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Eight new phenolic acids from the leaves of *Illicium dunnianum* and their osteoprotective activities†

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Eight previously unreported phenolic acids (1–8), including three new phenylpropanoid glycosides (1–3), and five undescribed shikimic acid derivatives (4–8), together with six known analogues (9–14), were obtained from the dried leaves of *Illicium dunnianum*. The structures of these new compounds were elucidated by extensive spectroscopic analyses (1D, 2D-NMR, HRESIMS, IR, UV) and chemical methods. Compounds 1, 2, 4, and 5 were tested for their promotion effect on osteoblastogenesis of pre-osteoblastic MC3T3-E1 cells and inhibitory effect on osteoclastogenesis of RANKL-induced RAW264.7 cells. As a result, 1 and 4 exerted a promotion effect on osteoblastogenesis, but without activity on osteoclastogenesis. Our studies not only enrich the structural diversity of phenolic acids in nature, but also discover new lead compounds from folk plants with activities on osteoblastogenesis or osteoclastogenesis.

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1 Introduction

Illicium dunnianum Tutcher, belonging to the genus *Illicium* of Magnoliaceae, is a folk plant found throughout Southern China and used as a medicine for bone injury and fractures, relieving pain, and treating rheumatism.^{1,2} Previous phytochemical investigations of *I. dunnianum* indicated the presence of phenylpropanoids,^{3–7} sesquiterpenes,^{3,8–11} phenolic glycosides,¹² flavonoids,^{1,13,14} triterpenes^{1,6} and other chemical components.^{1,3,13} Modern pharmacological studies have shown that the extracts of *I. dunnianum* and some of these types of compounds possess multiple biological activities, such as anti-inflammatory^{15,16} and analgesic,^{2,17} relieving gastrointestinal smooth muscle spasm,¹⁶ regulation immune,¹⁶ repairing bone fracture² and other pharmacological activities. In our previous research, 24 lignans (including nine previously uncharacterized compounds), one new phenolic glycoside, and one new benzofuran derivative with anti-inflammatory activities were isolated from the leaves of *I. dunnianum*.^{18,19} As a part of further

investigation of constituents with activities on osteoblastogenesis or osteoclastogenesis from the leaves of *I. dunnianum* has led to the isolation and structural elucidation of eight previously unreported phenolic acids (1–8), including three new phenylpropanoid glycosides (1–3), and five undescribed shikimic acid derivatives (4–8), together with six known analogues (9–14) (Fig. 1). In addition, compounds 1, 2, 4, and 5 were tested for their promotion effect on osteoblastogenesis of pre-osteoblastic MC3T3-E1 cells and inhibitory effect on osteoclastogenesis of RANKL-induced RAW264.7 cell. Herein, the isolation, structural identification, and bioactivity of these new compounds are discussed.

2 Results and discussion

2.1 Structural elucidation

Compound 1 was obtained as a brown amorphous powder. The sodium adduct ion at m/z 465.1373 [$M + Na$]⁺ (calcd. for 465.1373) by HR-ESI-MS demonstrated that the molecular formula of 1 was C₂₀H₂₆O₁₁. The ¹H NMR (Table 1) spectrum showed five aromatic proton signals at [δ_H 7.63 (2H, m, H-2, 6), 7.42 (3H, m, H-3, 4, 5)], a *trans*-substituted double bond [δ_H 7.81 (1H, d, J = 16.0 Hz, H-7), 6.58 (1H, d, J = 16.0 Hz, H-8)] and two anomeric proton signals [δ_H 5.58 (1H, d, J = 7.4 Hz, H-1'), 4.29 (1H, d, J = 7.5 Hz, H-1'')]; The ¹³C NMR spectrum displayed eight sp² carbon resonances (δ_C 147.8, 135.6, 131.8, 130.1 $\times$ 2, 129.4 $\times$ 2, 118.2) and a carbonyl carbon signal (δ_C 167.1), combined with the ¹H–¹H COSY correlations of H-2/H-3/H-4/H-5/H-6, H-7/H-8 and the key HMBC correlations (Fig. 2) of H-7/C-1,2,6,8,9; H-8/C-1, suggested the presence of a *trans*-cinnamoyl. In addition, two saccharide groups were determined to be a β -D-

