J. Food Sci. Nutr.
Vol. 4, No. 3, p. 163~166 (1999)

Lipoxygenase Inhibitors from *Paeonia lactiflora* Seeds

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Abstract

Previously, the methanolic extract of *Paeonia lactiflora* seeds was shown to have strong inhibitory activity against soybean lipoxygenase (SLO). Four phenolic compounds were isolated from the seeds by solvent fractionation, Sephadex LH-20 column chromatography and preparative HPLC, and three of them showed strong SLO inhibition and were characterized as *trans*-resveratrol, ε-viniferin and luteolin by UV, IR, ^{1}H-NMR, ^{13}C-NMR and MS spectrometry. *trans*-Resveratrol (IC_{50}=1.02 μM), ε-viniferin (IC_{50}=0.81 μM) and luteolin (IC_{50}=10.01 μM), first found in the above seeds, exhibited a potent SLO inhibitory activity although their activity was lower than that of a well-known lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA) (IC_{50}=0.57 μM). These results suggest that *Paeonia lactiflora* seeds, now an unused plant seed, may be developed into useful sources of anti-inflammatory drugs.

Key words: *Paeonia lactiflora* seed, soybean lipoxygenase inhibitor, *trans*-resveratrol, ε-viniferin, luteolin

INTRODUCTION

5-Lipoxygenase (5-LO) is a key enzyme in arachidonate cascade metabolism which catalyzes the first step of the biosynthesis of leukotrienes (LTs) (1). Leukotrienes are reportedly involved in the pathology of a variety of inflammatory and allergic diseases including asthma, psoriasis and rheumatic arthritis (2,3). Therefore, specific 5-LO inhibitor is expected to be a potential therapeutic drug for the prevention of these diseases. At present, the spectrophotometrical *in vitro* assay using soybean lipoxygenase (SLO) is widely used to screen natural 5-LO inhibitors due to structural and mechanistic similarities between SLO and human 5-LO (4,5).

trans-Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a well-known phytoalexin found in the grape vine, *Vitis vinifera*, as well as in other various families of plants (6-8). *trans*-Resveratrol has been shown to inhibit platelet aggregation (9-12), low-density lipoprotein oxidation (13) and carcinogenesis (14), and to protect the liver from lipid peroxidation (15). Recently, *trans*-resveratrol was found to have estrogenic properties (16) which may explain in part the cardiovascular benefits of wine drinking. In addition, resveratrol and its derivatives have been reported to inhibit melanin biosynthesis as a potent tyrosinase inhibitor (17,18), which can be used in cosmetics as skin-whitening agents. Thus, the plants containing resveratrol and its derivatives with a variety of biochemical and phamacological activities are very useful as potential sources of medicinal drugs.

Recently, much attention has been focused on the development of novel lipoxygenase inhibitors from plant seeds. We have screened SLO inhibitors from the oil cake of plant seeds, now an industrial waste product. As a result, several lignans and flavonoids were found to act as active principles of SLO inhibition (19-22). In a previous report, we found that the methanolic extract of *Paeonia lactiflora* seeds showed a potent SLO inhibitory activity, while that of its root, containing paeoniflorin and its derivatives known to be analgesic and antiinflammatory agents, exhibited less activity (21). This led us to examine different anti-inflammatory constituents in the *P. lactiflora* seeds.

The purpose of this study was to isolate and identify soybean lipoxygenase inhibitors from *Paeonia lactiflora* seeds.

MATERIALS AND METHODS

Materials and reagents

The seeds of *Paeonia lactiflora* Pall. were directly harvested in late August in Uisong, Kyongbuk, Korea. Soybean lipoxygenase (type V), linolenic acid, trifluoroacetic acid (TFA), and nordihydroguaiaretic acid (NDGA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other used for this study were analytical grade.

Soybean-lipoxygenase (SLO) assay

SLO assay was performed by a modified method of Block et al. described previously (19). 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, Sinco, Korea). The rates were measured from initial slopes of the linear portions of the curves. A sample containing all of the reagents except the enzyme solution was used as a blank sample. IC_{50} values were determined by linear regression analysis of the results at three different concentrations of the inhibitor.

