

## Lipoxygenase Inhibition and Antioxidative Activity of Flavonoids from *Paeonia moutan* Seeds

Hyo-Jin Kim, Shin-Kyo Chung\* and Sang-Won Choi†

Dept. of Food Science and Nutrition, Catholic University of Taegu-Hyosung, Hayang 712-702, Korea  
\*Dept. of Food Science and Technology, Kyungpook National University, Taegu 702-701, Korea

### Abstract

Previously, the methanolic extract of *Paeonia moutan* seeds was found to potently inhibit soybean lipoxygenase (SLO). Hence to isolate SLO inhibitor, the defatted methanolic extract of the seeds was consecutively partitioned with ether, ethyl acetate, n-butanol, and water. The ether soluble fraction showing strong inhibitory activity against SLO was further fractionated into a strongly acidic, a weakly acidic, and a neutral fractions. The strongly acidic components of the ether extract were successively subjected to chromatography on a silica gel, Sephadex LH-20, and preparative HPLC. Four phenolic compounds were isolated, and two of them showing a strong SLO inhibition activity were identified as luteolin (IC<sub>50</sub>=2.32 µg/ml) and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone (IC<sub>50</sub>=0.31 µg/ml) by UV, IR, <sup>1</sup>H- & <sup>13</sup>C-NMR, and MS spectroscopy. In addition, two flavonoids showed significantly antioxidative activity as strong as that of α-tocopherol (p<0.05) in the autoxidation system of linoleic acid. These results suggest that luteolin and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone may be used as a potential source of anti-inflammatory agents with antioxidative activity.

**Key words:** *Paeonia moutan* seeds, soybean lipoxygenase inhibitor, antioxidants, luteolin and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone

### INTRODUCTION

Lipoxygenase (linoleate: oxidoreductase, EC 1.13.11.12) catalyzes the oxidation of unsaturated fatty acids containing a *cis,cis*-1,4-pentadiene group (1). This enzyme widely exists in plants and mammals (2,3). In particular, soybean lipoxygenase (SLO) has extensively been studied in off-flavor and odor production (4). Meanwhile, 5-lipoxygenase (5-LO) is a key enzyme which catalyzes the first step in the biosynthesis of leukotrienes (LTs), which are intimately involved in the pathology of a variety of inflammatory and allergic diseases such as asthma, psoriasis and rheumatic arthritis (5-7). Therefore, the specific 5-LO inhibitors are expected to be potential therapeutic drug for the prevention of these diseases. At present, the spectrophotometrical *in vitro* assay using SLO is widely used to screen natural 5-LO inhibitors due to structural and mechanistic similarities between SLO and human 5-LO (8,9).

Recently, much attention has received on the development of novel natural 5-LO inhibitors. Lignans, coumarins, terpenoids, alkaloids and phenolic compounds from natural products have been found to act as LO inhibitors to possess anti-inflammatory actions (10-14). In particular, some flavonoids proved to be potent inhibitors of LO derived from several plants and mammals (15-18). In the course of our search for new SLO inhibitors, we have screened several SLO inhibitors from plant extracts, and some lignans and flavonoids isolated from plant seeds were found to play an especially important role in the inhibition of SLO (19-21).

The cortex radice parts of *Paeonia moutan* are widely used

as traditional Chinese medicines having anti-inflammatory, anti-allergy, and analgesic activity (22). Paeoniflorin, albiflorin, and their derivatives were shown to act as the major anti-inflammatory constituents (23). In a previous report, we found that the methanolic extract of *Paeonia moutan* seeds showed the most potent SLO inhibitory activity at low concentration, while that of its cortex radice exhibited less activity (24). The different activity prompted us to investigate the active components in the seeds of *Paeonia moutan*.

The purpose of this study was to isolate and identify soybean lipoxygenase inhibitors from *Paeonia moutan* seeds, and further to determine their antioxidative activity in a linoleic acid model system.