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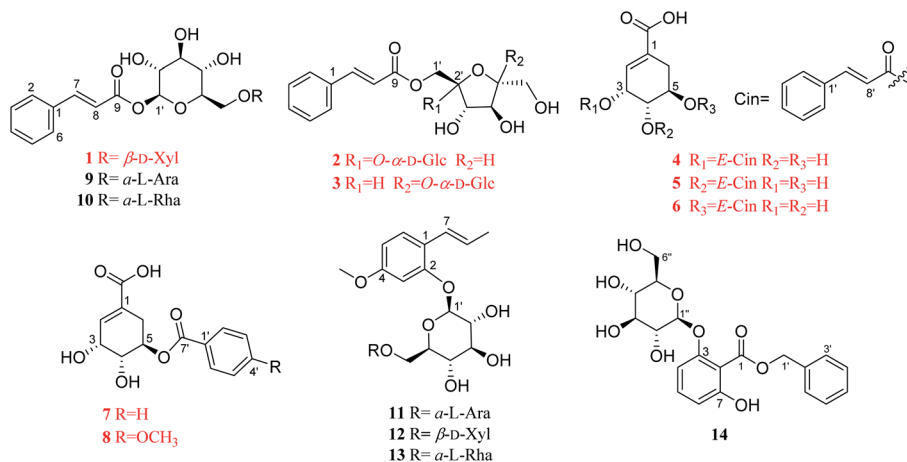


Fig. 1 Chemical structures of compounds 1–14.

glucopyranose and β -D-xylopyranose by HPLC analysis after acid hydrolysis and glycosyl derivatization, as well as the J value of the anomeric protons mentioned above. Furthermore, the HMBC correlations from H-1' to C-9 and H-1'' to C-6' (Fig. 2) suggested that the glucose group was connected to C-9 of the cinnamoyl group, and the xylosyl was linked to C-6' of glucose. Therefore, compound 1 was identified as (E)-1- O -cinnamoyl- β -D-xylopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside.

2 was obtained as a yellowish oil. The molecular formula of 2 was determined as C₂₁H₂₈O₁₂ by HR-ESI-MS (m/z 495.1471 [$M + Na$]⁺; calcd for C₂₁H₂₈O₁₂Na, 495.1478). The detailed analyses of 1D/2D NMR data of 2 determined a planar structure consisting of *trans*-cinnamoyl and a sucrose unit. ¹H-NMR (Table 1) spectrum displayed the presence of an anomeric proton signals [δ_H 5.45 (1H, d, J = 3.8 Hz, H-1'')]. The ¹³C NMR spectrum displayed 12 oxygen carbon signals, including two anomeric carbon

Table 1 ¹H and ¹³C NMR spectral data of compounds 1–3 (measured at 400 MHz for ¹H and 100 MHz for ¹³C in CD₃OD)^a

Pos.	1		2		3	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	135.6		135.7		135.7	
2	129.4	7.63	129.3	7.62	129.3	7.62
3	130.1	7.42	130.0	7.41	130.0	7.41
4	131.8	7.42	131.6	7.41	131.6	7.41
5	130.1	7.42	130.0	7.41	130.0	7.41
6	129.4	7.63	129.3	7.62	129.3	7.62
7	147.8	7.81, d (16.0)	146.9	7.74, d (16.0)	146.6	7.73, d (16.1)
8	118.2	6.58, d (16.0)	118.4	6.57, d (16.0)	118.7	6.56, d (16.1)
9	167.1		167.9		168.5	
1'	95.9	5.58, d (7.4)	64.2	4.47, d (12.1) 4.30, d (12.1)	63.8	3.64, s
2'	74.0	3.46	104.1		105.6	
3'	77.8	3.46	78.9	4.16, d (8.5)	78.9	4.13, d (8.2)
4'	71.0	3.46	75.1	4.07, t (8.0)	76.8	4.08, t (7.9)
5'	77.8	3.59	83.8	3.80, d (2.1)	80.7	4.0, td (7.5, 3.3)
6'	69.5	4.12, dd (11.1, 1.2) 3.75, dd (11.1, 5.3)	63.3	3.78	67.0	4.52, dd (11.7, 7.4) 4.45, dd (11.7, 3.3)
1''	105.2	4.29, d (7.5)	94.2	5.45, d (3.8)	93.5	5.40, d (3.7)
2''	74.9	3.21, d (9.2)	73.1	3.44, dd (9.8, 3.8)	73.3	3.41, dd (9.8, 3.8)
3''	77.7	3.31	74.6	3.7, d (9.5)	74.8	3.70, d (9.4)
4''	71.1	3.46	71.4	3.39, t (9.4)	71.6	3.35, d (9.4)
5''	66.9	3.84, dd (11.4, 5.1) 3.17, t (7.5)	74.4	3.87	74.3	3.87
6''			62.2	3.74, d (4.4) 3.84	62.5	3.75, d (5.1) 3.84

^a Multiplets and or overlapped signals are reported without designating multiplicity [the scale for ¹H-NMR (0–10 ppm), and ¹³C-NMR (0–200 ppm)].