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Isolation and purification of soybean lipoxygenase inhibitors

Dried *Paeonia lactiflora* seeds (100 g) were ground and extracted twice with hot n-hexane (2.0 l) to remove lipids. The residue was extracted twice with 80% aqueous MeOH (2.0 l) under reflux, and then filtered. The 80% aqueous methanolic layer was evaporated to a small volume *in vacuo*, and then partitioned with ether, ethyl acetate, and n-butanol, stepwise. The ether, ethyl acetate, n-butanol, and aqueous portion, each at a concentration of 10 μg/ml, showed SLO inhibitory activity of 77.5, 56.2, 24.5, and 10.4%, respectively. The ether soluble layer (3.1 g) was subjected to chromatography on a silica gel (70-230 mesh, Merck, Germany) using $CHCl_3$-MeOH (5:1) with increasing amounts of MeOH to give six fractions (fractions I-VI). Fractions IV and V consisted of a large amount of stilbene and flavonoid derivatives, which exhibited bluish-purple and yellow fluorescent spots on UV illumination of the TLC plates. These fractions were further subjected to column chromatography over Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) with MeOH as an eluent and then separated into five fractions (fractions I~V). The SLO inhibition activity of each fraction was 12.3, 78.3, 75.2, 31.3, 41.2%, respectively, in a concentration of 10 μg/ml. Fractions II and III were further purified by preparative HPLC (Waters Delta Prep 4000, USA) using a Waters RCM Prep Nova-pak HR C_{18} column (25 mm × 100 mm × 2 cartridge) monitored at 300 nm. The purification was conducted using a linear gradient of 100% solvent A (MeOH-H_2O-TFA=40:60:0.1) to 100% solvent B (100% MeOH) for 60 min with 5 ml/min of flow rate. Four phenolic compounds (compound **1**, 31.4 mg; compound **2**, 44.5 mg; compound **3**, 104.1 mg; compound **4**, 219.5 mg) were repeatedly isolated (Fig. 1). Among them, compound **2**, **3** and **4** with potent SLO inhibition activity were identified by UV, IR, NMR, and EI-MS spectroscopy.

Instrumental analysis

The UV and IR spectra were obtained with a photodiode array Sinco UV spectrophotometer and an IFS 120 HR FT-IR spectrometer (Bruker, Germany), repectively. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were measured in CD_3OD on a Unity Plus 500 spectrometer (Varian, USA) and chemical shifts are given as δ value with tetramethylsilane (TMS) as an internal standard. The electron impact-mass spectrometry (EI-MS) was determined with a Quattro II mass spectrometer (VG, U.K) at an ionization voltage of 70 eV.

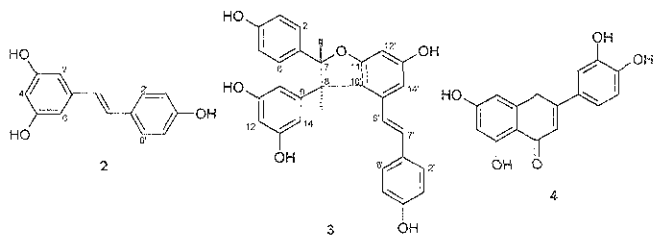

Fig. 1. Chemical structures of *trans*-resveratrol (2), ε-viniferin (3) and luteolin (4) isolated from *Paeonia lactiflora* seeds.