### MATERIALS AND METHODS

#### Materials and reagents

The seeds of *Paeonia moutan* Sims were directly collected in Chungsong, Kyungbuk, Korea. Soybean lipoxygenase (type V), linolenic acid, trifluoroacetic acid (TFA), apigenin, quercetin, kaempferol and nordihydroguaiaretic acid (NDGA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Linoleic acid, butylated hydroxyanisole (BHA) and α-tocopherol were purchased from Wako Pure Chemical Industries (Osaka, Japan). All others used for this study were of analytical grade.

#### Soybean lipoxygenase (SLO) assay

SLO assay was performed by the spectrophotometrical

†Corresponding author

method described previously (21). The reaction mixture containing Tris buffer (pH 8.5), samples and soybean lipoxygenase was incubated, and then lipid peroxidation was started by addition of linolenic acid. The change of absorbance at 234 nm was recorded as a function of time on a photodiode array spectrophotometer (S2030, Scinco, Korea). The rates were measured from the initial slopes of the linear portions of the curves. A sample containing all of the reagents except for the enzyme solution was used as a blank sample.  $IC_{50}$  values were determined by regression analysis of the results at three different concentrations of the inhibitor.

#### Antioxidative assay

Antioxidative activity was determined by a ferric thiocyanate method using linoleic acid as a substrate for autoxidation in the ethanol-water system (25).

Each sample (200  $\mu$ g) was added to a mixture solution containing linoleic acid, 99.9% distilled ethanol and 50 mM phosphate buffer (pH 7.0); the total volume was adjusted to 25 ml with distilled water. The solution was incubated at 40°C and hydroperoxide concentration was measured at intervals by the thiocyanate method.

#### Isolation and purification of soybean lipoxygenase inhibitors

*Paeonia moutan* seeds (350 g) were crushed into small pieces and extracted twice with hot n-hexane (2.0 L) to remove lipids. The residue was extracted twice with MeOH (2.0 L) under reflux, and then filtered. The concentrated methanolic extract was partitioned between n-hexane and 80% aqueous methanol. The 80% aqueous methanolic layer was evaporated to a low volume *in vacuo*, and then partitioned with ether, ethyl acetate, and n-butanol, stepwise. The ether, ethyl acetate, n-butanol, and aqueous portion, each in a concentration of 5.0  $\mu$ g/ml, showed SLO inhibitory activity of 82.2, 68.5, 17.4, and 5.4%, respectively. The ether soluble layer (8.34 g) was further fractionated into a strongly acidic (5%  $NaHCO_3$ ), a weakly acidic (5% NaOH) and a neutral fraction according to the usual method. The strongly acidic components of the ether extract were subjected to chromatography on a silica gel (70~230 mesh, Merck, Germany) using  $CHCl_3$ -MeOH (8:1) with increasing amounts of MeOH to give four fractions. The first and second active fractions were further purified by preparative HPLC (Waters Delta Prep 4000, USA) using a Waters RCM Prep Nova-pak  $C_{18}$  column (2.5 cm  $\times$  10 cm  $\times$  2 cartridge), and UV-visible detector set at 340 nm. Elution was carried out at 20°C using MeOH-H<sub>2</sub>O-TFA (60:40:0.1) as a mobile phase at 5 ml/min flow rate. Four phenolic compounds (compound 1, 5.4 mg; compound 2, 16.5 mg; compound 3, 34.3 mg; compound 4, 6.5 mg) were repeatedly isolated (Fig. 1). Among them, compound 3 and 4 showing potent SLO inhibitory activity were identified by UV, IR, NMR and EI-MS spectroscopy.

