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Inhibitors of osteoclastogenesis from *Lawsonia inermis* leaves

Nguyen Xuan Cuong [a,†], Nguyen Xuan Nhiem [a,b,†], Nguyen Phuong Thao [a], Nguyen Hoai Nam [a], Nguyen Tien Dat [a], Hoang Le Tuan Anh [a], Le Mai Huong [a], Phan Van Kiem [a,*], Chau Van Minh [a], Ji-Hee Won [c], Won-Yoon Chung [c], Young Ho Kim [b,*]

[a] Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Nghiado, Caugiay, Hanoi, Viet Nam
[b] College of Pharmacy, Chungnam National University, Daejeon 305 764, Republic of Korea
[c] Department of Oral Biology, Yonsei University College of Dentistry, Seoul 120 752, Republic of Korea

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ABSTRACT

Ten phenolic compounds (**1**–**10**) were isolated from a methanol extract of *Lawsonia inermis* leaves including two new ones, lawsoniasides A (**1**) and B (**2**). Their structures were elucidated by spectroscopic methods (NMR and FTICRMS) in combination with acid hydrolysis and GC analyses. Compounds **4** and **5** showed a significant inhibition on receptor activator for nuclear factor-κB ligand-induced osteoclast formation in murine bone-marrow macrophages.

Bone resorption is necessary in many physiological processes of the skeleton, and the physiological remodeling of bone in adults is strictly dependent on it. Normally, a balance exists between bone formation and bone resorption. When the balance is upset and bone resorption exceeds bone formation, metabolic bone diseases will occur, like osteoporosis, which is an important disease commonly found among elderly populations, especially postmenopausal women. Osteoporosis is characterized by excessive bone resorption, which causes changes in the microstructure of the bone matrix and makes bone prone to fracture. Costs of osteoporosis are staggering and increasing every year. Actually, antiresorptive therapies and medicines include the estrogen replacement therapies (ERT), selective estrogen receptor modulators (SERM), vitamin D, parathyroid hormone (PTH) analogues, and bisphosphonates, which show efficacy but reveal serious side effects. Thus, other nonhormonal and more specific therapies are needed.[1,2]

Lawsonia is a single-species genus of the Lythraceae family. In Vietnam, *Lawsonia inermis* L. (synonym *L. alba* Lamk, Vietnamese name: la mong tay), is used in traditional medicine to treat various diseases such as menstrual disorder, edema, rheumatism, bronchitis, and hemorrhoids.[3] In continuation of our investigations of Vietnamese medicinal plant constituents regarding antiosteoporosis effects,[4–7] this paper deals with the isolation, structural elucidation, and evaluation of anti-osteoclastogenic activity of ten phenolic compounds (Fig. 1), from a methanol extract of *L. inermis* leaves. Compounds **4** and **5** significantly inhibited receptor activator for nuclear factor-κB ligand (RANKL)-induced osteoclast formation of murine bone-marrow macrophages (BMMs).

The leaves of *Lawsonia inermis* L. was collected at Tam Dao National Park, Vinh Phuc, Vietnam, during February 2009 and identified by Dr. Ninh Khac Ban (Institute of Ecology and Biological Resources, VAST). A voucher specimen (INPC LI-0209) was deposited at the Herbarium of Institute of Natural Products Chemistry, VAST.

