Lignans from the Fruits of *Forsythia suspensa* (Thunb.) Vahl Protect High-Density Lipoprotein during Oxidative Stress

Min-Jung Chang, 1 Tran Manh Hung, 2 Byung-Sun Min, 1, 3 Jin-Cheol Kim, 3 Mi Hee Woo, 1 Jae Sue Choi, 4 Hyeong Kyu Lee, 2 and Ki Hwan Bae 5

1 College of Pharmacy, Catholic University of Daegu, Gyongsan 712-702, Korea
2 Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea
3 Korea Research Institute of Chemical Technology, Daejeon 305-600, Korea
4 Faculty of Food Science and Biotechnology, Pukyoung National University, Busan 608-737, Korea
5 College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea

Received June 11, 2008; Accepted July 7, 2008; Online Publication, October 7, 2008 [doi:10.1271/bbb.80392]

The objective of the present study was to investigate the beneficial properties lignan compounds obtained from the fruits of *Forsythia suspensa* (Thunb.) Vahl (Oleaceae) for protecting human high-density lipoprotein (HDL) against lipid peroxidation. The isolated compounds (1–8) inhibited the generation of thiobarbituric acid-reactive substances (TBARS) in a dose-dependent manner with IC₅₀ values from 8.5 to 18.7 μM, since HDL oxidation mediated by catalytic Cu²⁺. They also exerted an inhibitory effect against thermo-labile radical initiator (AAPH)-induced lipid peroxidation of HDL with IC₅₀ values from 12.1 to 51.1 μM. Compounds 1 and 5 exerted inhibitory effects against the Cu²⁺-induced lipid peroxidation of HDL, as shown by an extended lag time prolongation at the concentration of 3.0 μM. These results suggest that the antioxidative effects of *F. suspensa* are due to its lignans and that these constituents may be useful for preventing the oxidation of HDL.

**Key words:** Forsythia suspense; Oleaceae; lignan; human high-density lipoprotein; lipid peroxidation

The oxidative modification of low-density lipoprotein (LDL) and the accelerated uptake of oxidized LDL by artery wall macrophages have been implicated in the formation of atherosclerotic plaque. LDL oxidation is a crucial step in atherosclerosis. However, this process can be inhibited by high-density lipoprotein (HDL) through its oxidizable components or by associated enzymes like paraoxonase and platelet-activating factor acetylhydrolase. 11 The ability of HDL to protect LDL from oxidation may consequently influence some of the pathological processes mediating the development of atherosclerosis. Numerous prospective studies have demonstrated the protective nature of an elevated level of HDL and the high risk associated with a low level of this class of lipoproteins. 2, 3 However, similar to LDL, the lipids contained in HDL are susceptible to oxidation by a variety of pro-oxidants such as redox-active copper ions, metal ion-independent oxidants including reactive oxygen, and nitrogen species. 4 In general, the oxidation of HDL has been found to result in a loss of cardio-protective properties and loss of paraoxonase activity. 4 It is known that most of the measurable lipid peroxides in plasma can be found in the HDL fraction. 5 Surprisingly, in spite of having several important pathophysiological implications, studies on the HDL oxidation process have received less attention than those on LDL oxidation. 6

As a part of our screening program to find antioxidants from natural sources, we have attempted to determine the HDL oxidation inhibitory constituents from fruits of *Forsythia suspensa* (Thunb.) Vahl (Oleaceae). This species is a perennial herb that is cultivated for its beautiful yellow flowers, and is distributed throughout Korea, Japan, and China. The fruits have been used as a folk medicine for the treatment of inflammation, ulcers, pharyngitis, pyrexia, and tonsillitis. 7 Previous studies on this plant have reported the isolation of caffeoyl glycosides, cyclohexylethanes, flavonoids, iridoid glycosides, lignans and triterpenes, together with their anti-inflammatory, antibacterial, antioxidative, weight loss, blood pressure-reducing and cyclic adenosine monophosphate phos-
phodiesterase inhibitory effects. An ethanol extract of *F. suspensa* has recently protected against enzymatic and non-enzymatic lipid peroxidation in membranes and showed scavenging activity toward the superoxide radical. Even though the antioxidative activities of some lignans from this plant have been assessed by evaluating their protective effects against peroxynitrite-induced oxidative stress, no studies have specifically investigated the capacity of those components to protect HDL from oxidation. We therefore examined the susceptibility of HDL to *in vitro* copper (Cu²⁺) and thermo-labile radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced lipid peroxidation in the presence of an extract, fractions and isolated compounds from *F. suspensa*.

