Phenolic Glycosides from *Lindera fruticosa* Root and Their Inhibitory Activity on Osteoclast Differentiation

Myoung-Chong Song,[a] Fikru Nigussie,[b] Hye-Joung Yang,[a] Hong-Hee Kim,[c] Ji-Young Kim,[a] Dae-Kyun Chung,[a] and Nam-In Baek*,[a]

[a] *Graduate School of Biotechnology and Plant Metabolism Research Center, Kyung Hee University; Suwon 446701, South Korea:* [b] *Faculty of Veterinary Medicine, Addis Ababa University; Debre Zeit 34, Ethiopia: and* [c] *Department of Cell and Developmental Biology, College of Dentistry, Seoul National University; Seoul 110749, South Korea.*
Received September 28, 2007; accepted February 5, 2008; published online February 29, 2008

Two new compounds were found in the phenolic glycosides isolated from the roots of *Lindera fruticosa*: 5-*O*-[β-D-apiofuranosyl-(1″→2′)-*O*-β-D-xylopyranosyl]gentisic acid-7,5″-ester (3), named linderofruticoside A; and 5-*O*-[β-D-apiofuranosyl-(1″→3′)-*O*-β-D-xylopyranosyl]gentisic acid methyl ester (4), linderofruticoside B. Two previously known phenolic glycosides were also identified: β-D-(3,4-disinapoyl)fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (1) and β-D-(3-sinapoyl)fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (2). Compounds 1 and 2 inhibited osteoclast differentiation in a dose-dependent manner at concentrations higher than 1.04 μM and 0.132 μM, respectively.

Key words *Lindera fruticosa*; phenolic glycoside; linderofruticoside A; linderofruticoside B; osteoclast differentiation

Lindera fruticosa Hemsley is a shrub that grows in China, Nepal, India, and Ethiopia. Although the fruit and roots of this plant have been used in folk remedies in Ethiopia to treat diseases, little phytochemical and pharmacological research has been carried out on *L. fruticosa* to date.[1,2] This paper describes the isolation of two new phenolic glycosides, in addition to two known ones, from *L. fruticosa*, and demonstrates their inhibitory effect on osteoclast differentiation.

Results and Discussion

Dried powdered roots were extracted with 80% aqueous methanol (MeOH), and the extracts were then partitioned using water, ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). The EtOAc and *n*-BuOH extracts were subjected to ODS C_{18}, Sephadex G-15, and silica gel column chromatography. This resulted in four phenolic glycosides with sinapic acid or gentisic acid, including two new compounds. By comparing our findings with published spectroscopic data, we identified the known compounds as: β-D-(3,4-disinapoyl)fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (**1**)[3] and β-D-(3-sinapoyl)-fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (**2**).[3] The new compounds, which will subsequently be referred to as **3** and **4**, were identified based on spectroscopic and physiochemical evidence.