Air-dried leaves of *L. inermis* (2.5 kg) were exhaustively extracted (three times, each 1 h) with hot MeOH (50 °C) under ultrasonic condition to obtain 100 g MeOH residue. This was suspended in water (1 L) and partitioned in turn with $CHCl_3$ and EtOAc (each 3 × L) giving corresponding extracts: $CHCl_3$ (C, 26 g), EtOAc (E, 15 g), and a water layer (W). The water layer was passed through a Dianion HP-20 column using step wise elution with MeOH–H_2O (0:10, 2:10, 5:10, and 10:0, v/v) to give four corresponding fractions, W1–W4. Lawsoniaside B (**2**, 15 mg),[8] (+)-pinoresinol di-*O*-β-D-glucopyranoside (**8**, 17 mg),[9] and syringaresinol di-*O*-β-D-glucopyranoside (**9**, 19 mg),[10] were isolated from fraction W2 (7 g) by using a YMC RP-18 CC with H_2O–MeOH (5:1, v/v) as eluent, followed by a silica gel CC eluting with $CHCl_3$–MeOH–H_2O (4:1:0.1, v/v/v). Fraction W3 (5 g) was further separated on a silica gel CC eluting with $CHCl_3$–acetone–H_2O (1:6:0.2, v/v/v), followed by YMC RP-18 CC with H_2O–acetone (4:1, v/v) as eluent to furnish syringinoside (**3**, 7.0 mg),[11] daphneside (**4**, 5.0 mg),[12] daphnorin (**5**, 4.5 mg),[13]

* Corresponding authors. Tel.: +82 42 821 5933; fax: +82 42 823 6566 (Y.H.K.), tel.: +84 4 38363375; fax: +84 4 37564390 (P.V.K.).
E-mail addresses: phankiem@vast.ac.vn (P.V. Kiem), yhk@cnu.ac.kr (Y.H. Kim).
† These authors contributed equally to this work.

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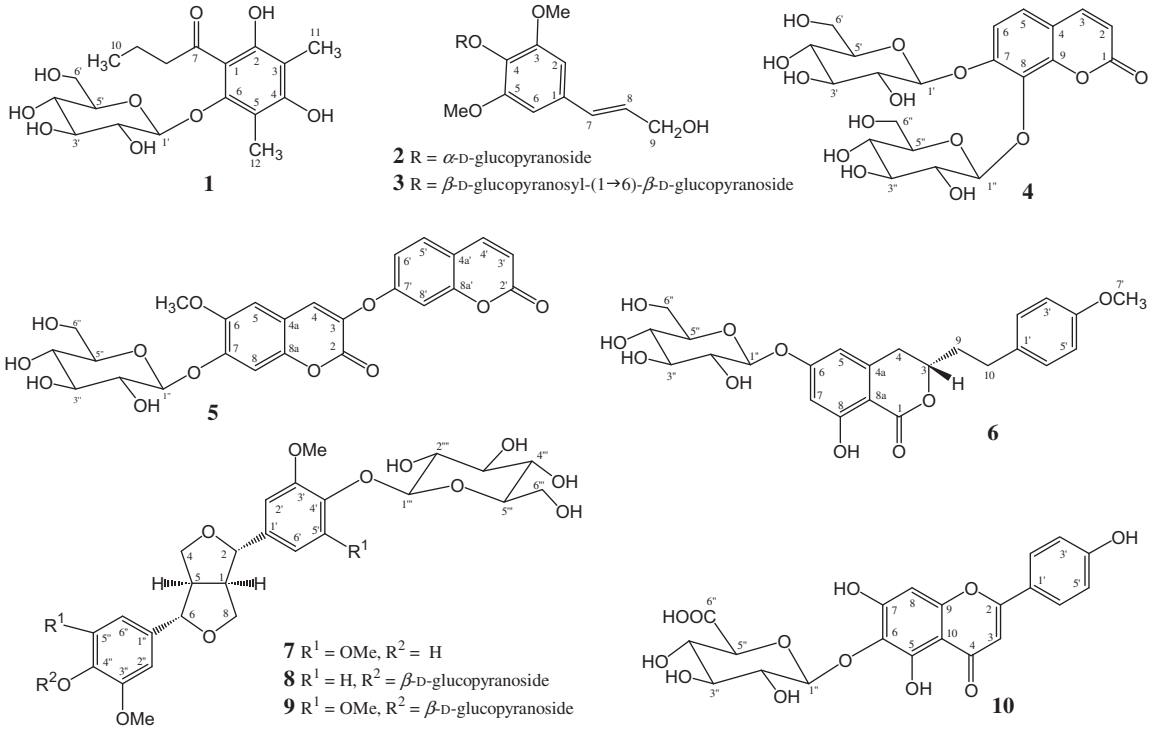

