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

Hai-bo Li, †‡ Sen-ju Ma, †‡ Ying-xin Shan, † a Ting Li, † a Zhen-zhong Wang, †*b Zuo-cheng Qiu *b and Yang Yu *a

Eight previously unreported phenolic acids (1–8), including three new phenylpropenoid 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, HR-ESIMS, 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.

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.†‡ Previous phytochemical investigations of *I. dunnianum* indicated the presence of phenylnpropanoids,† 1–7 sesquiterpenes,† 8–11 phenolic glycosides,† 12 flavonoids,† 13–14 triterpenes† 15 and other chemical components.† 1,6 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,† 18 regulation immune,† 16 repairing bone fracture† 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 phenylpropenoid 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 cells. Herein, the isolation, structural identification, and bioactivity of 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 C_{26}H_{35}O_{11}. 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 anomic 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 sp^2 carbon resonances (δ_{C} 147.8, 135.6, 131.8, 130.1 × 2, 129.4 × 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-3/C-4/C-5/C-6/C-7/C-8/C-9/C-10. The suggested presence of a trans-cinnamoyl. In addition, two saccharide groups were determined to be a β-D-C-1,2,6,8,9-H-8/C-1, suggested the presence of a trans-cinnamoyl.

The two saccharides were determined to be a β-D-xylopyranosyl and a β-D-glucopyranosyl residue. The anomeric proton signals of the two saccharides were observed at δ_{H} 4.79 (1H, d, J = 7.2 Hz, H-1) and δ_{H} 4.96 (1H, d, J = 7.4 Hz, H-1) and the key HMBC correlations of H-1/C-2/C-3/C-4/C-5/C-6/C-7/C-8/C-9/C-10. The two saccharides were determined to be a β-D-xylopyranosyl and a β-D-glucopyranosyl residue. The two saccharides were determined to be a β-D-xylopyranosyl and a β-D-glucopyranosyl residue. The two saccharides were determined to be a β-D-xylopyranosyl and a β-D-glucopyranosyl residue. The two saccharides were determined to be a β-D-xylopyranosyl and a β-D-glucopyranosyl residue.
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”0 to C-6”0 (Fig. 2) suggested that the glucose group was connected to C-9 of the cinnamoyl group, and the xylosyl was linked to C-6”0 of glucose. Therefore, compound 1 was identified as (E)-1-O-cinnamoyl-β-D-xylopyranosyl-(1→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 [δH 5.45 (1H, d, J = 3.8 Hz, H-1”0)]. The 13C NMR spectrum displayed 12 oxygen carbon signals, including two anemic 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)α

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α Multiplets and or overlapped signals are reported without designating multiplicity [the scale for 1H-NMR (0–10 ppm), and 13C-NMR (0–200 ppm)].
signals (δc 104.1, 94.2), three methylene carbon signals (δc 64.2, 63.3, 62.2) and seven methine carbon signals (δc 74.6, 74.4, 73.1, 71.4). Comparing the above data with the literature,36 indicated the presence a sucrose unit, which was confirmed by acid hydrolysis and TLC analysis. Furthermore, the HMBC correlations from H-1 to H-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 [M + Na]+ (calcd. for 495.1478). The NMR spectra (Table 1) of 3 are 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 C16H16O6 based on HR-ESI-MS (m/z 305.1024 [M + H]+, calcd. for 305.1025). Its NMR data (Table 2) indicated the presence of a trans-cinnamoyl [δH 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′)] and 6.60 (1H, d, J = 16.0 Hz, H-8′); δc 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 δH 6.73 (s, H-2), the signals of a CH2-group [δH 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 [δH 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,37 which was further confirmed by 1H-1H COSY correlations of H-2/H-3/H-4/H-5/H2-6 and HMBC correlation from H-3 to C-9 of the trans-cinnamoyl. Furthermore, the key HMBC correlation from H-4 to C-9′ suggested that the hydroxyl group at C-6′ of the trans-cinnamoyl was connected to C-4 of the shikimic acid unit; therefore, the structure of 4 was assigned as a trans-cinnamoyl sucrose glycoside

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4. Compound 2 was isolated as a yellow amorphous powder, their molecular formulas were consistent with 4 by HRESIMS at m/z 305.1029 [M + H]+, and 305.1024 [M + 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;
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. 279.0869), corresponding to the molecular formula of C14H14O6. 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 (δH 5.40) to C-7 (δC 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 C15H16O7 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 methoxy group (δH 3.86) was substituted at C-4', which was confirmed by HMBC correlation from OCH3-4' (δH 3.86, s) to C-4' (δC 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-methoxypheynylpropene-2-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (9), (E)-2-hydroxy-4-methoxypheynylpropene-2-O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (10), (E)-2-Hydroxy-4-methoxybenzene-propene-2-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (11), (E)-1-O-cinnamoyl-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside (12), gleditschiaside A (13) and benzyl-3-O-β-D-glucopyranosyl-7-hydroxybenzoate (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 μM. For the osteooclasis activity, all the tested compounds exerted no effect on the number of multinucleated osteoclasts induced by RANKL (Fig. 3B and C).