**Materials and Methods**

**Plant materials.** The fruits of *F. suspensa* were purchased from a local market in Daegu, Korea in March 2006, and were identified by Prof. Byung-Sun Min. A voucher specimen (CUD-2477-2) has been deposited in the herbarium of the College of Pharmacy, Catholic University of Daegu, Korea.

**Extraction and isolation.** Dried plant material (10.0 kg) was extracted with 70% EtOH at room temperature (4 × 5 liters) to obtain 1.85 kg of a solid extract. The 70% EtOH extract was suspended in *H*₂O and successively extracted with hexane (3 × 3 liters), CHCl₃ (3 × 3 liters), EtOAc (3 × 3 liters), and BuOH (3 × 3 liters) to give the hexane-(520 g), CHCl₃-(320 g), EtOAc-(250 g), and BuOH-soluble fractions (504 g), respectively. These fractions were screened for antioxidative activity, and the CHCl₃ and EtOAc active fractions were subjected to chromatography in a silica gel column, eluting with CHCl₃-MeOH (50:1 to 4:1) to afford fourteen fractions (C1–14). Fraction C13 was chromatographed in a silica gel column, eluting with a gradient of hexane–EtOAc (7:1 to 3:1) and CHCl₃–MeOH (50:1 to 4:1) to give eleven fractions (E1–11). Fraction E9 was re-chromatographed in an RP-C₁₈ column eluting with a gradient of MeOH–H₂O (1:1.7 to 3:1) to afford fifteen subfractions (E9.1–15). Subfraction E9.3 was applied to preparative HPLC-C₁₈, using a gradient of acetonitrile–H₂O (1:3 to 1:1) to yield compound 7 (5.1 mg). Further chromatography of E10 in a silica gel column, eluting with CHCl₃–MeOH (8:1) gave three sub-fractions (E10.1–3). Compound 10 (48.8 mg) was obtained from E10.3 after chromatographed on a silica gel column with CHCl₃–MeOH (7:1).

**Pinoresinol (1):** yellow amorphous powder; [α]₂₂° +88.5° (c 0.18, MeOH); UV (MeOH) λ<sub>max</sub> nm (log ε): 232 (4.51), 281 (4.14); EI-MS (rel. int.) m/z: 358 [M]+ (39), 327 (65), 221 (6), 205 (14), 151 (100), 137 (68), 131 (23); mol. formula: C₂₀H₂₂O₅.

**Phillygenin (2):** yellow crystals; mp: 136–138°C; [α]₂₂° +90.0° (c 0.2, MeOH); UV (MeOH) λ<sub>max</sub> nm (log ε): 232 (4.12), 280 (3.66); EI-MS (rel. int.) m/z: 372 [M]+ (80), 341 (9), 151 (100), 137 (41), 131 (16); mol. formula: C₂₁H₂₃O₇.

**8-Hydroxy pinoresinol (3):** white amorphous powder; [α]₂₂° +23.9° (c 0.16, MeOH); UV (MeOH) λ<sub>max</sub> nm (log ε): 233 (4.45), 281 (4.04); EI-MS (rel. int.) m/z: 374 [M]+ (45), 226 (22), 207 (34), 193 (20), 165 (41), 151 (100), 137 (67), 131 (46); mol. formula: C₂₁H₂₂O₇.