Compound **3** was isolated as a yellowish, amorphous powder. Its IR spectrum showed absorptions characteristic of an H-bonded hydroxyl group (3398 cm^{-1}), phenyls (3140, 1682, 1609, 1480 cm^{-1}), and an ester (1745 cm^{-1}). Negative FAB-MS exhibited a quasi-molecular ion at *m/z* 399 $[M-H]^-$, corresponding to a molecular formula of $C_{17}H_{20}O_{11}$. This molecular formula ($C_{17}H_{20}O_{11}$) was determined by negative HR-FAB-MS ($[M-H]^-$, *m/z* 399.0926, Calcd 399.0927 for $C_{17}H_{19}O_{11}$). The ^{1}H-NMR spectrum of compound **3** contained signals of a 1,2,4-trisubstituted benzene ring [olefinic protons at δ_H 7.63 (1H, d, *J*=2.8 Hz, H-6), δ_H 7.37 (1H, dd, *J*=9.2, 2.8 Hz, H-4), and δ_H 6.07 (1H, d, *J*=9.2 Hz, H-3)]. In the oxygenated sp^3 region, we detected two hemiacetal proton signals at δ_H 6.50 (1H, br s, H-1″) and δ_H 5.51 (1H, d, *J*=8.0 Hz, H-1′), four oxygenated methine signals [δ_H 4.43 (1H, dd, *J*=8.0, 8.8 Hz, H-2′), δ_H 4.42 (1H, br s, H-2″), δ_H 4.36 (1H, dd, *J*=8.0, 8.8 Hz, H-3′), δ_H 4.23 (1H, ddd, *J*=10.4, 10.4, 8.0 Hz, H-4′)], and three oxygenated methylene signals [δ_H 4.98 (1H, d, *J*=9.2 Hz, H-4″a), δ_H 4.48 (1H, d, *J*=9.2 Hz, H-4″b), δ_H 4.21 (1H, dd, *J*=10.4, 10.4 Hz, H-5′a), and δ_H 3.87 (1H, dd, *J*=10.4, 10.4 Hz, H-5′b)] including downfield-shifted signals at δ_H 4.82 (1H, d, *J*=10.8 Hz, H-5″a) and δ_H 4.72 (1H, d, *J*=10.8 Hz, H-5″b) caused by an esterification effect between C-5″ and C-7. On the basis of these observations, we assumed compound **3** was a phenolic glycoside with two pentoses.

The ^{13}C-NMR spectrum indicated signals of a carbonyl carbon at δ_C 169.5 (C-7), two oxygenated olefinic quaternary carbons at δ_C 156.6 (C-2) and δ_C 149.3 (C-5), an olefinic quaternary carbon at δ_C 112.6 (C-1), and three olefinic methine carbons [δ_C 122.2 (C-4), δ_C 118.5 (C-3), and δ_C 116.3 (C-6)]. The spectroscopic data implied that the compound was a trioxygenated benzoic acid with a 1,2,4-trisubstituted benzene ring and supported the supposition that compound **3** had gentisic acid as an aglycone. In the oxygenated sp^3 area, we observed two hemiacetal signals as anomeric carbon [δ_C 109.1 (C-1″) and δ_C 99.0 (C-1′)], an oxygenated quaternary carbon at δ_C 78.7 (C-3″), five oxygenated methine carbons [δ_C 79.1 (C-3′), δ_C 78.2 (C-2″), δ_C 76.0 (C-2′), δ_C 71.0 (C-4′)], and three oxygenated methylene carbons [δ_C 74.5 (C-4″) and δ_C 67.2 (C-5′), including a downfield-shifted carbon signal at δ_C 69.8 (C-5″) caused by an esterification effect between C-7 and C-5″]. From this evidence, we expected the two pentoses of compound **3** to be an apiose and a xylose with an ester bond between the pentose and gentisic acid. Additionally, on acidic hydrolysis, D-xylose and D-apiose were identified as component sugars of compound **3**. The absolute configuration of D-xylose and D-apiose was identified by GC analysis of their trimethylsilyl derivatives. The specific rotation of the obtained D-apiose was $[\alpha]_D$ +5.8° (*c*=0.165, MeOH), which corresponds to the value of $[\alpha]_D$ +6.4° found by Gorin and Perlin[4] for D-apiose. The chemical shifts and coupling constants of H-1′ (δ_H 5.51, d, *J*=8.0 Hz) and of H-1″ (δ_H 6.50, br s), and the chemical

* To whom correspondence should be addressed. e-mail: nibaek@khu.ac.kr.

