7 and $2.3\pm0.3\ \mu g/ml$, respectively. *W. subsessilis* (Caprifoliaceae), an endemic species in Korea, is a deciduous shrub that grows in sunny mountainous districts.[5] It usually grows to 2—3 m in height and has opposite leaves. A yellowish green flower blooms on an axil in spring, which changes to red.[5] Although flavonoids and coumarins have been reported as constituents of the genus *Weigela*,[6,7] up to the present, few studies on the chemical constituents and biological activities have been carried out on this plant. Our phytochemical study on the plant has led to the isolation of four coumarins, scopoletin (**1**), cleomiscosin A (**2**), scopolin (**3**) and fraxin (**4**). In the present study, we investigated the antioxidant activity of *W. subsessilis* and its coumarin constituents against LDL oxidation mediated by either catalytic copper ions (Cu^{2+}) or free radicals generated with the azo compound 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH).

MATERIALS AND METHODS

Plant Material The leaves and stems of *W. subsessilis* were collected at Mt. Gyeryong, Korea in April, 2000. A voucher specimen (CNU 2009) was identified by Prof. Ki-Hwan Bae and deposited in the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Extraction and Isolation The air-dried leaves and stems of *W. subsessilis* (6.7 kg) were extracted with MeOH at room temperature for one month and the solution was dried *in vacuo*. The MeOH extract (684 g) was resuspended in water and partitioned with hexane, EtOAc, and BuOH, sequentially. The EtOAc fraction (163 g) was subjected to silica gel column chromatography using a hexane–acetone gradient (from 10 : 1 to 0 : 1) to yield eight fractions (Fr. 1—8). Fr. 4 and 5 were further chromatographed over a silica gel column, eluted with hexane–acetone (3 : 1), to afford compound **1** (452 mg). Compound **2** (158 mg) was obtained from Fr. 7 using LH-20 column, eluting with MeOH–H_2O (1 : 1), and then recrystallized in MeOH–EtOAc. The BuOH fraction (164 g) was chromatographed over a silica gel column eluted with mixtures of $CHCl_3$–MeOH–H_2O (80 : 20 : 1) to give three fractions (Fr. 1—3). Fr. 2 (71 g) was separated by RP-18 column chromatography eluted with MeOH–H_2O (1 : 2) to afford compounds **3** (22 mg) and **4** (560 mg).

Scopoletin (**1**): Yellow crystal, mp: 202—204 °C; IR (KBr) $\nu_{max}\ cm^{-1}$: 3340, 3106, 3031, 2990, 1710, 1600; UV λ_{max} (MeOH) nm: 230, 254, 260, 298, 346; FAB-MS *m/z*: 193 $[M+H]^+$; ^1H- and ^{13}C-NMR spectral data were in accordance with published data.[8]

Cleomiscosin A (**2**): White needles, mp: 245—247 °C; IR (KBr) $\nu_{max}\ cm^{-1}$: 3423, 1702, 1614, 1447, 1419, 1140; UV λ_{max} (MeOH) nm: 207, 232; FAB-MS *m/z*: 387 $[M+H]^+$; ^1H- and ^{13}C-NMR spectral data were in accordance with published data.[9]

Scopolin (**3**): White amorphous powder, mp: 217—219 °C; IR (KBr) $\nu_{max}\ cm^{-1}$: 3440, 1710, 1610, 1190; UV λ_{max} (MeOH) nm: 203, 228; FAB-MS *m/z*: 355 $[M+H]^+$; ^1H- and ^{13}C-NMR spectral data were in accordance with published data.[7]

Fraxin (**4**): Yellow crystal, mp: 203—205 °C; IR (KBr)

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ν_{max} cm^{-1}: 3400, 1750, 1583, 1161; UV λ_{max} (MeOH) nm: 210, 230, 344; FAB-MS *m/z*: 371 $[M+H]^+$; ^{1}H- and ^{13}C-NMR spectral data were in accordance with published data.[7]