**7-epi-8-Hydroxy pinoresinol (4):** white amorphous powder; [α]₂₂° +70.7° (c 0.16, MeOH); UV (MeOH) λ<sub>max</sub> nm (log ε): 232 (4.45), 281 (4.05); EI-MS (rel. int.) m/z: 374 [M]+ (57), 222 (28), 207 (61), 193 (20), 165 (48), 151 (99), 137 (100), 131 (61); mol. formula: C₂₀H₂₂O₇.

**Lariciresinol (5):** yellow amorphous powder; [α]₂₂° +25.3° (c 0.21, MeOH); UV (MeOH) λ<sub>max</sub> nm (log ε): 231 (4.41), 282 (4.05); EI-MS (rel. int.) m/z: 360 [M]+ (69), 311 (3), 194 (30), 175 (18), 137 (100), 122 (14); mol. formula: C₂₀H₂₂O₆.

**Isolariciresinol (6):** yellow amorphous powder; [α]₂₂° +24.0° (c 0.18, MeOH); UV (MeOH) λ<sub>max</sub> nm (log ε): 232 (sh), 284 (4.15); EI-MS (rel. int.) m/z: 360 [M]+ (25), 311 (1), 267 (72), 137 (44), 98 (100); mol. formula: C₂₀H₂₂O₆.

**Olivil (7):** white amorphous powder; [α]₂₂° −46.5° (c 0.17, MeOH); UV (MeOH) λ<sub>max</sub> nm (log ε): 232 (4.67), 281 (4.32); EI-MS (rel. int.) m/z: 376 [M]+ (10), 326 (6), 311 (2), 137 (100); mol. formula: C₂₀H₂₂O₇.

**Cedrusin (8):** brown amorphous powder; [α]₂₂° +10.0° (c 0.16, MeOH); UV (MeOH) λ<sub>max</sub> nm (log ε): 232 (sh), 283 (4.21); EI-MS (rel. int.) m/z: 346 [M]+ (31), 328 (79), 316 (100), 296 (17), 137 (35); mol. formula: C₁₉H₂₂O₅.

**HDL preparation.** Blood from healthy normolipemic donors was obtained by venipuncture and collected in EDTA-containing vacutainer tubes. To isolate HDL, plasma was prepared by centrifugation at 3,000 rpm for 10 min and thereafter used for the preparation of plasma lipoproteins. HDL was isolated from the plasma by ultracentrifugation for 1.5 h with a vertical rotor as described previously. After dialyzing at 4°C for 24 h against 10 mM phosphate-buffered saline (PBS) at


HDL oxidation. The oxidation of HDL was assessed by the formation of conjugated dienes which was determined as the change in UV absorbance at 232 nm at 10-min intervals over 5 h at 37 °C, using a UV-1240 spectrophotometer (Shimadzu, Tokyo, Japan). The lag time was measured as the intercept between the baseline and the tangent to the absorbance curve during the propagation phase. The oxidation of HDL to malondialdehyde (MDA) was measured by using a thiobarbituric acid reactive substances (TBARS) assay. HDL in PBS (pH 7.4) was pre-incubated with each compound, and then Cu²⁺ or one of the thermo-labile radical initiators (AAPH) was added to initiate the oxidation process. The reaction mixture was incubated at 37 °C for 2 h. At the end of this incubation, the reaction was terminated by adding 20% trichloroacetic acid (TCA) and 1% thiobarbituric acid (TBA). After boiling at 95 °C for 15 min, the mixture was centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. In this experiment, vitamin C and vitamin E were used as positive controls.

Statistical analysis. Student’s t-test and a two-way analysis of variance were used to determine the statistical significance of differences between values for the experimental and control groups. Each result is expressed as the mean value ± S.D. of three experiments conducted in triplicate. A p < 0.05 value was considered statistically significant.

Results and Discussion

The fruits of *F. suspensa* were extracted with 70% EtOH at room temperatures and the alcoholic extract obtained was partitioned into hexane-, CHCl₃-, EtOAc-, BuOH- and aqueous fractions. Repeated column chromatography led to the isolation of eight compounds (1–8). A spectroscopic analysis and comparison of physical constants with those in the literature allowed us to identify these compounds as pinoresinol (1), phillygenin (2), 8-hydroxy-pinoresinol (3), 7′-epi-8-hydroxypinoresinol (4), lariresinol (5), isolariciresinol (6), olivil (7) and cedrusin (8) (Fig. 1).