shifts of C-1′ (δ_C 99.0) and C-1″ (δ_C 109.1) supported a β-configuration for the anomeric carbons.[5] We subsequently confirmed the whole structure of compound **3** with HMBC spectroscopic data. The correlation of H-1′ (δ_H 5.51) with C-2′ (δ_C 76.0) and C-5 (δ_C 149.3) confirmed the attachment of a xylose at C-5 of the gentisic acid moiety. C-2′ (δ_C 76.0) showed cross peaks with H-1′ (δ_H 5.51), H-3′ (δ_H 4.36), and H-1″ (δ_H 6.50) proving that an apiose group was linked to C-2′ of the xylosyl residue. The oxygenated quaternary C-3″ (δ_C 78.7) showed cross peaks with H-2″ (δ_H 4.42), H-4″a (δ_H 4.98), H-4″b (δ_H 4.48), H-5″a (δ_H 4.82), and H-5″b (δ_H 4.72) by *J*2 correlation. H-1″ (δ_H 6.50) also displayed cross peaks with C-3″ (δ_C 78.7) and C-4″ (δ_C 74.5) by *J*3 correlation to reveal an apiofuranoside. H-5″a (δ_H 4.82) and H-5″b (δ_H 4.72) exhibited cross peaks with C-7 (δ_C 169.5) to confirm ester formation between the carbonyl group of the gentisic acid moiety and the hydroxy group at C-5″ of the apiofuranose through an ester bond. In the ^{1}H-NMR data, the significant downfield shift of H-5″ also supported acylation of the hydroxy group at C-7 with C-5″ of apiose. Ultimately, we identified the new compound **3** as 5-*O*-[β-D-apiofuranosyl-(1″→2′)-*O*-β-D-xylopyranosyl] gentisic acid-7,5″-ester, and named it linderofruticoside A.

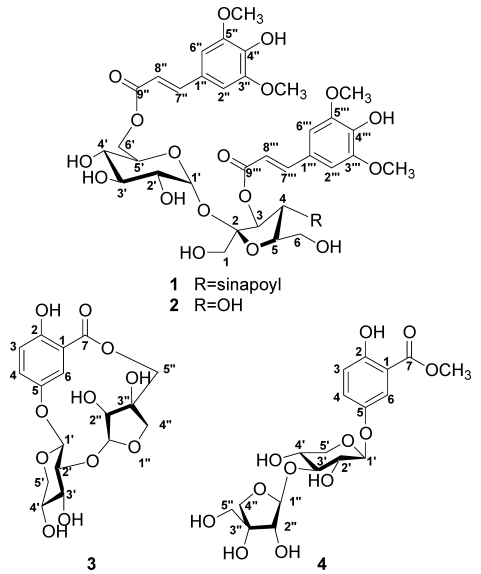

Fig. 1. Chemical Structures of Compounds **1**—**4** Extracted from *Lindera fruticosa*

Compound **4** was isolated as a yellowish, amorphous powder. Its IR spectrum showed absorptions characteristics of an H-bonded hydroxyl group (3386 cm^{-1}), phenyls (3120, 1630, 1605, 1472 cm^{-1}), and an ester (1749 cm^{-1}). Negative FAB-MS exhibited a quasi-molecular ion at *m/z* 431 [M−H]$^-$ corresponding to a molecular formula of $C_{18}H_{24}O_{12}$. A molecular formula of $C_{18}H_{24}O_{12}$ was determined by negative HR-FAB-MS ([M−H]$^-$, *m/z* 431.1203, calcd 431.1189 for $C_{18}H_{23}O_{12}$). The ^{1}H- and ^{13}C-NMR spectra revealed compound **4** to be similar to compound **3**, with the exception of a downfield shift of C-7 (δ_C 172.0), an upfield shift of H-5″ (δ_H 3.55) and C-5″ (δ_C 65.8), and the presence of a methoxy proton (δ_H 3.94) and carbon (δ_C 53.0). We subsequently confirmed the whole structure of compound **4** with heteronuclear multiple bonding correlation (HMBC) spectroscopic data.