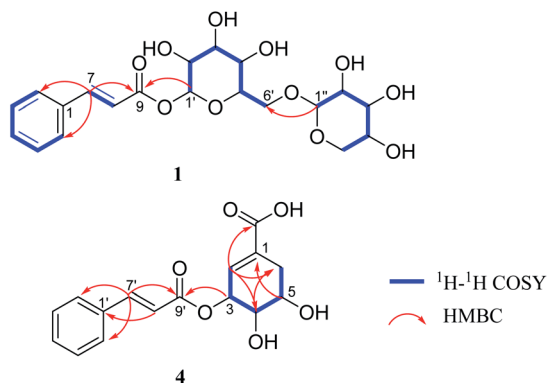


Fig. 2 Key ^1H – ^1H COSY and HMBC correlations of compounds 1 and 4.

signals (δ_{C} 104.1, 94.2), three methylene carbon signals (δ_{C} 64.2, 63.3, 62.2) and seven methine carbon signals (δ_{C} 83.8, 78.9, 75.1, 74.6, 74.4, 73.1, 71.4). Comparing the above data with the literature,²⁰ indicated the presence a sucrose unit, which was confirmed by acid hydrolysis and TLC analysis. Furthermore, the HMBC correlations from H-1' to C-9 suggested that the sucrose unit was connected to C-9 of the *trans*-cinnamoyl. Thus, the structure of 2 was assigned as (*E*)-1'-*O*-cinnamoyl sucrose glycoside.

Compound 3, a yellowish oil, its molecular formula was consistent with 2 by the molecular ion at m/z 495.1476 [$\text{M} + \text{Na}$]⁺ (calcd. for 495.1478). The NMR spectra (Table 1) of 3 were highly similar to 2, both have a *trans*-cinnamoyl and a sucrose unit. In the HMBC spectrum, the correlations from H-6' to C-9

suggesting that the hydroxyl group at C-6' of sucrose forms an ester bond with the cinnamoyl group. Therefore, the structure was named as (*E*)-6'-*O*-cinnamyl sucrose glycoside.

Compound 4 was isolated as a yellowish oil, gave the molecular formula of $\text{C}_{16}\text{H}_{16}\text{O}_6$ based on HR-ESI-MS (m/z 305.1024 [$\text{M} + \text{H}$]⁺, calcd. for 305.1025). Its NMR data (Table 2) indicated the presence of a *trans*-cinnamoyl [δ_{H} 7.62 (2H, m, H-2', 6'), 7.41 (3H, m, H-3', 4', 5'), 7.77 (1H, d, $J = 16.0$ Hz, H-7'), 6.60 (1H, d, $J = 16.0$ Hz, H-8'); δ_{C} 135.8 (C-1'), 129.3 (C-2', 6'), 130.0 (C-3', 5'), 131.6 (C-4'), 146.7 (C-7'), 118.8 (C-8'), 168.0 (C-9')]. The remaining olefinic proton signal at δ_{H} 6.73 (s, H-2), the signals of a CH_2 -group [δ_{H} 2.30 (1H, dd, $J = 18.4, 5.1$ Hz, H-6a), 2.78 (1H, dt, $J = 18.4, 2.3$ Hz, H-6b)] and the three methine protons appearing at [δ_{H} 3.95 (1H, dd, $J = 7.1, 4.1$ Hz, H-4), 4.09 (1H, dd, $J = 12.1, 5.1$ Hz, H-5) and 5.70 (s, H-3)], were attributed to a shikimic acid residue,²¹ which was further confirmed by ^1H – ^1H COSY correlations of H-2/H-3/H-4/H-5/H-6 and HMBC cross peaks from H-2 to C-4, 6, 7; H-4 to C-2, 6; H-5 to C-1; H-6 to C-2, 4 (Fig. 2). In addition, the ester linkage of both units *via* the hydroxyl group at C-3 of the shikimic acid unit could be deduced by the key HMBC correlation from H-3 to C-9'. Accordingly, the structure of 4 could be established as 3-*O*-cinnamoyl shikimic acid.

Compounds 5 and 6 were yield as a yellow amorphous powder, their molecular formulas were consistent with 4 by HRESIMS at m/z 305.1029 [$\text{M} + \text{H}$]⁺, and 305.1024 [$\text{M} + \text{H}$]⁺ (calcd. for 305.1025), respectively. The NMR spectroscopic data of 5 and 6 were very similar to those of 4, except for the substitute position of a *trans*-cinnamoyl. Furthermore, the key HMBC correlation from H-4 to C-9' in 5, suggested that the *trans*-cinnamoyl was connected to C-4 of the shikimic acid unit;