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

Compounds 1, 2, 4, and 5 were also evaluated for their osteooclasis activities using pre-osteoclastic 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 μM) and 4 (1 μM) could significantly deepen the staining colour of ALP staining, which indicated that 1 and 4 could promote the osteooclasis activity of pre-osteoclastic MC3T3-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 various 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 osteoclastogenesis of pre-osteoclastic MC3T3-E1 cells and inhibitory effect on osteoclastogenesis of RANKL-induced RAW264.7 cell. As a result, 1 and 4 could promote osteoclastogenesis 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_{3}OD: 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 C_{18} column (5 μm, Φ 4.6 × 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 × 250 mm; Phenomenex Inc., Los Angeles, USA) and Preparative HPLC using a Cosmosil Packed C18 column (5 μm, Φ 20.0 × 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, Shandong, 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 (Gothenburg, Sweden), and Semi-preparative HPLC using a Cosmosil Packed C18 column (5 μm, Φ 20.0 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan).

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 (2018D101) 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–H_{2}O (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 CH_{3}Cl_{2}–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 CH_{3}OH–H_{2}O 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 CH_{3}Cl_{2}–MeOH gradient (95:5, 5:1, 1:0, 100:0) to give 9 subfractions (Fr. 3L3A–3L3I), Fr. 3L3D and Fr. 3L3E was isolated using semipreparative HPLC [30% CH_{3}OH–H_{2}O (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–H_{2}O gradient elution (10:90–40:60, 95:5) to give 7 subfractions (Fr. 3L4A–3L4G). Fr. 3L4A was isolated using preparative HPLC [35% CH_{3}OH–H_{2}O (containing 0.1% HCOOH), 8 mL min^{-1}] to give fractions 3L4A2–3L4A9. Fr. 3 L4A2 was isolated using semipreparative HPLC [15% CH_{3}CN–H_{2}O (0.1% HCOOH), 3 mL min^{-1}] to yield 2 (14.9 mg). Fr. 3 L4A3 was isolated using preparative HPLC [19% CH_{3}CN–H_{2}O (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 preparative HPLC [16% CH_{3}CN–H_{2}O (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 CH_{3}OH–H_{2}O gradient elution (25:75–100:0) to give 9 subfractions (Fr. 3G1–3G9). Fr. 3G9 was isolated using semipreparative HPLC [20% CH_{3}CN–H_{2}O (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 CH_{3}OH to give six subfractions (Fr. 3G7A–3G7F). Fr. 3G7F was isolated using semipreparative HPLC [27% CH_{3}CN–H_{2}O (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% CH_{3}CN–H_{2}O (0.1% HCOOH), 3 mL min^{-1}] to yield 14 (47.9 mg).
4.4.6 5-O-Cinnamoyl shikimic acid (6). Yellowish amorphous powder; $[\alpha]_D^{25} = -55.9 (c 0.34, MeOH); UV (MeOH) $\lambda_{\text{max}}$ (log e): 206 (4.78), 217 (4.76), 278 (4.73) nm; IR (KBr) $r_{\text{max}}$ 3394, 2921, 1704, 1599, 1402, 1172, 1045 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 C14H24O6, 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) $\lambda_{\text{max}}$ (log e): 205 (5.0), 227 (4.99), 278 (3.95) nm; IR (KBr) $r_{\text{max}}$ 3369, 2933, 2624, 2497, 1716, 1659, 1274, 1102, 1033 cm$^{-1}$; the 1H and 13C NMR spectra data are shown in Table 2; HR-ESI-MS $m/z$: 279.0870 [M + H]$^+$ (calcd for C14H20O6, 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) $\lambda_{\text{max}}$ (log e): 207 (5.19), 257 (5.04) nm; IR (KBr) $r_{\text{max}}$ 3000, 2964, 2910, 2842, 2627, 2497, 2208, 2044, 1707, 1608, 1511, 1271, 1102 cm$^{-1}$; the 1H and 13C NMR spectra data are shown in Table 2; HR-ESI-MS $m/z$: 309.0975 [M + H]$^+$ (calcd for C15H17O7, 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 the concentration was measured. Then, the hydrolysates were reacted with 2.5 mg L-cysteine methyl ester hydrochloride and then reacted with 5 mL of 1 M NaOH. The reaction products were filtered with a 0.45 μm filter membrane for HPLC analysis, detected by a UV detector at 250 nm. Authentic samples of D-Glc, L-Fuc, L-Rha, L-Xyl, D-Xyl, and D-Rha were treated following the same procedure.

4.6 Cell culture and viability
Murine pre-osteoblastic MC3T3-E1 cells (ATCC, USA) and RAW264.7 cells (ATCC, USA) were routinely cultured in modified Eagle medium alpha (z-MEM, Gibco, USA), containing 10% fetal bovine serum (Gibco, USA), 100 U mL$^{-1}$ penicillin, and 100 μg mL$^{-1}$ streptomycin. They were cultured at 37 °C in a humified atmosphere of 5% CO$_2$. Cells were digested with 0.05% trypsin–EDTA (Gibco, USA) and cultured 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$^3$ 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 (z-MEM with 10% FBS and 50 μg mL$^{-1}$ of ascorbic acid and 10 mM of β-glycerophosphate) in the present or absent of compound 1, 2, 4, and 5 (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 (z-MEM with 10% FBS and 10 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. The number of multinucleated osteoclasts were quantified under microscopy (Olympus CKX53).

4.9 Data analysis
The data obtained are presented as the means ± 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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Notes and references