#### Instrumental analysis

The UV spectra were recorded using UV-Visible spectro-

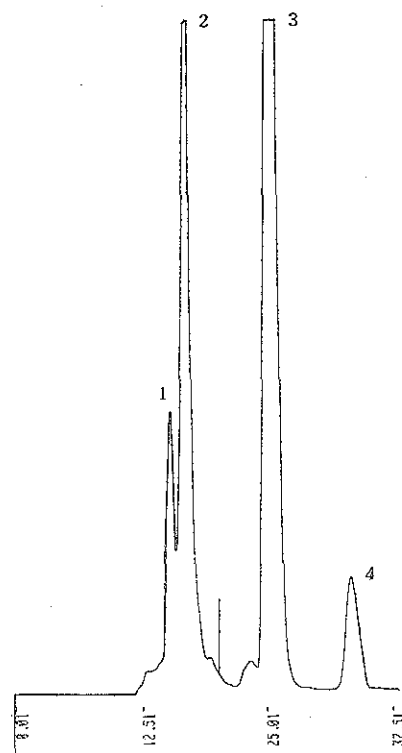


Fig. 1. HPLC chromatogram of four compounds isolated from *Paeonia moutan* seeds. 1 & 2, unknown compounds; 3, luteolin; 4, 5,6,4'-trihydroxy-7,3'-dimethoxyflavone. HPLC condition: column, RCM Prep Nova-Pak  $C_{18}$  (2.5 cm  $\times$  10 cm  $\times$  2 cartridge); solvent, MeOH-H<sub>2</sub>O-TFA (60:40:0.5); flow rate, 5 ml/min; detection, 340 nm

photometer equipped with photodiode array and 1-cm cells in MeOH. The IR spectra were measured with an FT-IR spectrometer (IFS 120 HR, Bruker, Germany) with KBr as the dilutant. The NMR spectra were measured in CD<sub>3</sub>OD on a spectrometer (Unity Plus 300, Varian, USA) and chemical shifts are given as  $\delta$  value with tetramethylsilane (TMS) as an internal standard. The electron impact-mass spectrometry (EI-MS) was determined with a mass spectrometer (Quattro II, VG, UK) at an ionization voltage of 70 eV.

#### Statistical analysis

Statistical analysis was accomplished with the Statistical Analysis System (26) software package on replicated test data. Significant differences among the means were determined by Duncan's multiple range tests.

## RESULTS AND DISCUSSION

#### SLO inhibitory activity of four isolated compounds

The SLO inhibitory activity of four isolated compounds and their related flavonoids are given in Table 1. Compound 4 ( $IC_{50}$ =0.31  $\mu$ g/ml) showed the most potent inhibitory activity, comparable to that of a well known lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA,  $IC_{50}$ =0.36  $\mu$ g/ml). Compound 3 also exhibited an inhibitory activity stronger than quercetin

on an equimolar basis, although the activity was weaker than NDGA. However, the inhibitory effects of compound 1 and 2 on SLO were considerably less effective than those of 3 and 4. Thus, the strong SLO inhibitory effect of the ether soluble acidic components of *Paeonia moutan* seeds is considered to be mainly attributable to compound 3 and 4.

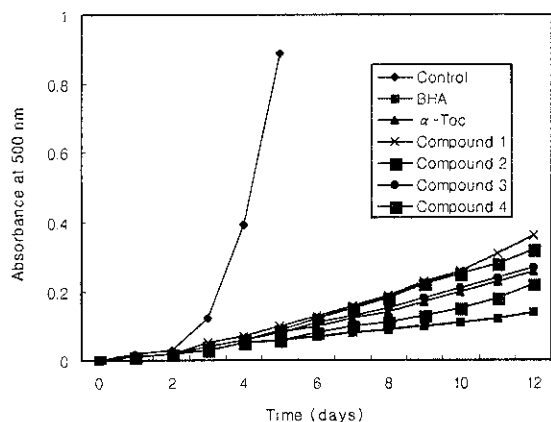
#### Antioxidative activity of four compounds

Antioxidative activity of four isolated compounds in the linoleic acid model system is shown in Fig. 2. Compound 4 showed the highest antioxidative activity, followed by compound 3, compound 2 and compound 1, in descending order. 3 and 4 exhibited especially strong antioxidative activity, comparable to that of  $\alpha$ -tocopherol ( $p < 0.05$ ), although their activity was significantly weaker than that of BHA, a reference compound. Thus, 3 and 4 were found to be strong SLO inhibitors with antioxidative activity. The two above results suggest that compound 3 and 4 from the defatted *Paeonia moutan* seeds can be potentially useful as a SLO inhibitor with antioxidative activity.