Table 2 ^1H and ^{13}C NMR spectral data of compounds 4–8^c

	4 ^a		5 ^b		6 ^a		7 ^b		8 ^b	
Pos.	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	134.0		131.2		131.5		130.2		130.7	
2	133.6	6.73	138.0	6.85, d (3.1)	137.6	6.82	139.0	6.90, d (1.5)	138.6	6.87
3	71.0	5.70, s	65.6	4.63	67.4	4.41	67.4	4.45, s	67.5	4.44, s
4	70.8	3.95, dd (7.1, 4.1)	75.3	5.04, dd (7.8, 4.2)	70.3	3.91, dd (7.2, 3.9)	69.9	4.0, dd (7.4, 4.2)	70.0	3.98, dd (7.4, 4.1)
5	68.7	4.09, dd (12.1, 5.1)	65.7	4.22, dd (13.2, 5.6)	71.8	5.27, dd (12.2, 6.0)	72.1	5.40, dd (12.5, 5.5)	71.8	5.36, dd (12.6, 5.2)
6	31.8	2.78, dt (18.4, 2.3)	32.2	2.75, dd (18.4, 5.0)	29.7	2.90, dd (18.4, 3.7)	29.1	2.92, dd (18.3, 4.7)	29.2	2.90, dd (18.4, 5.0)
		2.30, dd (18.4, 5.1)		2.32, dd (18.4, 5.8)		2.33, dd (18.4, 5.1)		2.41, dd (18.3, 5.2)		2.38, dd (18.4, 5.3)
7	170.5		169.9		170.1		169.6		170.1	
1'	135.8		135.8		135.8		131.4		123.5	
2'	129.3	7.62	129.3	7.60	129.3	7.61	130.6	8.02, d (7.9)	132.7	7.96, d (8.9)
3'	130.0	7.41	130.0	7.40	130.0	7.41	129.6	7.48, t (7.6)	114.8	6.99, d (8.9)
4'	131.6	7.41	131.5	7.40	131.6	7.41	134.4	7.61, t (7.4)	165.3	
5'	130.0	7.41	130.0	7.40	130.0	7.41	129.6	7.48, t (7.6)	114.8	6.99, d (8.9)
6'	129.3	7.62	129.3	7.60	129.3	7.61	130.6	8.02, d (7.9)	132.7	7.96, d (8.9)
7'	146.7	7.77, d (16.0)	146.5	7.74, d (16.0)	146.6	7.72, d (16.0)	167.4		167.3	
8'	118.8	6.60, d (16.0)	118.9	6.60, d (16.0)	118.9	6.56, d (16.0)				
9'	168.0		168.3		168.1					
4'-OCH ₃									56.0	3.86, s

^a Measured at 600 MHz for ^1H and 150 MHz for ^{13}C in CD_3OD . ^b Measured at 400 MHz for ^1H and 100 MHz for ^{13}C in CD_3OD . ^c Multiplets and overlapped signals are reported without designating multiplicity [the scale for ^1H -NMR (0–10 ppm), and ^{13}C -NMR (0–200 ppm)].



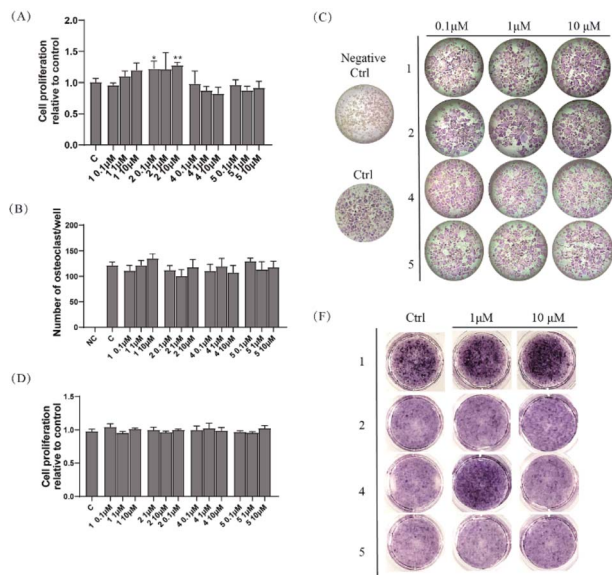


Fig. 3 The osteoblastogenesis and osteoclastogenesis activities of compounds 1, 2, 4, and 5. (A) Cell proliferation activity of RAW264.7 cells; (B) quantitative of osteoclasts (TRAP positive multi-nucleated cells (MNCs), more than 3 nuclei) induced from RAW264.7 cells by osteoclast differentiation medium for 4 days ($n = 3$). (C) Representative images of TRAP staining osteoclast at day 4 in each well of 96-well plate. (D) Cell proliferation activity of pre-osteoblast MC3T3-E1 cells; (E) representative morphological pictures of ALP staining on the MC3T3-E1 cells induced by osteoblast differentiation medium at day 6. NC: negative control, 0.1% DMSO without osteoclast differentiation medium (OCM); C: control, 0.1% DMSO with OCM; results were obtained from three independent experiments in triplicate and expressed are means $\pm$ SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different from the control group.

the key HMBC correlation between H-5 and C-9' in **6**, indicated that the *trans*-cinnamoyl was connected to C-5 of the shikimic acid unit. Thus, the structures of **5** and **6** were determined and named as 4-*O*-cinnamoyl shikimic acid and 5-*O*-cinnamoyl shikimic acid, respectively.