Figure 1. Structures of **1**–**10**.

and (+)-syringaresinol *O*-β-D-glucopyranoside (**7**, 10 mg).[14] The ethyl acetate extract (E, 15 g) was separated into five fractions, E1–E5, by a silica gel CC using gradient elution of $CHCl_3$–MeOH (30:1–1:1, v/v). Lawsoniaside A (**1**, 10 mg),[8] agrimonolide 6-*O*-β-D-glucopyranoside (**6**, 5.0 mg),[15] and isoscutellarin (**10**, 7.5 mg)[16] were isolated from fraction E4 (2 g) by using a silica gel CC with $CHCl_3$–MeOH–H_2O (4:1:0.1, v/v/v) as eluent, followed by a YMC RP-18 CC eluting with H_2O–MeOH (5:1, v/v).

Lawsoniaside A (**1**) was obtained as a white powder. Its molecular formula, $C_{18}H_{26}O_9$, was determined by the FTICRMS peak at *m*/*z* 409.14679 $[M+Na]^+$ (calcd for $C_{18}H_{26}O_9Na$, 409.14745). The ^1H NMR spectrum of **1** (see Table 1) showed typical signals of two aryl methyls and one terminal methyl at δ 2.05 (s, H-11), 2.21 (s, H-12), and 0.94 (t, J = 7.5 Hz, H-10), respectively. An anomeric proton at δ 4.52 (1H, d, J = 8.0 Hz, H-1′) confirmed a β-glycosidic linkage. The ^{13}C NMR spectrum of **1** revealed 18 carbon signals, including seven quaternary, five methine, three methylene, and three methyl carbons, detected by DEPT experiments. Of these, a fully substituted aromatic ring was suggested by six quaternary carbon signals at δ 112.72 (C-1), 159.96 (C-2), 109.18 (C-3), 161.82 (C-4), 111.61 (C-5), and 154.88 (C-6). The presence of a butanoyl moiety was identified by carbon signals at δ 210.09 (C, C-7), 46.95 (CH_2, C-8), 19.62 (CH_2, C-9), and 14.33 (CH_3, C-10), and two aryl methyls were at δ 8.27 (C-11) and 9.81 (C-12).[17,18] Moreover, sugar carbon signals at δ 105.72 (CH, C-1′), 75.70 (CH, C-2′), 77.88 (CH, C-3′), 71.91 (CH, C-4′), 78.02 (CH, C-5′), and 62.93 (CH_2, C-6′), together with the spin-coupling pattern of the sugar proton signals ($J_{1'-2'}$ = 8.0 Hz, $J_{2'-3'}$ = 9.0 Hz, and $J_{3'-4'}$ = 9.0 Hz) indicated a β-D-glucopyranosyl unit. The D-glucose was confirmed by acid hydrolysis (see Supplementary data).

Whole structure of **1** was elucidated by HSQC and HMBC experiments. The aryl methyl protons H-11 (δ 2.05) showed HMBC correlations with C-2 (δ 159.96)/C-3 (δ 109.18)/C-4 (δ 161.82), and H-12 (δ 2.21) correlated with C-4 (δ 161.82)/C-5 (δ 111.61)/C-6 (δ 154.88), confirming carbon positions of the aromatic ring (Fig. 2). Comparison of the ^{13}C NMR chemical shifts for the aromatic ring of **1** with those of dryopteroside[18] and eucalmainoside E,[19] indicated position of the butanoyl moiety at C-1 and two hydroxyl groups at C-2 and C-4, confirming the aglycone of **1** as dimethylphlorobutyrophenone.[20] In addition, attachment of the glucose at C-6 was assigned by an HMBC cross peak between H-1′ (δ 4.52) and C-6 (δ 154.88). Thus, **1** was elucidated as 1-butanoyl-3,5-dimethylphloroglucinyl-6-*O*-β-D-glucopyranoside, named lawsoniaside A.