LDL Preparation Blood was drawn from healthy normolipidemic volunteers and human LDL was prepared from plasma by sequential flotation ultracentrifugation as described previously.[10—12] For Cu^{2+}-mediated oxidation experiments, LDL was dialysed for 20 h at 4 °C against EDTA-free, phosphate buffered saline (PBS) to remove EDTA.[11,12] For azo-initiated oxidation experiments, LDL was dialysed overnight against the same PBS containing 1 mM EDTA.[11,12] The purity of LDL evaluated by agarose gel electrophoresis was > 97 %. The LDL protein was determined by the bicinchoninic acid method using bovine serum albumin as a standard.[13]

Cu^{2+}-Mediated LDL Oxidation The oxidation of LDL induced by copper ion was measured as described previously.[10—12] Briefly, LDL (100 μg/ml) in PBS (pH 7.4, final volume of 1 ml) was pre-incubated with samples, and then 5 μM $CuSO_4$ was added to initiate the oxidation at 37 °C. The reaction was terminated by the addition of 1 mM EDTA and 500 μM butylated hydroxy toluene (BHT). The oxidation of LDL was assessed by the formation of conjugated dienes determined as the change in UV absorbance at 234 nm.[10,14] Absorbance was continuously monitored at 5 min intervals for 4 h at 37 °C. The oxidation of LDL to malondialdehyde (MDA) was measured using the thiobarbituric acid reactive substances (TBARS) assay after 3 h incubation.[10]

Azo-Initiated LDL Oxidation The oxidation of LDL mediated by an azo compound was determined as previously described.[10—12] Briefly, LDL (100 μg/ml) in PBS (pH 7.4, final volume of 1 ml) was pre-incubated with samples, and then 5 mM of an aqueous AAPH was added to initiate the oxidation at 37 °C for 3 h. The reaction was stopped by the addition of 500 μM BHT and stored at 4 °C. The oxidation of LDL was quantified by the generation of TBARS.[10]

Electrophoresis of Apolipoprotein B-100 (apoB-100) Fragmentation The fragmentation of apoB-100 following Cu^{2+}-induced oxidation was determined using SDS-polyacrylamide gel electrophoresis (PAGE) system. The oxidized LDL solution was denatured with 3 % SDS, 10 % glycerol and 5 % bromophenol at 95 °C for 10 min. The electrophoresis was run on SDS-PAGE (4 %) at 100 V for 80 min, and then stained with Coomassie Brilliant Blue R250.[10]

RESULTS AND DISCUSSION

Oxidation of LDL is considered to be essential in the pathogenesis of atherosclerosis.[1—4] Although it is not clear how LDL is oxidized *in vivo*, several lines of evidence suggest that antioxidants could have beneficial effects in the prevention of cardiovascular disease.[1—4] Since the MeOH extract of *W. subsessilis* exhibited antioxidant activity in our recent study, we set out to demonstrate the inhibitory activity of this plant against the LDL oxidation.

Phytochemical investigation on the leaves and stems of *W. subsessilis* led to the isolation of four coumarins, scopoletin (**1**), cleomiscosin A (**2**), scopolin (**3**) and fraxin (**4**) (Fig. 1). These compounds were identified by physical and spectroscopic data measurement (MS, ^{1}H-, ^{13}C-NMR, and 2D NMR) and by comparing the data obtained with those of published values.[7—9] Two coumarins, **1** and **2**, were first isolated from this plant. Although the antioxidant properties of coumarins have been reported,[15,16] little is known as to whether the compounds inhibit LDL oxidation.