RESULTS AND DISCUSSION

Structural elucidation of isolated compound 2, 3 and 4

As shown in Fig. 1, four compounds were isolated from *Paeonia lactiflora* seeds on the final purification step by preparative HPLC. The chemical structures of compound **2**, **3** and **4** with potent SLO inhibitory activity were characterized by UV, IR, NMR and MS spectrometry. Compounds **2** and **3** emitted a blue fluorescence under UV lamp (365 nm), and **3** especially gave an orange color which subsequently turned to dark brown, then to green on a TLC plate when sprayed with ceric sulphate. The UV (λ_{max} at 308 & 320 nm) and IR spectra (ν_{max} at 3200-3300, 1590, 1509, 1151, 967 cm^{-1}) data of **2** indicated the presence of a *trans*-stilbene moiety (23). The ^{1}H-NMR spectrum of **2** (in CD_3OD) showed the presence of two independent aromatic rings and a *trans*-olefinic group. One aromatic ring showed AX_2-type signals (δ 6.15, 1H, t, J=2Hz; δ 6.43, 2H, d, J=2Hz) which were assignable to protons on a 1,3,5-trisubstituted aromatic ring, while the other showed A_2X_2-type signals at δ 6.75 and 7.33 (each 2H, d, J=8Hz) assignable to protons on a 4′-substituted system. Furthermore, two proton signals appeared at comparatively low field as a pair of doublets (δ 6.94, 6.79, each 1H, d), and its coupling constant (J=16Hz) suggested the presence of a *trans*-olefinic group in **2**. The ^{13}C-NMR spectrum of **2** is very similar to the previously reported data (24). The EI mass spectrum of **2** exhibited a molecular ion peak at *m/z* 228, together with a minor peak at *m/z* 114 and 181 suggestive of the presence of stilbene group. From these spectral data, compound **2** was determined to be *trans*-resveratrol (Fig. 2). Meanwhile, the UV spectrum with the absorption maxima at 312 and 324 nm and the IR spectrum with the band at 3230, 1594, 964 cm^{-1} of **3** indicated the presence of a *trans*-stilbene moiety (23). No shifts were observed with added sodium acetate-boric acid, indicating the absence of *ortho*-dihydroxy groups. The ^{1}H-NMR spectrum of **3** (in Me_2CO-d_6) showed two doublets at δ_H 4.34 and 5.35 (1H each, J=6.5Hz) and the presence of 12 aromatic protons which definitely belong to two sets, one consisting of eight and the other of four protons, to account for the A_2B_2 quartet centered at δ 6.85 and the meta coupled doublets at 6.25 and 6.16. The coupled protons at δ_H 6.82 (1H, d, J=16.5Hz) and at δ_H 6.62 (1H, d, J=16.5Hz) are due to *trans*-olefinic protons. The ^{13}C-NMR spectrum of **3** showed four singlets (δ_C 157-163 ppm) for six phenolic carbon atoms, eight doublets (97-129 ppm) for aromatic carbon atoms, two doublets (126.6 and 131.6 ppm) for olefinic carbon atoms, and two further doublets (57.7 and 94.9 ppm) for aliphatic carbon atoms. Further, **3** exhibited a molecular ion peak at *m/z* 454 in agreement with the molecular formula $C_{28}H_{22}O_6$, even though did not elucidate fragment ion peaks in this study. On the basis of these data, compound **3** was deduced to be *trans*-ε-viniferin (a resveratrol dimer) (Fig. 2) reported previously from several plant families including Vitaceae, Dipterocarpaceae and Cyperaceae (25-27). Finally, the ^{1}H-& ^{13}C-NMR and EI mass spectrum of

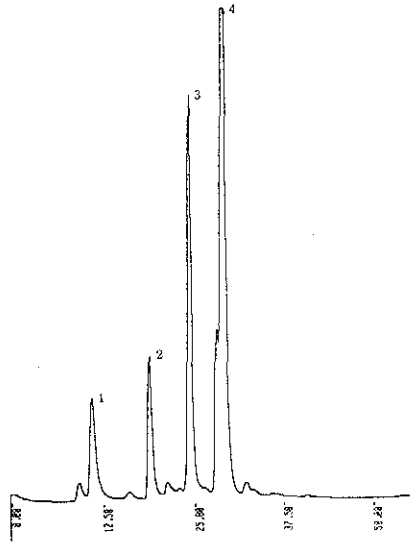

Fig. 2. HPLC chromatogram of four compounds isolated from *Paeonia lactiflora* seeds. **1**, unknown compounds; **2**, *trans*-resveratrol; **3**, ε-viniferin; **4**, luteolin. HPLC condition: column, RCM Prep Nova-Pak C_{18} (2.5 cm × 10 cm × 2 cartridge); linear gradient elution from solvent A (0.1% TFA in 40% MeOH) to solvent B (100% MeOH) for 60 min; flow rate, 5 ml/min; detection, 300 nm.

compound **4** exhibited those of luteolin (Fig. 2) which was already isolated from *Paeonia moutan* seed (22). The detailed assignments of compound **2**, **3** and **4** for UV, IR, NMR and MS spectra are shown in Table 1. It is very interesting to note that *Paeonia lactiflora* seeds contain higher concentration of *trans*-resveratrol and ε-viniferin, which were first isolated and characterized from Paeony roots and seeds.

SLO inhibitory activity of isolated compounds 2, 3 and 4

The SLO inhibitory activity of *trans*-resveratrol, ε-viniferin and luteolin isolated from *P. lactiflora* seed is given in Table 2. *trans*-Resveratrol (IC_{50}=1.02 μM), ε-viniferin (IC_{50}=0.81 μM) and luteolin (IC_{50}=10.01 μM) showed potent SLO inhibitory activity, although their activity was weaker than a known SLO inhibitor, nordihydroguaiaretic acid (NDGA, IC_{50}=0.57 μM). These results in part supported earlier reports that resveratrol and its oligomeric stilbenes had strong anti-inflammatory activity (28,29). Thus, *trans*-resveratrol, ε-viniferin and luteolin may be mainly responsible for potent SLO inhibitory effect of the methanolic extracts of *Paeonia lactiflora* seeds, which can be used as potential sources of anti-inflammatory drugs. Further study on the identification of compound **1** and other minor compounds from *P. lactiflora*, and