The molecular formula of lawsoniaside B (**2**) was determined to be $C_{17}H_{24}O_9$ by FTICRMS result at *m*/*z* 395.13183 $[M+Na]^+$ (calcd for $C_{17}H_{24}O_9Na$, 395.13180). The ^{13}C NMR data of **2** (see Table 1) were identical to those of syringin,[21] except for signals of the glucosyl moiety. The small coupling constant of the anomeric proton at δ 4.91 (1H, d, J = 5.0 Hz, H-1′) and sugar carbon signals at δ 102.58 (C-1′), 74.15 (CH, C-2′), 76.51 (CH, C-3′), 69.93 (CH, C-4′), 77.14 (CH, C-5′), and 60.89 (CH_2, C-6′), indicated an α-D-glucopyranosyl unit.[22] Moreover, acid hydrolysis of **2** afforded D-glucose (see Supplementary data). The anomeric proton H-1′ (δ 4.91) showed HMBC correlation with C-4 (δ 133.92), confirming attachment of the glucose at C-4 (Fig. 2). Consequently, the new structure 3-(4-*O*-α-D-glucopyranosyl-3,5-dimethoxy) phenyl-2*E*-propenol was elucidated for **2**, named lawsoniaside B.

Since potentiated bone resorption by osteoclasts is one of the major causes of osteoporosis, inhibitors of osteoclasts may therefore present useful agents to prevent the excessive bone resorption associated with osteoporosis. Thus, an inhibitory activity of all compounds on TRAP-positive osteoclast formation was assayed (see Supplementary data). Among isolated compounds, **4** and **5** significantly suppressed osteoclast formation by 20% and 13% at a 10 μM concentration, respectively, as revealed by the decreased number of TRAP-positive multinuclear osteoclasts (Fig. 3). These compounds may reduce osteoclastogenesis by blocking the fusion of RANKL-treated BMMs rather than cell viability. In addition, the compounds did not show cytotoxic activity on BMMs at 10 μM. These results suggest that **4** and **5** have anti-osteoclastogenic activities. This is the first report on anti-osteoclastogenic constituents of *Lawsonia inmermis*, suggesting potential usage of this plant and its secondary metabolites for prevention and treatment osteoporosis and related bone diseases.

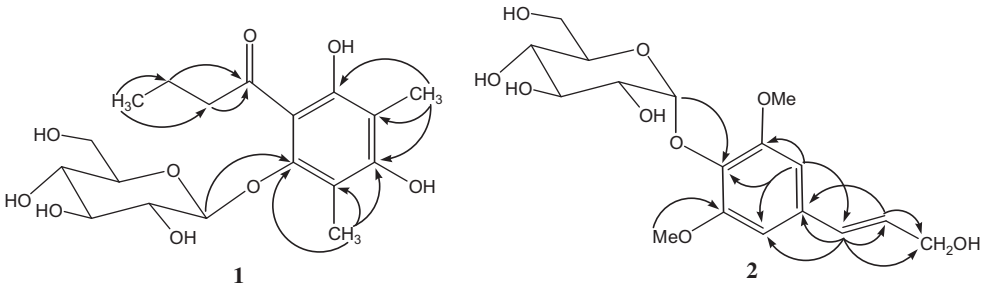

Figure 2. Key HMBC correlations of **1** and **2**.