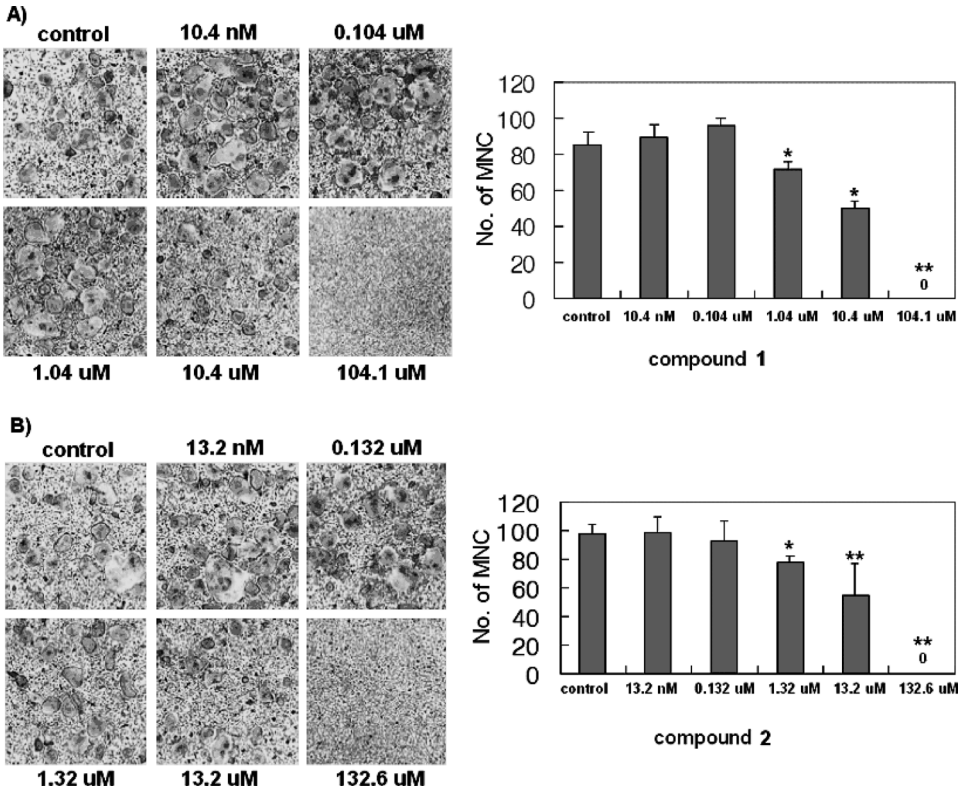

Fig. 2. Effect of Compounds **1** and **2** on Osteoclast Differentiation

Bone marrow-derived macrophages were cultured in 48-well plates in the presence of 30 ng/ml M-SCF and 50 ng/ml RANKL for 5 d with the indicated concentrations of compound **1** or **2**. Photographs of TRAP-stained osteoclasts generated from BMMs (image to the left of A and B). Cells were fixed and stained for TRAP, and TRAP-positive MNC were counted (image to the right of A and B). Data from one experiment are presented as mean±S.E. of triplicate samples, and similar results were obtained in two other experiments. $*p<0.05$, $**p<0.01$, significantly different from the control.

The H-1′ (δ_H 4.80) showed correlation peaks with C-2′ (δ_C 78.0) and C-5 (δ_C 151.0), confirming the linkage of a xylose at C-5 of the gentisic acid moiety. The C-3′ (δ_C 79.0) showed cross peaks with H-2′ (δ_H 3.54), H-4′ (δ_H 3.55), and H-1″ (δ_H 5.41), proving that an apiofuranosyl group was linked to C-3′ of the xylosyl residue. The methoxy protons (δ_H 3.94) exhibited a cross peak with C-7 (δ_C 172.0), confirming the methyl esterification of the C-7 carboxylic acid of the gentisic acid moieties. The chemical shifts and coupling constants of H-1′ (δ_H 4.80, d, J=6.8 Hz) and of H-1″ (δ_H 5.41, d, J=2.0 Hz), and the chemical shifts of C-1′ (δ_C 102.9) and C-1″ (δ_C 111.0) supported a β-configuration for their anomeric carbons.[5] The new compound **4** was therefore identified as 5-*O*-[β-D-apiofuranosyl-(1″→3′)-*O*-β-D-xylopyranosyl] gentisic acid methyl ester and was named linderofruticoside B.