In the primary study, we tested the inhibitory activity of the 70% EtOH extract and fractions (hexane-, CHCl₃-, EtOAc-, and BuOH-soluble) at the concentration of 100 μg/ml against the oxidation of HDL, which was initiated by Cu²⁺ to form the malondialdehyde (MDA), by using a thiobarbituric acid reactive substances (TBARS) assay. The results showed that the CHCl₃- and EtOAc-soluble fractions were approximately 2.5- and 2.3-fold more potent than the alcoholic extract, while the hexane- and BuOH-soluble fractions showed very weak activity at the same tested concentration (Table 1). Considering that the CHCl₃ and EtOAc fractions were the most potent, these were selected for isolation of the active constituents.

In the next stage of this study, we tested the inhibitory activity of all the isolated compounds against the oxidation of HDL initiated by both Cu²⁺ and AAPH. As shown in Table 1, the tested compounds markedly reduced the formation of TBARS. Under Cu²⁺-mediated oxidation, compounds 1–8 showed HDL-antioxidative activities in a dose-dependent manner, the concentration required for 50% inhibition (IC₅₀) ranging from 8.5 to 18.7 μM. Under AAPH-mediated oxidation, isolated compounds 1, 2, 4–6 and 8 also exhibited HDL oxidation activities, with IC₅₀ values ranging from 12.1 to 51.1 μM. Vitamin C and vitamin E were used as the positive controls, vitamin C showing inhibitory activity with IC₅₀ values of 11.7 and 18.9 μM, while vitamin E showed inhibitory activity with IC₅₀ values of 2.0 and 5.8 μM, under Cu²⁺- and AAPH-mediated oxidation.

Since the formation of conjugated dienes represents the propagation phase of HDL oxidation, the extent of the lag time indicates the oxidation resistance. As shown in Fig. 2, a spectrophotometric analysis of Cu²⁺-induced HDL oxidation based on the formation of conjugated diene indicated the presence of unsaturated lipids. When HDL was incubated with Cu²⁺ alone, the lag time was 48 min, whereas, in the presence of pinoresinol (1, 3.0 μM) and lariresinol (5, 3.0 μM), the lag phase was delayed to 87 and 90 min, respectively. The lag phases of vitamin C (3.0 μM) and vitamin E (3.0 μM) were delayed to 85 and 128 min, respectively.

Certain forms of oxidized HDL may actually enhance protection by stimulating the delivery of intracellular cholesterol to cell surface sites where it becomes available for removal by other (non-oxidized) HDL particles. Lipid oxidation in HDL is promoted by a variety of factors. Although there have been a number of reports on the design and development of synthetic peroxidation inhibitors, only a few studies have been reported on HDL oxidation inhibitors derived from plants. Our results clearly demonstrate that the HDL oxidation inhibitors of isolated lignans significantly inhibited the lipid oxidation of HDL which had been exposed to such sources of oxidant stress as metal ion-independent (Cu²⁺) and peroxy radicals (AAPH). Interestingly, such lignans as pinoresinol (1), 8-hydroxy-pinoresinol (3), 7′-epi-8-hydroxypinoresinol (4), lariresinol (5), isolariciresinol (6) and olivil (7) were more effective than vitamin C, but less effective than vitamin E from evidence obtained by the TBARS method (Table 1). Pinoresinol (1) and lariresinol (5) were selected to examine the inhibitory ability in the propagation phase through a delay in the lag time oxidation. Despite the important role of HDL, the absence of a lag phase before the initiation of oxidation in HDL is associated with several antioxidative enzymes, this suggests that HDL may protect LDL from
Pinoresinol (1)  Phillygenin (2)  8-Hydroxyphenoxyresinol (3)

7’-epi-8-Hydroxyphenoxyresinol (4)  Lariciresinol (5)  Isolariciresinol (6)