7 was obtained as a yellow needle crystal. The HRESIMS of **7** showed a quasimolecular peak at m/z 279.0870 $[M + H]^+$ (calcd for $C_{14}H_{14}O_6$, 279.0869), corresponding to the molecular formula of $C_{14}H_{14}O_6$. Its NMR data (Table 2) possessed similar signals to those of **6** except for the loss of the *trans*-substituted double bond. The benzoyl group was located at C-5 of the shikimic acid unit, which was evidenced by HMBC correlations from H-5 (δ_H 5.40) to C-7 (δ_C 167.4). Therefore, the structure of **7** was assigned, and named 5-*O*-benzoyl shikimic acid.

Compound **8** was isolated as a yellow needle crystal. The molecular formula of **8** was determined as $C_{15}H_{16}O_7$ by HRESIMS (m/z 309.0975 $[M + H]^+$; calcd. 309.0974). A comparison of the spectral data (Table 2) of **8** and **7** revealed that an additional methoxyl group (δ_H 3.86) was substituted at C-4', which was confirmed by HMBC correlation from OCH_3 -4' (δ_H 3.86, s) to C-4' (δ_C 165.3). To sum up, **8** was identified as 5-*O*-*p*-methoxybenzoyl shikimic acid.

The other six known compounds were identified as (*E*)-2-hydroxy-4-methoxyphenylpropene-2-*O*- α -L-arabifuranosyl-(1 $\rightarrow$

6)- β -D-glucopyranoside (**9**),²² (*E*)-2-hydroxy-4-methoxyphenylpropene-2-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (**10**).²² (*E*)-2-Hydroxy-4-methoxybenzene-propene-2-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside²³ (**11**), (*E*)-1-*O*-cin-namoyl- α -L-arabifuranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside^{24,25} (**12**), gleditschiaside A²⁶ (**13**) and benzyl-3-*O*- β -D-glucopyranosyl-7-hydroxybenzoate²⁷ (**14**). Their structures were established by comparison of their spectroscopic data recorded in the literature. Among them, compounds **12** and **13** were firstly isolated from the genus *Illicium*.

2.2 Osteoclastogenesis inhibitory effects of compounds 1, 2, 4, 5

Compounds **1**, **2**, **4**, and **5** were evaluated for their osteoclastogenesis effect on the pre-osteoclastic RAW264.7 cells. As shown in Fig. 3A, **2** increased the cell proliferation of RAW 264.7 cells about 20% at the test concentration, although no statistic significant was indicated at the concentration of 1 μ M. For the osteoclastogenesis activity, all the tested compounds exerted no effect on the number of multinucleated osteoclasts induced by RANKL (Fig. 3B and C).

2.3 Osteoblastogenesis promoting effects of compounds 1, 2, 4, and 5

Compounds **1**, **2**, **4**, and **5** were also evaluated for their osteoblastogenesis activities using pre-osteoblastic MC3T3-E1 cells. As shown in Fig. 3D, all the tested compounds exhibited no cytotoxicity or cell proliferation effect on the MC3T3-E1 cells. However, **1** (1 and 10 μ M) and **4** (1 μ M) could significantly deepen the staining colour of ALP staining, which indicated that **1** and **4** could promote the osteoblastogenesis activity of pre-osteoblast MC-3T3 E1 cells.

3 Conclusions

In summary, eight previously unreported phenolic acids, including three new phenylpropenoid glycosides (**1**–**3**), and five undescribed shikimic acid derivatives (**4**–**8**), together with six known analogues (**9**–**14**) were isolated and elucidated *via* varies of chromatographic and spectroscopic methods from the dried leaves of *Illicium dunnianum*. In addition, compounds **1**, **2**, **4**, and **5** were tested for their promotion effect on osteoblastogenesis of pre-osteoblastic MC3T3-E1 cells and inhibitory effect on osteoclastogenesis of RANKL-induced RAW264.7 cell. As a result, **1** and **4** could promote osteoblastogenesis activity without affect osteoclastogenesis activity, which will be helpful for gaining a deep insight into the biological role of such phenolic acids in bone fracture repair.