Table 1. UV, IR, NMR and EI-MS spectral data of *trans*-resveratrol and ε-viniferin isolated from *Paeonia lactiflora* seeds

Instrumental analysis	*trans*-Resveratrol	ε-Viniferin
UV λ_{max} nm (log ε)	219 (4.28), 308 (4.02), 320 (3.34)	218 (4.52), 312 (4.40), 324 (4.48)
IR ν_{max} (cm^{-1})	3,200-3,300, 1,589, 1,507, 1,150, 966	3,230, 1,594, 1,510, 1,440, 1,002, 964
^{1}H-NMR	6.43 (1H, *d*, *J*=2.5 Hz, H-2)	7.19 (2H, *d*, *J*=8.5 Hz, H-2 & 6)
	6.14 (1H, *t*, *J*=2.5 Hz, H-4)	6.83 (2H, *d*, *J*=8.5 Hz, H-3 & 5)
	6.43 (1H, *d*, *J*=2.5 Hz, H-6)	5.41 (1H, *d*, *J*=5.5 Hz, H-7)
	7.33 (1H, *d*, *J*=6.5 Hz, H-2′)	4.47 (1H, *d*, *J*=5.5 Hz, H-8)
	7.33 (1H, *d*, *J*=6.5 Hz, H-6′)	6.32 (2H, *d*, *J*=2.0 Hz, H-10 & 14)
	6.75 (1H, *d*, *J*=6.5 Hz, H-3′)	6.29 (1H, *t*, *J*=2.0 Hz, H-12)
	6.75 (1H, *d*, *J*=6.5 Hz, H-5′)	7.15 (2H, *d*, *J*=8.5 Hz, H-2′ & 6′)
	6.79 (1H, *d*, *J*=16.5 Hz, H α)	6.75 (2H, *d*, *J*=8.5 Hz, H-3′ & 5′)
	6.94 (1H, *d*, *J*=16.5 Hz, H β)	6.91 (1H, *d*, *J*=16.5 Hz, H-7′)
		6.71 (1H, *d*, *J*=16.5 Hz, H-8′)
		6.24 (1H, *d*, *J*=1.0 Hz, H-12′)
		6.03 (1H, *d*, *J*=1.0 Hz, H-14′)
^{13}C-NMR	141.31 (C-1)	133.69 (C-1)
	105.76 (C-2)	127.93 (C-2 & 6)
	159.37 (C-3)	116.27 (C-3 & 5)
	102.64 (C-4)	158.27 (C-4 & C-4′)
	159.37 (C-5)	94.13 (C-7)
	108.20 (C-6)	57.01 (C-8)
	127.02 (C α)	147.17 (C-9)
	130.42 (C β)	106.90 (C-10 & 14)
	131.40 (C-1′)	159.83 (C-11 & 13)
	129.38 (C-2′)	101.76 (C-12)
	115.84 (C-3′)	129.87 (C-1′)
	158.37 (C-4′)	128.72 (C-2′ & 6′)
	116.48 (C-5′)	116.27 (C-3′ & 5′)
	128.79 (C-6′)	130.88 (C-7′)
		126.12 (C-8′)
		137.18 (C-9′)
		120.03 (C-10′)
		162.56 (C-11′)
		96.53 (C-12′)
		159.51 (C-13′)
		106.72 (C-14′)
EI-MS (*m/z*)	228 [M^+], 181, 114	454 [M^+], 438, 360, 342, 256, 239

Coupling constants (*J* in Hz) in parentheses
d, doublet; t, triplet

Table 2. Inhibitory effects of *trans*-resveratrol, ε-viniferin and luteolin isolated from *Paeonia lactiflora* seeds on a soybean lipoxygenase (SLO)

Compound	SLO inhibitory activity (IC_{50}, μM)[1)]
trans-Resveratrol	1.02
ε-Viniferin	0.81
Luteolin	10.01
NDGA	0.57

[1)]IC_{50} value, the concentration of sample causing 50% inhibition of SLO activity, was calculated by linear regression analysis. NDGA, nordihydroguaiaretic acid, was used as reference compounds.

the inhibitory effects of four isolated compounds on 5-LO derived from peritoneal polymorphonuclear leukocytes (PMNL) of the rat are now in progress.

ACKNOWLEDGEMENTS

This research was partly funded by a special Grants Research Program (No. 296076) of the High-Technology Development Project for Agriculture, Forestry, and Fisheries in Korea. The authors are indebted to Dr. Kim Sung-Hong and Ms. Chae, Seen-Ae, Korea Basic Science Institute, Taegu, Korea, for ^{1}H-NMR and ^{13}C-NMR and EI-MS measurements.

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(Received July 7, 1999)