All four compounds were evaluated for inhibitory activity on osteoclast differentiation. Osteoclasts are multinucleated cells (MNC) formed by multiple steps of cell differentiation from progenitor cells of hematopoietic origin. Intervention in osteoclast differentiation is considered an effective therapeutic approach for the treatment of bone disease involving osteoclasts.[6] Each of the isolated compounds from *L. fruticosa* was tested for inhibitory effects on osteoclast differentiation in cultures of mouse bone marrow cells and osteoblasts. Compounds **1** and **2** effectively reduced the formation of tartrate resistant acid phosphatase positive (TRAP+) MNC. Compound **1** decreased osteoclast generation by 9.63±2.35% at 1.04 μM, 40.72±2.44% at 10.4 μM, and 100% at 104 μM, and slightly increased generation at concentrations lower than 1.04 μM in a co-culture system (Fig. 2A right). Compound **2** reduced osteoclast generation by 5.15±1.36% at 0.132 μM, 19.59±2.27% at 1.32 μM, 42.27±2.32% at 13.2 μM, and 100% at 132.6 μM (Fig. 2B right). Specifically, compounds **1** and **2** did not show any cytotoxicity at concentrations lower than 100 μM. Compounds **1** and **2** inhibited osteoclast differentiation more than the naturally occurring inhibitor, tanshinone IIA isolated from *Salvia miltiorrhiza*, which inhibited differentiation by 100, 93.62, and 79.70% at 5.88, 2.94, and 1.47 μM, respectively.[7] In summary, compounds **1** and **2** are effective inhibitors of osteoclast differentiation in bone marrow-derived macrophage culture systems.

Experimental

General Experimental Procedures and Plant Material Details are provided in a previous paper.[2,8]

Extraction and Isolation Dried, powdered roots (1 kg) were extracted with 80% aqueous methanol (MeOH–H_2O) (20 l×3), and concentrated *in vacuo*. The extracts were partitioned with H_2O (2 l), EtOAc (2 l×3), and *n*-BuOH (2 l×3). The concentrated EtOAc fraction (LFE, 14 g) was subjected to silica gel column chromatography (150 g, Φ 6.5×12 cm) and eluted with a gradient of $CHCl_3$–MeOH (10 : 1→7 : 1, 1 l of each), resulting in 12 fractions (LFE1—LFE12). Fraction LFE4 [1.3 g, Ve/Vt (elution volume/total volume) 0.15—0.18] was subjected to silica gel column chromatography (150 g, Φ 6×10 cm) and eluted with $CHCl_3$–MeOH (10 : 1→8 : 1→6 : 1→4 : 1, 2.2→1.8→1.4→1.0 l of each), yielding compound **1** [231 mg, Ve/Vt 0.40—0.70; TLC (Keiselgel 60 F_{254}) *Rf* 0.4, $CHCl_3$–MeOH, 5 : 1]. Fraction LFE7 (584 mg, Ve/Vt 0.27—0.53) was separated by silica gel column chromatography (75 g, Φ 3.5×15 cm) and eluted with $CHCl_3$–MeOH (5 : 1, 1.8 l), yielding compound **2** [210 mg, Ve/Vt 0.20—0.30; TLC (Keiselgel 60 F_{254}) *Rf* 0.5, $CHCl_3$–MeOH, 3 : 1]. The concentrated *n*-BuOH fraction (LFB, 14 g) was subjected to silica gel column chromatography (150 g, Φ 6×14.5 cm) and eluted with a gradient of $CHCl_3$–MeOH–H_2O (7 : 3 : 1→6 : 4 : 1, 2.2 l of each lower layer), resulting in 10 fractions (LFB1—LFB10). Fraction LFB5 (617 mg, Ve/Vt 0.40—0.50) was separated by ODS column chromatography (100 g, Φ 3×10 cm) and eluted with MeOH–H_2O (1 : 1→3 : 2, 1.4→1.0 l of each), resulting in 8 fractions (LFB5-1—LFB5-8). Fraction LFB5-6 (70 mg, Ve/Vt 0.51—0.61) was subjected to Sephadex G-15 column chromatography (100 g, Φ 3.5×30 cm) and eluted with MeOH–H_2O (1 : 2, 1.5 l), yielding compound **3** [35 mg, Ve/Vt 0.73—1.00; TLC (RP-18 F_{254S}) *Rf* 0.5, MeOH–H_2O, 3 : 1]. Fraction LFB7 (15.4 g, Ve/Vt 0.65—0.80) was separated by ODS column chromatography (75 g, Φ 5×8.5 cm) and eluted with MeOH–H_2O (1 : 1→3 : 2, 1.4→1.0 l of each), resulting in 8 fractions (LFB7-1—LFB7-8). Fraction LFB7-2 (410 mg, Ve/Vt 0.10—0.20) was subjected to silica gel column chromatography (100 g, Φ 3×7 cm) and eluted with $CHCl_3$–MeOH–H_2O (12 : 3 : 1, lower layer), yielding compound **4** [35 mg, Ve/Vt 0.13—0.16; TLC (Keiselgel 60 F_{254}) *Rf* 0.7, $CHCl_3$–MeOH–H_2O, 12 : 3 : 1].