**Table 1.** Inhibitory effects of four compounds isolated from *Paeonia moutan* seeds on a soybean lipoxygenase (SLO)

Compound	SLO inhibitory activity ( $IC_{50}$ , $\mu\text{g/ml}$ ) <sup>1)</sup>
Compound 1	24.21 $\pm$ 0.12 <sup>f</sup>
Compound 2	21.54 $\pm$ 0.14
Compound 3	2.32 $\pm$ 0.08 <sup>b</sup>
Compound 4	0.31 $\pm$ 0.13 <sup>a</sup>
Apigenin	5.16 $\pm$ 0.15 <sup>d</sup>
Quercetin	3.94 $\pm$ 0.11 <sup>c</sup>
Kaempferol	8.63 $\pm$ 0.21 <sup>e</sup>
NDGA	0.36 $\pm$ 0.07 <sup>a</sup>

<sup>1)</sup> $IC_{50}$  represent the concentration of flavonoids causing 50% reduction of enzyme activity. Values in a column with the same letters are not significantly different ( $p < 0.05$ ) by Duncan's multiple range tests. NDGA, nordihydroguaiaretic acid; TDF, 5,6,4'-trihydroxy-7,3'-dimethoxyflavone. NDGA, quercetin, kaempferol and apigenin were used as positive references.



**Fig. 2.** Antioxidative activity of four compounds isolated from *Paeonia moutan* seeds. The activity was determined by a ferric thiocyanate method.

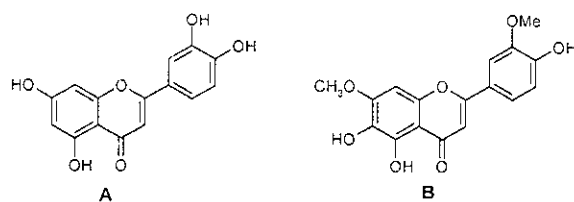
#### Structural elucidation of compound 3 and 4

The structural elucidation of compound 3 and 4 with antioxidative SLO inhibitors was performed by UV, IR, NMR and MS spectroscopy. The UV ( $\lambda_{\text{max}}$  at 253 & 349 nm and 279 & 343 nm) and IR spectra ( $\nu_{\text{max}}$  3421, 2927, 2853, 1680, 1629, 1453, 1203  $\text{cm}^{-1}$ ) of 3 and 4 suggested that two compounds were flavone or flavonol (27). The bathochromic shifts of the UV absorption maximum of 3 at 349 nm induced by NaOAc ( $\lambda_{\text{II}} = +16$  nm) and  $\text{AlCl}_3$  ( $\lambda_{\text{I}} = +77$  nm), indicated the presence of 5- and 7-hydroxy group and B-ring *ortho*-dihydroxy substitution (Table 2). The <sup>1</sup>H-NMR spectrum of 3 showed aromatic proton signals at  $\delta$  6.69 (1H, s, H-3), 6.22 (1H, d,  $J = 2.0$  Hz, H-6) and 6.47 (1H, d,  $J = 2.0$  Hz, H-8), as well as ABX-pattern proton signals at  $\delta$  6.92 (1H, d,  $J = 8.8$  Hz, H-5'), 7.44 (1H, dd,  $J = 8.8, 2.0$  Hz, H-6') and 7.59 (1H, d,  $J = 2.0$  Hz, H-2'), which can be assigned to the B-ring with two hydroxy groups at the C-3' and C-4' positions. The <sup>13</sup>C-NMR of 3 was very similar to the previously reported data (28). The electron impact-mass spectrometry (EI-MS) gave its molecular ion peak [M<sup>+</sup>] at 286  $m/z$ . Furthermore, the prominent fragment ion peak at  $m/z$  258 [286-28 (C=O)], 230 [258-28 (C=O)], 153 (benzene A ring fragment,  $\text{C}_7\text{H}_6\text{O}_4$ ), and 134 (benzene B ring fragment,  $\text{C}_8\text{H}_6\text{O}_2$ ) was observed from high-resolution EI-MS spectrum. Based on the above evidence, compound 3 was readily identified as luteolin (Fig. 3). Meanwhile, the UV and IR spectra of compound 4 were similar to those of compound 3, indicative of a flavone skeleton. The bathochromic shifts of the UV absorption maximum of 4 with NaOMe ( $\lambda_{\text{I}} = +64$  nm),  $\text{AlCl}_3$  ( $\lambda_{\text{I}} = +15$  nm), NaOAc ( $\lambda_{\text{II}} = +1$  nm), and NaOAc/ $\text{H}_3\text{BO}_3$  ( $\lambda_{\text{I}} = +1$  nm) indicated the presence of 5- & 4'-hydroxy group and 7- & 3'-substituted group in flavone (Table 2). The <sup>1</sup>H-NMR spectrum of 4 displayed the presence of flavone derivatives substituted with two methoxy groups, and the two singlet signals at  $\delta$  6.67 and 6.28 which were assigned to H-3