4 Experimental

4.1 General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter with a 1 cm cell at room temperature. IR spectra were obtained using a JASCO FT/IR-480 plus spectrometer. UV spectra were recorded on a JASCO V-550 UV/Vis spectrometer. HRESIMS



spectra were acquired using a Waters Synapt G2 mass spectrometer. 1D and 2D NMR spectra were acquired with Bruker AV 600/400 using solvent signals (CD_3OD : δ_{H} 3.30/ δ_{C} 49.0) as the internal standard. Analytical HPLC was conducted on a Shimadzu HPLC system with an LC-20AB solvent delivery system and an SPD-20A UV/vis detector using a Phenomenex Gemini C₁₈ column (5 μm , Φ 4.6 $\times$ 250 mm; Phenomenex Inc., Los Angeles, USA). Semi-preparative HPLC was carried out on a Shimadzu LC-6AD liquid chromatography system equipped with a SPD-20A detector on a Phenomenex Gemini C₁₈ column (5 μm , Φ 10.0 $\times$ 250 mm; Phenomenex Inc., Los Angeles, USA) and Preparative HPLC using a Cosmosil Packed C₁₈ column (5 μm , Φ 20.0 $\times$ 250 mm, Nacalai Tesque Inc., Kyoto, Japan).

Silica gel (200–300 mesh, Qingdao Marine Chemical Ltd, China), Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) and ODS (YMC Ltd, Japan) were used for column chromatography (CC). TLC was performed on precoated silica gel plate (SGF254, 0.2 mm, Yantai Chemical Industry Research Institute, China). HPLC-grade methanol and acetonitrile were bought from Oceanpack Alexative Chemicals Co. Ltd (Gothenburg, Sweden). All analytical grade reagents were from Concord Chemicals Co. Ltd, (Tianjin, China). Modified eagle medium alpha (α -MEM), fetal bovine serum (FBS), penicillin and streptomycin, trypsin-EDTA were purchased from Gibco (Gaithersburg, MD, USA). Murine sRANKL and MCSF were the products of Peprotech (Rocky Hill, NJ, USA). CCK-8 assay kit was purchased from Dojindo (Kumamoto, Japan). Leucocyte acid phosphatase staining kit was purchased from Sigma (St Louis, MO, USA).

4.2 Plant material

The leaves of *I. dunnianum* were purchased from Ji'an County, Jiangxi, China, in 2018, and were identified by Prof. Zhou Wu (Jiangsu Kanion Pharmaceutical Co. Ltd). A sample (2018ID101) was deposited in Institute of Traditional Chinese Medicine & Natural Products, college of pharmacy, Jinan University, Guangzhou, China.

4.3 Extraction and isolation

The air-dried leaves of *I. dunnianum* (ID, 15.5 kg) were extracted with 50% EtOH by heat reflux for 3 times (2 h each). Total extracts (IDEs, 2 kg) were yielded by evaporation under reduced pressure. IDEs were separated by HP-20 resin column chromatography (CC) eluted with EtOH–H₂O (0 : 100, 30 : 70, 50 : 50, 95 : 5) gradient to afford 4 fractions (ID-1 to ID-4). Fr. ID-3 (180 g) was separated over a silica gel column eluting with a CH₂Cl₂–MeOH gradient (100 : 0, 98 : 2, 95 : 5, 90 : 10, 85 : 15, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 0 : 100) to afford 15 fractions (Fr. 3A to 3O). Fr. 3 L (17.4 g) was chromatographed by ODS CC using a CH₃OH–H₂O gradient elution (30 : 70–70 : 30, 100 : 0) to give 9 subfractions (Fr. 3L1–3L9). Fr. 3L3 (3.1 g) was chromatographed by silica gel column eluting with a CH₂Cl₂–MeOH gradient (95 : 5–1 : 1, 0 : 100) to give 9 subfractions (Fr. 3L3A–3L3I). Fr. 3L3D and Fr. 3L3E was isolated using semipreparative HPLC [30% CH₃OH–H₂O (0.1% HCOOH), 3 mL min^{−1}] to yield 12 (7.5 mg), 1 (12.3 mg) and 13 (14 mg). Fr. 3L4 was further

chromatographed by polyamide CC using a EtOH–H₂O gradient elution (10 : 90–40 : 60, 95 : 5) to give 7 subfractions (Fr. 3L4A–3L4G). Fr. 3L4A was isolated using preparative HPLC [35% CH₃OH–H₂O (containing 0.1% HCOOH), 8 mL min^{−1}] to give fractions 3L4A2–3L4A9. Fr. 3 L4A2 was isolated using semipreparative HPLC [15% CH₃CN–H₂O (0.1% HCOOH), 3 mL min^{−1}] to yield 2 (14.9 mg). Fr. 3 L4A4 was isolated using semipreparative HPLC [19% CH₃CN–H₂O (0.1% HCOOH), 3 mL min^{−1}] to yield 9 (10 mg), 10 (14.6 mg) and 3 (4.4 mg). Fr. 3L4A6 was isolated using semipreparative HPLC [16% CH₃CN–H₂O (0.1% HCOOH), 3 mL min^{−1}] to yield 11 (7.1 mg). Fr. 3G (7.1 g) was chromatographed by ODS CC using a CH₃OH–H₂O gradient elution (25 : 75–100 : 0) to give 9 subfractions (Fr. 3G1–3G9). Fr. 3G9 was isolated using semipreparative HPLC [20% CH₃CN–H₂O (0.1% HCOOH), 3 mL min^{−1}] to yield 4 (148.7 mg) and 5 (73.2 mg). Fr. 3G7 was chromatographed by Sephadex LH-20 eluting with a CH₃OH to give six subfractions (Fr. 3G7A–3G7F). Fr. 3G7F was isolated using semipreparative HPLC [27% CH₃CN–H₂O (0.1% HCOOH), 3 mL min^{−1}] to yield 6 (3.5 mg), 7 (3.7 mg) and 8 (5.5 mg). Fr. 3G7E was isolated using semipreparative HPLC [18% CH₃CN–H₂O (0.1% HCOOH), 3 mL min^{−1}] to yield 14 (47.9 mg).