5-*O*-[β-D-Apiofuranosyl-(1″→2′)-*O*-β-D-xylopyranosyl]gentisic Acid-7,5″-ester (Linderofruticoside A, **3**): Yellow amorphous powder: $[\alpha]_D^{26}$ −100° (c=0.21, MeOH); IR (CaF_2 window in MeOH) ν_{max} 3398, 3140, 1745, 1682, 1609, 1480, 1320, 1240, 1078, 1036, 1008 cm^{-1}; neg. FAB-MS m/z 399.1 $[M-H]^-$; neg. HR-FAB-MS m/z 339.0926 $[M-H]^-$ (Calcd for $C_{17}H_{19}O_{11}$=399.0927); ^{1}H-NMR (400 MHz, pyridine-d_5) δ 7.63 (1H, d, J=2.8 Hz, H-6), 7.37 (1H, dd, J=9.2, 2.8 Hz, H-4), 6.50 (1H, br s, H-1″), 6.07 (1H, d, J=9.2 Hz, H-3), 5.51 (1H, d, J=8.0 Hz, H-1′), 4.98 (1H, d, J=9.2 Hz, H-4″a), 4.82 (1H, d, J=10.8 Hz, H-5″a), 4.72 (1H, d, J=10.8 Hz, H-5″b), 4.48 (1H, d, J=9.2 Hz, H-4″b), 4.43 (1H, dd J=8.0, 8.8 Hz, H-2′), 4.42 (1H, br s, H-2″), 4.36 (1H, dd, J=8.8, 8.0 Hz, H-3′), 4.23 (1H, ddd, J=10.4, 10.4, 8.0 Hz, H-4′), 4.21 (1H, dd, J=10.4, 10.4 Hz, H-5′a), 3.87 (1H, dd, J=10.4, 10.4 Hz, H-5′b), ^{13}C-NMR (100 MHz, pyridine-d_5) δ 169.5 (C-7), 156.6 (C-2), 149.3 (C-5), 122.2 (C-4), 118.5 (C-3), 116.3 (C-6), 112.6 (C-1), 109.1 (C-1″), 99.0 (C-1′), 79.1 (C-3′), 78.7 (C-3″), 78.2 (C-2″), 76.0 (C-2′), 74.5 (C-4″), 71.0 (C-4′), 69.8 (C-5″), 67.2 (C-5′).