**Table 2.** UV absorption spectra of two flavonoids isolated from *Paeonia moutan* seeds

	UV absorption spectra ( $\lambda_{\text{max}}$ , nm)					
	MeOH	NaOMe	$\text{AlCl}_3$	$\text{AlCl}_3/\text{HCl}$	NaOAc	NaOAc/ $\text{H}_3\text{BO}_3$
Luteolin	253	266	274	275	269	259
	267	329(s)	328	355	326(s)	370
	349	401	426	385	384	430(s)
TDF <sup>1)</sup>	279	277	280	285	281	270
	342	410	359	350	341	346

<sup>1)</sup>TDF: 5,6,4'-trihydroxy-7,3'-dimethoxyflavone



**Fig. 3.** Chemical structures of luteolin (A) and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone (B) isolated from *Paeonia moutan* seeds.

and H-6, respectively. The methoxy signals at  $\delta$  3.97 and 3.94 were attributed to the C-7 and C-3' positions, which were deduced from previous UV chemical shift data. The other two aromatic proton signals at  $\delta$  6.67 (1H, s, H-3),  $\delta$  6.28 (1H, s, H-8) and ABX-pattern signals of the B-ring, 6.96 (1H, *d*, *J*=8.8 Hz, H-5'), 7.56 (1H, *d*, *J*=2.0 Hz, H-2') and 7.60 (1H, *dd*, *J*=2.0 & 8.8 Hz, H-6') indicated luteolin skeleton with a hydroxy group at the C-6 position. As  $^{13}\text{C}$ -NMR spectrum of **4** was compared with that of **3**, the signal of C-6 was shifted downfield by 31.08 ppm, whereas the signals of C-7, C-5, C-9 were displaced upfield by 9.38, 11.45 and 11.02 ppm, respectively. However, the signal of C-4 and C-10 remained unaffected. This indicated that a hydroxy group was attached to C-6 of the aglycone. The other  $^{13}\text{C}$ -NMR signals of **4** was likely consistent with the previous data with the literature values (29). The electron impact-mass spectrometry (EI-MS) gave its molecular ion peak [ $M^+$ ] at 330 *m/z*, and several prominent fragment ion peaks at *m/z* 312 [330-18 (H<sub>2</sub>O)], 284 [312-28 (C=O)], 182 (benzene A ring fragment, C<sub>6</sub>H<sub>6</sub>O<sub>5</sub>), and 148 (benzene B ring fragment, C<sub>6</sub>H<sub>6</sub>O<sub>2</sub>) was also observed. On the basis of the above spectral data, the structure of **4** was deduced to be 5,6,4'-trihydroxy-7,3'-dimethoxyflavone (Fig. 3). The detailed assignments of compound **3** and **4** for UV,

IR, NMR and MS spectra are shown in Table 3. Luteolin and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone were first isolated and identified here from *Paeonia moutan* barks and seeds, although luteolin has already been reported in other plants (20,30).

