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

Phuong Thien ThUONG, a MinKyun NA, a Nguyen Duy Su, a Rack Seon SEONG, b Young Mi LEE, c Dai Eun SOK, a and KiHwan BAE, a,*

a College of Pharmacy, Chungnam National University; Daejeon 305–764, Korea; b Korea Food & Drug Administration; Seoul 122–704, Korea; and c College of Pharmacy, Wonkwang University; Iksan, Cheonbuk 540–749, Korea.

Received October 18, 2004; accepted January 24, 2005

Oxidation of low density lipoprotein (LDL) is thought to be a major factor in the pathophysiology 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 atherosclerosis.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 IC50 values of 27.0±0.7 and 2.3±0.3 μg/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 (Cu2+) 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–H2O (1 : 1), and then recrystallized in MeOH–EtOAc. The BuOH fraction (164 g) was chromatographed over a silica gel column eluted with mixtures of CHCl3–MeOH–H2O (80 : 20 : 1) to give three fractions (Fr. 1—3). Fr. 2 (71 g) was separated by RP-18 column chromatography eluted with MeOH–H2O (1 : 2) to afford compounds 3 (22 mg) and 4 (560 mg).

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

Cleomiscosin A (2): White needles, mp: 245—247°C; IR (KBr) νmax cm−1: 3423, 1702, 1614, 1447, 1419, 1140; UV λmax (MeOH) nm: 207, 232; FAB-MS m/z: 387 [M+H]+; 1H- and 13C-NMR spectral data were in accordance with published data.8)

Scopolin (3): White amorphous powder, mp: 217—219°C; IR (KBr) νmax cm−1: 3440, 1710, 1610, 1190; UV λmax (MeOH) nm: 203, 228; FAB-MS m/z: 355 [M+H]+; 1H- and 13C-NMR spectral data were in accordance with published data.8)

Fraxin (4): Yellow crystal, mp: 203—205°C; IR (KBr)
Determined as the change in UV absorbance at 234 nm.10,14) Of LDL was assessed by the formation of conjugated dienes (NMR) and by comparing the data obtained with those of.

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, 1H-, 13C-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.

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
are more hydrophilic, which makes it more difficult for them to protect the fragmentation of apoB-100. Moreover, the aglycone of apoB-100 was inhibited in a dose-dependent manner. Under the same condition used, compound \( \text{a}-\text{Tocopherol} \) was examined at 5 \( \mu \)M, respectively, followed by compound \( \text{a} \). Since compounds \( \text{a} \) and \( \text{a} \)-mediated LDL oxidation with an IC\(_{50} \) value of 22.9 \( \mu \)M, and BHT was found to be effective in both Cu\(^{2+}\)- and AAPH-mediated LDL oxidation (IC\(_{50} \) 2.3, 10.1 \( \mu \)M, respectively). It has been reported that products of lipid peroxidation such as oxidized phosphatidylethanolamine or MDA could cause fragmentation of apoB-100, a major component of LDL. \( \text{a} \) Meanwhile, \( \alpha \)-tocopherol was effective in only Cu\(^{2+}\)-mediated LDL oxidation with an IC\(_{50} \) value of 22.9 \( \mu \)M, and BHT was found to be effective in both Cu\(^{2+}\)- and AAPH-mediated LDL oxidation (IC\(_{50} \) 2.3, 10.1 \( \mu \)M, respectively).

In conclusion, we found that the coumarins scopoletin (1) and cleomiscosin A (2) isolated from \( W. \text{subsessilis} \) 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 radicals generated with an azo compound (AAPH). The effect was assessed by conjugated diene formation, TBARS analysis. As shown in Fig. 3, SDS-PAGE analysis revealed that incubation of LDL with Cu\(^{2+}\) led to a loss of LDL (100 \( \mu \)g/ml) was incubated with 5 \( \mu \)g 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 \( \mu \)g/ml), lane 4: 2 (10 \( \mu \)g/ml), lane 5: 2 (5 \( \mu \)g/ml), lane 6: 2 (2 \( \mu \)g/ml), lane 7: 2 (1 \( \mu \)g/ml), lane 8: 2 (0.5 \( \mu \)g/ml).

### Acknowledgements
This work was supported by grant No. (R01-2002-000-00276-0) from the Basic Research Program of the Korea Science and Engineering Foundation.

## REFERENCES


### Table 1. Effects of compounds 1—4 isolated from \( W. \text{subsessilis} \) on LDL Oxidation Mediated by Cu\(^{2+}\) and AAPH

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lag-time((^a)) (min)</th>
<th>TBARS, IC(_{50} )((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{a} )</td>
<td>42</td>
<td>57.4±4.4</td>
</tr>
<tr>
<td>( \text{b} )</td>
<td>112</td>
<td>13.1±2.5</td>
</tr>
<tr>
<td>( \text{c} )</td>
<td>25</td>
<td>—</td>
</tr>
<tr>
<td>( \text{d} )</td>
<td>29</td>
<td>—</td>
</tr>
<tr>
<td>MeOH ex.</td>
<td>&gt;240</td>
<td>22.9±1.2</td>
</tr>
</tbody>
</table>

\( ^a \) Each compound was treated at a concentration of 5 \( \mu \)g/ml, while the MeOH extract was examined at 5 \( \mu \)g/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.