4.4 Structural characterization of undescribed compounds

4.4.1 (*E*)-1-*O*-Cinnamoyl- β -D-xylopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (1). Brown amorphous powder; $[\alpha]_{\text{D}}^{25}$ −12.35 (*c* 0.51, MeOH); UV(MeOH) λ_{max} (log ϵ): 205 (4.23), 217 (4.07), 223 (4.0), 280 (4.08) nm; IR (KBr) ν_{max} 3281, 2927, 2890, 1716, 1637, 1509, 1455, 1374, 1333, 1271, 1172, 1068 cm^{−1}; The ¹H and ¹³C NMR spectra data are shown in Table 1; HR-ESI-MS *m/z*: 465.1373 [*M* + Na]⁺ (calcd for C₂₀H₂₆O₁₁Na, 465.1373).

4.4.2 (*E*)-1'-*O*-Cinnamoyl sucrose glycoside (2). Yellowish oil; $[\alpha]_{\text{D}}^{25}$ +41.72 (*c* 0.58, MeOH); UV (MeOH) λ_{max} (log ϵ): 205 (4.23), 217 (4.0), 222 (4.16), 279 (4.36) nm; IR (KBr) ν_{max} 3317, 2927, 2887, 1710, 1637, 1450, 1381, 1333, 1160, 1051 cm^{−1}; the ¹H and ¹³C NMR spectra data are shown in Table 1; HR-ESI-MS *m/z*: 495.1471 [*M* + Na]⁺ (calcd for C₂₁H₂₈O₁₂Na, 495.1478).

4.4.3 (*E*)-6'-*O*-Cinnamoyl sucrose glycoside (3). Yellowish oil; $[\alpha]_{\text{D}}^{25}$ +38 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ): 205 (4.39), 216 (4.3), 223 (4.2), 278 (4.31) nm; IR (KBr) ν_{max} 3312, 2933, 2882, 1707, 1634, 1596, 1498, 1453, 1320, 1283, 1329, 1065 cm^{−1}; The ¹H and ¹³C NMR spectra data are shown in Table 1; HR-ESI-MS *m/z*: 495.1476 [*M* + Na]⁺ (calcd for C₂₁H₂₈O₁₂Na, 495.1478).

4.4.4 3-*O*-Cinnamoyl shikimic acid (4). Yellow oil; $[\alpha]_{\text{D}}^{25}$ −144.3 (*c* 0.27, MeOH); UV (MeOH) λ_{max} (log ϵ): 206 (4.34), 217 (4.33), 222 (4.25), 278 (4.32) nm; IR (KBr) ν_{max} 3258, 2902, 1704, 1639, 1554, 1402, 1175 cm^{−1}; the ¹H and ¹³C NMR spectra data are shown in Table 2; HR-ESI-MS *m/z*: 305.1024 [*M* + H]⁺ (calcd for C₁₆H₁₇O₆, 305.1025).

4.4.5 4-*O*-Cinnamoyl shikimic acid (5). Yellowish sand crystal; $[\alpha]_{\text{D}}^{25}$ −149.7 (*c* 0.53, MeOH); UV(MeOH) λ_{max} (log ϵ): 205 (4.27), 216 (4.28), 278 (4.26) nm; IR (KBr) ν_{max} 3383, 3069, 1719, 1696, 1424, 1277, 1065 cm^{−1}; the ¹H and ¹³C NMR spectra data are shown in Table 2; HR-ESI-MS *m/z*: 305.1029 [*M* + H]⁺ (calcd for C₁₆H₁₇O₆, 305.1025).

4.4.6 5-O-Cinnamoyl shikimic acid (6). Yellowish amorphous powder; $[\alpha]_D^{25} -55.9$ (c 0.34, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ): 206 (4.78), 217 (4.76), 278 (4.73) nm; IR (KBr) $\nu_{\max}$ 3394, 2921, 1704, 1599, 1402, 1172, 1045 cm^{-1} ; the ^1H and ^{13}C NMR spectra data are shown in Table 2; HR-ESI-MS m/z : 305.1024 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{16}\text{H}_{17}\text{O}_6$, 305.1025).