Recently, luteolin and dimethoxyflavone from natural products were found to be potent anti-inflammatory compounds (30,31), and the methanolic extract of Moutan Seed have a much higher SLO inhibitory effect than that of Moutan Cortex (24). Therefore, the defatted methanolic extract of *Paeonia moutan* seeds containing luteolin and 5,6,4'-trihydroxy-7,3'-dimethoxyflavone can be used as a potential source of natural anti-inflammatory substances. Further study on the inhibitory effects of compounds **1-4** on 5-LO derived from peritoneal polymorphonuclear leukocytes (PMNL) of rat are now in progress.

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**Table 3.** UV, IR, NMR and EI-MS spectral data of compound **3** and **4** isolated from *Paeonia moutan* seeds

Instrumental analysis	Compound <b>3</b>	Compound <b>4</b>
UV $\lambda_{\text{max}}$ nm (log $\epsilon$ )	253 (4.13), 267 (s), 349 (4.17)	278 (4.12), 342 (4.07)
IR $\nu_{\text{max}}$ (cm <sup>-1</sup> )	3421 (OH), 2927, 2853, 1680 (C=O), 1629, 1562, 1500, 1464, 1203	3423 (OH), 2929, 2876, 1682 (C=O), 1612, 1567, 1453 (OMe), 1211
$^1\text{H}$ -NMR	6.69 (1H, s, H-3) 6.22 (1H, <i>d</i> , <i>J</i> =2.0 Hz, H-6) 6.47 (1H, <i>d</i> , <i>J</i> =2.0 Hz, H-8) 6.92 (1H, <i>d</i> , <i>J</i> =8.8 Hz, H-5') 7.44 (1H, <i>dd</i> , <i>J</i> =2.0 & 8.8 Hz, H-6') 7.59 (1H, <i>d</i> , <i>J</i> =2.0 Hz, H-2')	6.67 (1H, s, H-3) 6.28 (1H, s, H-8) 6.96 (1H, <i>d</i> , <i>J</i> =8.8 Hz, H-5') 7.56 (1H, <i>d</i> , <i>J</i> =2.0 Hz, H-2') 7.60 (1H, <i>dd</i> , <i>J</i> =2.0 & 8.8 Hz, H-6')
-OCH <sub>3</sub>		3.97 (3H, s)
-OCH <sub>3</sub>		3.94 (3H, s)
$^{13}\text{C}$ -NMR	166.32 (C-2) 103.92 (C-3) 183.92 (C-4) 163.34 (C-5) 100.15 (C-6) 166.02 (C-7) 94.94 (C-8) 159.48 (C-9) 105.37 (C-10) 123.75 (C-1') 114.16 (C-2') 147.02 (C-3') 150.93 (C-4') 116.84 (C-5') 120.37 (C-6')	166.05 (C-2) 104.37 (C-3) 184.39 (C-4) 151.89 (C-5) 131.23 (C-6) 156.64 (C-7) 91.41 (C-8) 148.46 (C-9) 105.61 (C-10) 122.63 (C-1') 112.42 (C-2') 148.22 (C-3') 150.24 (C-4') 116.12 (C-5') 119.02 (C-6')
-OCH <sub>3</sub>		56.61
-OCH <sub>3</sub>		55.77
EI-MS ( <i>m/z</i> )	286 [ $M^+$ ], 258, 230	330 [ $M^+$ ], 312, 284

Coupling constants (*J* in Hz) in parentheses

S, singlet; d, doublet; dd, double doublet

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