5-*O*-[β-D-Apiofuranosyl-(1″→3′)-*O*-β-D-xylopyranosyl]gentisic Acid Methyl Ester (Linderofruticoside B, **4**): Yellow amorphous powder: $[\alpha]_D^{27}$ −41° (c=0.03, MeOH); IR (CaF_2 window in MeOH) ν_{max} 3386, 3120, 1749, 1630, 1605, 1472, 1335, 1230, 1088, 1032, 1010 cm^{-1}; pos. FAB-MS m/z 455.3 $[M+Na+H]^+$; neg. FAB-MS m/z 431.2 $[M-H]^-$; neg. HR-FAB-MS m/z 431.1203 $[M-H]^-$ (Calcd for $C_{18}H_{23}O_{12}$ = 431.1189); ^{1}H-NMR (400 MHz, CD_3OD) δ 7.49 (1H, d, J=3.2 Hz, H-6), 7.24 (1H, dd, J=8.8, 3.2 Hz, H-4), 6.88 (1H, d, J=8.8 Hz, H-3), 5.41 (1H, d, J=2.0 Hz, H-1″), 4.80 (1H, d, J=6.8 Hz, H-1′), 4.03 (1H, d, J=9.2 Hz, H-4″a), 3.96 (1H, d, J=2.0 Hz, H-2″), 3.94 (3H, s, H-OCH_3), 3.90 (1H, dd, J=11.2, 4.8 Hz, H-5′a), 3.77 (1H, d, J=9.2 Hz, H-4″b), 3.57 (1H, dd, J=8.4, 8.0 Hz, H-3′), 3.55 (1H, ddd, J=10.4, 8.0, 4.8 Hz, H-4′), 3.55 (2H, s, H-5″), 3.54 (1H, dd, J=8.4, 6.8 Hz, H-2′), 3.30 (1H, dd, J=11.2, 10.4 Hz, H-5′b), ^{13}C-NMR (100 MHz, CD_3OD) δ 113.2 (C-1), 158.2 (C-2), 119.1 (C-3), 127.2 (C-4), 151.0 (C-5), 118.3 (C-6), 172.0 (C-7), 102.9 (C-1′), 78.0 (C-2′), 79.0 (C-3′), 71.0 (C-4′), 66.7 (C-5′), 111.0 (C-1″), 78.0 (C-2″), 80.6 (C-3″), 75.3 (C-4″), 65.8 (C-5″), 53.0 (–OCH_3).

Acidic Hydrolysis and Determination of the Absolute Configuration of Sugars in 3 and 4 Solutions of **3** (15.1 mg) and **4** (17.0 mg) in 1 M HCl (H_2O–MeOH=1 : 1) were refluxed for 2 h, at which time TLC ($CHCl_3$–MeOH=5 : 1) indicated completion of the reaction. After the reaction mixture was neutralized with Ag_2CO_3 and filtrated, the solution was extracted with EtOAc (1 ml×3) to remove the aglycon. The aqueous layer was concentrated under reduced pressure to dryness to analyze the residue of the sugar fraction. A portion of the residue (1.1 mg and 1.2 mg) was trimethylsilylated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 0.5 ml) for 2 h at 80 °C. The mixture was partitioned between *n*-hexane and H_2O (0.3 ml each) and the *n*-hexane extract was analyzed by gas chromatography. A Shimadzu gas chromatograph Model GC-14B (Japan), equipped with an on-column injection system and FID, was used. The column was fused silica DB-5HT (30 m×0.32 mm i.d., film thickness 0.1 μm; J&W, Folsom, CA, U.S.A.). The operating conditions were the following: carrier gas flow (N_2), 1.3 ml/min; H_2, 60 kPa; air 50 kPa; make-up gas (N_2), 29 ml/min; injector, 250 °C; detector, 300 °C. The oven temperature was held at 230 °C. 3 μl of each sample was injected directly into the inject port. In the acidic hydrolysate of **3** and **4**, D-xylose and D-apiose were confirmed by comparison of the retention times: 5.17 and 5.44 min for **3** derivative; and 5.18 and 5.43 min for **4** derivative. The L-xylose, D-xylose and D-apiose derivatives were prepared in a similar way, and showed retention times of 5.02, 5.18 and 5.44 min, respectively. Another portion of the residue (7.2 mg and 7.0 mg) was subjected to silica gel column chromatography (20 g, Φ 1×8 cm) and eluted with the lower layer of $CHCl_3$–MeOH–H_2O (8 : 3 : 1, 0.3 l), yielding apiose [1.7 mg and 2.1 mg, Ve/Vt 0.21—0.51; TLC (Keiselgel 60 F_{254}) *Rf* 0.53, $CHCl_3$–MeOH–H_2O, 7 : 3 : 1]. P-1020 (Jasco, Tokyo, Japan) took the $[\alpha]_D$ of apiose.