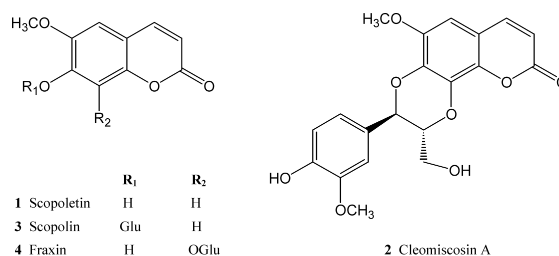


Fig. 1. Structures of Compounds **1**—**4** Isolated from *W. subsessilis*

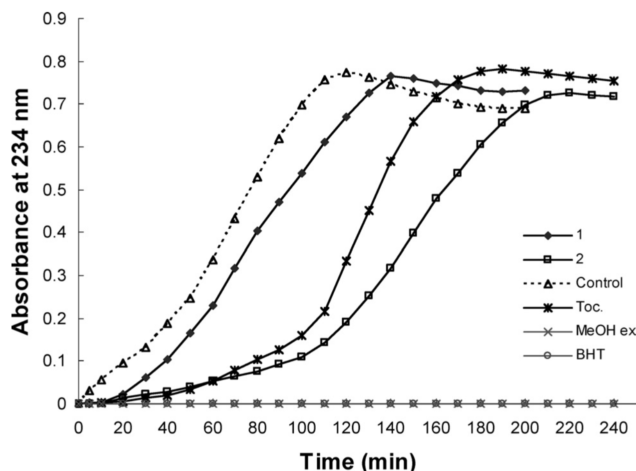


Fig. 2. Effect of *W. subsessilis* and Its Coumarins on Cu^{2+}-Mediated LDL Oxidation

LDL (100 μg/ml) in PBS (pH 7.4) was incubated with 5 μM $CuSO_4$ at 37 °C in the present or absence of 5 μM compound. The MeOH extract was treated at a concentration of 5 μg/ml. Conjugated diene formation was measured by determining the absorbance at 234 nm every 5 min for 4 h. BHT and α-tocopherol (Toc.) were used as positive controls.

In this study, we examined the inhibitory activity of the isolated coumarins against the LDL oxidation mediated by Cu^{2+} and an azo compound. The oxidation of LDL was assessed by the formation of conjugated dienes. As presented in Fig. 2, the reaction kinetics of diene formation consists of a lag phase characterized by a low oxidation rate due to the consumption of endogenous antioxidants, a second phase of maximal rate of oxidation that starts when the antioxidants are consumed, and a third terminal phase with a plateau in diene formation.[17] Of the coumarins tested, **1** and **2** were found to be effective in extending the lag phase of Cu^{2+}-mediated LDL oxidation. In the presence of 5 μM of compounds **1** and **2**, the lag phase was retarded to 42 and 112 min, respectively, compared with the control (25 min) (Fig. 2). In the presence of 5 μM of α-tocopherol and BHT used as positive controls, it increased to 97 and >240 min, respectively (Fig. 2). Thus at the same concentration, compound **2** is more effective than α-tocopherol in inhibiting LDL oxidation (Fig. 2). In contrast, coumarin glycosides **3** and **4** did not inhibit the formation of conjugated dienes in Cu^{2+}-mediated LDL oxidation.

Subsequently, the oxidation of LDL initiated by Cu^{2+} and the azo compound was measured by the formation of malondialdehyde (MDA) using the TBARS assay. As shown in

Table 1. Effects of compounds **1**—**4** Isolated from *W. subsessilis* on LDL Oxidation Mediated by Cu^{2+} and AAPH

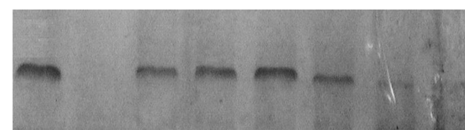
Compound[a]	Lag-time[b] (min)	TBARS, IC_{50}[c] (μM)	
		Cu^{2+}-mediated	AAPH-mediated
1	42	57.4±4.4	37.3±2.6
2	112	13.1±2.5	26.8±1.8
3	25	—	—
4	29	>200	>200
MeOH ex.	>240	ND[d]	ND
α-Tocopherol[e]	97	22.9±1.2	—
BHT[e]	>240	2.3±0.1	10.1±1.4

a) Each compound was treated at a concentration of 5 μM, while, the MeOH extract was examined at 5 μg/ml; *b*) the lag-time of control was estimated to be 25 min; *c*) the values represent mean±S.D. of triplicate experiments; *d*) ND: not determined; *e*) compounds used as positive controls.