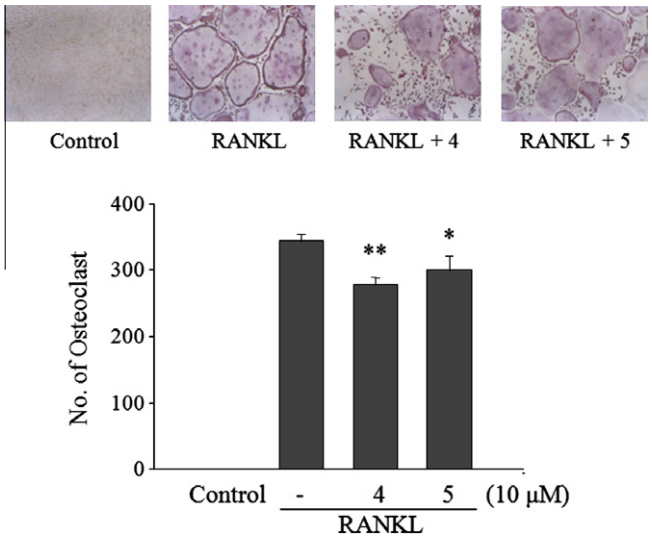

Figure 3. Effect of **4** and **5** on RANKL-induced osteoclast formation. BMMs were cultured with 10 μM of compound **4** or **5**, 30 ng/mL of M-CSF and 100 ng/mL of RANKL. After 5 days, cells were stained for TRAP activity, and the TRAP-positive multinucleated cells with three or more nuclei/cell were counted by light microscopy. $^{*}p$ <0.05, $^{**}p$ <0.005 versus RANKL-treated cells.

Table 1
The NMR spectroscopic data of **1** and **2**

Position	**1**[a]		**2**[b]	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
Aglycone				
1	112.72	—	132.57	—
2	159.96	—	104.51	6.72 s
3	109.18	—	152.67	—
4	161.82	—	133.92	—
5	111.61	—	152.67	—
6	154.88	—	104.51	6.72 s
7	210.09	—	128.41	6.47 d (16.0)
8	46.95	3.17 dd (7.0, 13.5)	130.12	6.33 dt (16.0, 5.0)
9	19.62	1.66 m	61.41	4.11 t (5.0)
10	14.33	0.94 t (7.5)		
11	8.27	2.05 s		
12	9.81	2.21 s		
OMe			56.34	3.77 s
Glucose				
1′	105.72	4.52 d (8.0)	102.58	4.91 d (5.0)
2′	75.70	3.56 dd (8.0, 9.0)	74.15	3.21[c]
3′	77.88	3.42 t (9.0)	76.51	3.20[c]
4′	71.91	3.35 t (9.0)	69.93	3.15 m
5′	78.02	3.12 m	77.14	3.05 m
6′	62.93	3.60 dd (5.5, 12.0)	60.89	3.42 dd (5.0, 12.0)
		3.72 dd (2.0, 12.0)		3.59 dd (2.0, 12.0)

[a] Measured in CD_3OD.
[b] Measured in DMSO-d_6.
[c] Overlapped signals, assignments were done by HSQC and HMBC experiments.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.118.

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8. *Lawsoniaside A* (**1**): amorphous white powder, $[\alpha]_D^{25}$ +1.5 (*c* 0.50, MeOH); IR(KBr) ν_{max} 3428 (OH), 2925 (CH), 1730 (C=O), 1645 (aromatic ring), 1075 (C–O–C) cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) are given in Table 1; ESIMS *m*/*z* 409 $[M+Na]^+$; FTICRMS *m*/*z* 409.14679 $[M+Na]^+$ (calcd for $C_{18}H_{26}O_9Na$, 409.14745). *Lawsoniaside B* (**2**): amorphous white powder, $[\alpha]_D^{25}$ −15.5 (*c* 0.50, MeOH); IR(KBr) ν_{max} 3435 (OH), 2917 (CH), 1652 (aromatic ring), 1084 (C–O–C) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) are given in Table 1; ESIMS *m*/*z* 395 $[M+Na]^+$; FTICRMS *m*/*z* 395.13183 $[M+Na]^+$ (calcd for $C_{17}H_{24}O_9Na$, 395.13180).
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