4.4.7 5-O-Benzoyl shikimic acid (7). Yellow needle crystal; $[\alpha]_D^{25} -80.2$ (c 0.52, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ): 205 (5.0), 227 (4.99), 278 (3.95) nm; IR (KBr) $\nu_{\max}$ 3369, 2933, 2624, 2497, 1716, 1659, 1274, 1248, 1102, 1033 cm^{-1} ; the ^1H and ^{13}C NMR spectra data are shown in Table 2; HR-ESI-MS m/z : 279.0870 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{14}\text{H}_{15}\text{O}_6$, 279.0869).

4.4.8 5-O-*p*-Methoxybenzoyl shikimic acid (8). Yellow needle crystal; $[\alpha]_D^{25} -89.7$ (c 0.51, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ): 207 (5.19), 257 (5.04) nm; IR (KBr) $\nu_{\max}$ 3300, 2964, 2910, 2842, 2627, 2497, 2208, 2044, 1707, 1608, 1511, 1271, 1102 cm^{-1} ; the ^1H and ^{13}C NMR spectra data are shown in Table 2; HR-ESI-MS m/z : 309.0975 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{15}\text{H}_{17}\text{O}_7$, 309.0974).

4.5 Acid hydrolysis and sugar analysis

The compounds (1.0 mg) were hydrolyzed with 2 mL of 2 M HCl for 2 h at 90 °C. The hydrolysates were extracted with equal volume of ethyl acetate twice. The aqueous layer was dried, and then reacted with 2.5 mg L-cysteine methyl ester hydrochloride in 1 mL of pyridine for 1 h at 60 °C. After 1 h, a total of 5 μL of *o*-tolyl isothiocyanate was added to the reaction mixture and further reacted at 60 °C for 1 h. The reaction products were filtered by a 0.45 μm filter membrane for HPLC analysis, detected by a UV detector at 250 nm. Authentic samples of D-Glc, L-Glc, D-Xyl, L-Xyl, D-Rha, L-Rha and D-Fuc, L-Fuc were treated following same procedure.

4.6 Cell culture and viability

Murine pre-osteoblastic MC3T3-E1 cells (ATCC, USA) and RAW 264.7 cells (ATCC, USA) were routinely cultured in modified Eagle medium alpha (α -MEM, Gibco, USA), containing 10% fetal bovine serum (Gibco, USA), 100 U mL^{-1} penicillin, and 100 $\mu\text{g mL}^{-1}$ streptomycin. They were cultured at 37 °C in a humidified atmosphere of 5% CO_2 . Cells were digested with 0.05% trypsin-EDTA (Gibco, USA) and culture in a new culture dish every three days. MC3T3-E1 cells and RAW264.7 cells were seeded in 96-well plates at a density of 5×10^3 or 3×10^3 cells per well, respectively. After drug treatment for 48 h, CCK-8 assay was used as an indirect colorimetric measurement of cell viability.

4.7 Effect of compounds 1, 2, 4, and 5 on the osteoblastogenesis of MC3T3-E1 cells

Alkaline phosphatase (ALP) activity was commonly used as a marker of osteoblast differentiation. Cells were seeded in 12-well plates with a cell density of 5×10^4 cells per well. After 2 days, the medium was changed to osteoblast differentiation medium (α -MEM with 10% FBS and 50 $\mu\text{g mL}^{-1}$ of ascorbic acid and 10 mM of β -glycerophosphate) in the present or absent of compound 1, 2, 4, and 5 (1, 10 μM). Upon treatment for 7 days,

ALP activity was measured by staining with the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, China) following the instruction of manufacturer. The cells were rinsed with PBS, and images of each well were captured.

4.8 Effect of compounds 1, 2, 4, and 5 on the osteoclastogenesis of RANKL-induced RAW264.7 cells

RAW264.7 cells were seeded at 3000 cells per well in 96 well plates. After 24 h of incubation, cells were treated with different concentrations of 1, 2, 4, and 5 in osteoclast differentiation medium (α -MEM with 10% FBS and 100 ng mL^{-1} of RANKL and 10 ng mL^{-1} of M-CSF). After 4 days, TRAP staining was performed according to our previous method.²⁸ The number of multinucleated osteoclasts were quantified under microscopy (Olympus CKX53).

4.9 Data analysis

The data obtained are presented as the means $\pm$ SD of three independent experiments. A one-way analysis of variance (ANOVA) test was used for statistical analysis, followed by a Dunnett's post hoc test for multiple comparisons. GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA) was used to perform the analyses.

Conflicts of interest

There are no conflicts to declare.

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