Osteoclast Differentiation Assay The inhibition of osteoclast differen-

tiation was determined using a method previously described in the literature.[6,7] Mouse bone marrow cells and calvarial osteoblasts were cultured in 48-well plates and incubated for 6—7 d in the presence of 10^{-8} M VtD_3 and 10^{-6} M PGE_2. Alternatively, osteoclasts were generated by culturing bone marrow-derived macrophages in the presence of M-CSF and RANKL (Peprotech EC). Bone marrow cells were cultured for 24 h in α-MEM/10% FBS. Nonadherent cells were collected and cultured for 3 d in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL for 7 d. Osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) with a leukocyte acid phosphatase kit (Sigma, St. Louis, MO, U.S.A.). Bone marrow-derived macrophages prepared as previously described were suspended at 1×10^6 cells/ml in α-MEM/10% FBS and cultured in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL for 3 d. These cells were pretreated with each of the four compounds for 1 h, incubated with Giemsa solution for 5 min to stain nuclei, and then washed with 1% sodium carbonate. TRAP-positive MNC containing five or more nuclei were counted as osteoclasts. Fluorescence was captured under a confocal microscope (Olympus-FV300). In experiments with diphenyleneiodomium (DPI), the mean fluorescence intensity was measured by the confocal system. Data from one experiment are presented as mean±S.D. of triplicate samples, and similar results were obtained in two other experiments.

Statistical Analysis All data are presented as mean±standard error (S.E.). Data were analyzed by one-way ANOVA, and then differences among means were analyzed using Student's *t*-test. Differences were considered significant at $*\ p<0.05$, $**\ p<0.01$ significantly different from the control.

Acknowledgments This study was supported by the SRC program of MOST/KOSEF (R11-2000-081) through the Plant Metabolism Research Center, Kyung Hee University, and a grant from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

References

1) Manadhar N. P., *Fitoterapia*, **65**, 7—13 (1994).
2) Song M. C., Nigussie F., Jeong T. S., Lee C. Y., Regassa F., Markos T., Baek N. I., *J. Nat. Prod.*, **69**, 853—855 (2006).
3) Tommasi N. D., Piacente S., Simone F. D., Pizza C. *J. Nat. Prod.*, **56**, 134—137 (1993).
4) Gorin P. A. J., Perlin A. S., *Can. J. Chem.*, **36**, 480—485 (1958).
5) Takeda Y., Ooiso Y., Masuda T., Honda G., Otsuka H., Sezik E., Yesilada E., *Phytochemistry*, **49**, 787—791 (1998).
6) Kwak H. B., Lee S. W., Li Y. J., Kim Y. A., Han S. Y., Jhon G. J., Kim H. H., Lee Z. H., *Biochem. Pharmacol.*, **67**, 1239—1248 (2004).
7) Kim H. H., Kim J. H., Kwak H. B., Huang H., Han S. H., Ha H. I., Lee S. W., Woo E. R., Lee Z. H., *Biochem. Pharmacol.*, **67**, 1647—1656 (2004).
8) Lee D. Y., Lyu H. N., Kwak H. Y., Jung L., Lee Y. H., Kim D. K., Chung I. S., Kim S. H., Baek N. I., *J. Appl. Biol. Chem.*, **50**, 144—147 (2007).