Olivin (7)  Cedrusin (8)

![Chemical structures of isolated compounds](image)

![Figure 1](image)

**Table 1.** Effects of the Extracts and Isolated Compounds on the Oxidation of HDL

<table>
<thead>
<tr>
<th>Extract/Compound</th>
<th>Lag time (min)*</th>
<th>TBARS, IC_{50} (μM)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% EtOH extract</td>
<td>ND</td>
<td>42.6 ± 4.2</td>
</tr>
<tr>
<td>Hexane fraction</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CHCl₃ fraction</td>
<td>ND</td>
<td>88.3 ± 4.1</td>
</tr>
<tr>
<td>EtOAc fraction</td>
<td>ND</td>
<td>75.5 ± 5.6</td>
</tr>
<tr>
<td>n-BuOH fraction</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pinoresinol (1)</td>
<td>87</td>
<td>9.7 ± 0.5*</td>
</tr>
<tr>
<td>Phillygenin (2)</td>
<td>ND</td>
<td>14.5 ± 1.1*</td>
</tr>
<tr>
<td>8-Hydroxyphenoxyresinol (3)</td>
<td>ND</td>
<td>10.5 ± 0.5*</td>
</tr>
<tr>
<td>7’-epi-8-Hydroxyphenoxyresinol (4)</td>
<td>ND</td>
<td>10.2 ± 0.3*</td>
</tr>
<tr>
<td>Lariciresinol (5)</td>
<td>90</td>
<td>8.5 ± 0.4*</td>
</tr>
<tr>
<td>Isolariciresinol (6)</td>
<td>ND</td>
<td>8.8 ± 0.2*</td>
</tr>
<tr>
<td>Olivin (7)</td>
<td>ND</td>
<td>8.8 ± 1.0*</td>
</tr>
<tr>
<td>Cedrusin (8)</td>
<td>ND</td>
<td>18.7 ± 1.3*</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>85</td>
<td>11.7 ± 0.5</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>128</td>
<td>2.0 ± 0.1</td>
</tr>
</tbody>
</table>

*The lag time of the blank was estimated to be 48 min.

*Each value represents the mean ± S.D. of five experiments performed on different days.

*% inhibition at a concentration of 100 μg/ml. (->) weak activity.

*ND, not determined.

*P < 0.05 vs. vitamin C

*P < 0.05 vs. vitamin E
oxidation in part by acting as a sacrificial target for oxidation until the antioxidants have been depleted from LDL. Even at a low concentration (3.0 mM), 1 and 5 could exert a protective effect against Cu²⁺-induced lipid peroxidation of HDL, as shown by the delayed lag time of the conjugated diene process in comparison with the control (87 and 90 min versus 48 min, Fig. 2). The results from the TBARS method implicate that those lignans were more effective antioxidants in the metal-dependent pro-oxidant system than in the peroxyl radical system; metal ion-chelating properties may underlie their apparent antioxidative effect towards HDL oxidation in vitro. Incubating HDL in the presence of Cu²⁺-oxidized erythrocyte membranes increased the content of lipid hydroperoxides in HDL, this suggests transport of the phospholipids containing hydroperoxides from oxidized erythrocyte membranes to lipoprotein. It has previously been reported that the 4-hydroxy-3-methoxy substitution pattern of such guaiacyl lignans as pinoresinol (1), 8-hydroxypinoresinol (3), 7'-epi-8-hydroxypinoresinol (4), lariciresinol (5), isolariciresinol (6), and olivil (7) showed slightly stronger anti-lipid peroxidation capacity. 25, 26) Their antioxidative effects via the initial stage might involve the reversible donation of a phenolic hydrogen radical. 26) The results demonstrate that, by reducing oxidative stress, the lignan constituents of the fruits of *F. suspensa* may prevent the development and progression of HDL oxidation. However, the cardio-protective ability of the HDL lipoprotein fraction in combination with active compounds to prevent atherogenic modification should be investigated.

**Acknowledgment**

This research was supported by a grant (PF06219-00) from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology of the Korean government.

**References**