Table 1, comparable results were obtained. Compound **2** exhibited the strongest inhibitory activity against Cu^{2+} and AAPH-mediated LDL oxidation with IC_{50} values of 13.1 and 26.8 μM, respectively, followed by compound **1** (IC_{50} 57.4, 37.3 μM, respectively). Compounds **3** and **4** did not show inhibitory activity in LDL oxidation. In general, the aglycone scopoletin was more effective than its corresponding glycosides against LDL oxidation. This is because the glycosides are more hydrophilic, which makes it more difficult for them to react with lipid radicals inside the LDL.[18] Meanwhile, α-tocopherol was effective in only Cu^{2+}-mediated LDL oxidation with an IC_{50} value of 22.9 μM, and BHT was found to be effective in both Cu^{2+}- and AAPH-mediated LDL oxidation (IC_{50} 2.3, 10.1 μM, respectively).

It has been reported that products of lipid peroxidation such as oxidixed phosphatidylcholine or MDA could cause fragmentation of apoB-100, a major component of LDL.[19] Since compounds **1** and **2** exhibited inhibitory activity against LDL oxidation, we examined the effect of these compounds on the fragmentation of apoB-100 using electrophoretic analysis. As shown in Fig. 3, SDS-PAGE analysis revealed that incubation of LDL with Cu^{2+} led to a loss of apoB-100. When the LDL (100 μg/ml in PBS) was incubated with Cu^{2+} alone, the apoB-100 band completely disappeared, whereas, in the presence of compound **2**, the fragmentation of apoB-100 was inhibited in a dose-dependent manner. Under the same condition used, compound **1** also displayed a protective effect on the fragmentation of apoB-100, albeit to a relatively low extent compared with **2** (data not shown). It is known that transition metal ions and free radicals are involved in LDL oxidation *in vivo*.[1—4,20] We subjected LDL to oxidation by copper ions and free radicals directly generated by AAPH. The mechanism by which coumarins **1** and **2** inhibit LDL oxidation is unknown. Since they could inhibit both Cu^{2+} and AAPH-mediated LDL oxidation, they may act as antioxidants by chelating metal ions and scavenging free radicals. Furthermore, we found that **1** and **2** had the capacity to protect the fragmentation of apoB-100.

The MeOH extract of *W. subsessilis* was more effective than its coumarin constituents, even more than α-tocopherol, in retarding the lag phase of Cu^{2+}-mediated LDL oxidation. Taken together with the data of radical scavenging activities, it is clear that *W. subsessilis* contains other antioxidants which have not been elucidated. Therefore, it is worthwhile to investigate the active principles in this plant.



	1	2	3	4	5	6	7	8
LDL	+	+	+	+	+	+	+	+
Cu2+	–	+	+	+	+	+	+	+
Compound 2 (μM)	–	–	20	10	5	2	1	0.5

Fig. 3. Inhibition of Cu^{2+}-Mediated apoB-100 Fragmentation in LDL by Compound **2**

LDL (100 μg/ml) was incubated with 5 μM $CuSO_4$ at 37 °C. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250. Lane 1: native LDL (absence of $CuSO_4$), lane 2: oxidized LDL, lane 3: **2** (20 μM), lane 4: **2** (10 μM), lane 5: **2** (5 μM), lane 6: **2** (2 μM), lane 7: **2** (1 μM), lane 8: **2** (0.5 μM).

In conclusion, we found that the coumarins scopoletin (**1**) and cleomiscosin A (**2**) isolated from *W. subsessilis* inhibited LDL oxidation mediated by both metal ions (Cu^{2+}) and free radicals generated with an azo compound (AAPH). The effect was assessed by conjugated diene formation, TBARS assay, and the fragmentation of apoB-100. The results suggest that *W. subsessilis* and its active coumarin constituents may be beneficial to prevent the LDL oxidation involved in the pathogenesis of atherosclerosis.

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