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Chemical Constituents of *Acanthus ilicifolius* L. and Effect on Osteoblastic MC3T3E1 Cells

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A new coumaric acid derivative called acancifoliuside (**1**) and six known compounds as acteoside (**2**), isoacteoside (**3**), acanthaminoside (**4**), (+)-lyoniresinol 3a-*O*-β-glucopyranoside (**5**), (-)-lyoniresinol (**6**), and α-amyrin (**7**), were isolated from the methanolic extract of the leaves of *Acanthus ilicifolius* L. (Acanthaceae). Their structures were determined by spectroscopic methods and a comparison with the spectral data reported in the literature. The effects of the compounds on the function of osteoblastic MC3T3-E1 cells were tested. Compounds **2, 3,** and **5** (30 μM) increased the growth and differentiation of osteoblasts significantly (P<0.05), indicating that *A. ilicifolius* leaves may help prevent osteoporosis.

Key words: *Acanthus ilicifolius*, Acanthaceae, Acancifoliuside, Osteoblast

INTRODUCTION

Acanthus ilicifolius L. (Acanthaceae) typically has spinose margins on its evergreen leaves and stipular spines at the stem nodes. It grows particularly well in areas with higher freshwater input. The plant can sometimes cover large areas and form thickets, especially in disturbed mangroves, and also grows along river banks. The whole plant is used in traditional Vietnamese medicine to treat excitation, backache, paralysis, cough, and asthma. The roots are useful in treating dropsy, strangury, and rheumatism (Bich *et al*., 2004; Chi, 1999). In traditional Chinese medicine, *A. ilicifolius* is used to treat inflammation, hepatitis, swelled-spleen, asthma, gastralgia, and malignant-tumours (Chi, 1999). Previous pharmacological studies on this plant reported that the crude alcoholic extract of its leaves showed antioxidant, hepatoprotective, antitumor, and anti-carcinogenic activity (Babu *et al*., 2001, 2002). Preliminary studies reported the isolation of the following compounds: a triterpenoidal saponin (Minocha and Tiwari, 1981), 2-benzoxazolinone (Kapil and Sharma, 1994), benzoxazinoid glucosides, lignan glucosides, phenylethanoid glycosides, aliphatic alcohol glycosides, and coumaric acid glycosides (Kanchanapoom *et al*., 2001a, 2001b; Wu *et al*., 2003a, 2003b). Our phytochemical studies on the *A. ilicifolius* growing in Vietnam resulted in the isolation of a new coumaric acid derivative called acancifoliuside (**1**) as well as six known compounds, acteoside (**2**), isoacteoside (**3**), acanthaminoside (**4**), (+)-lyoniresinol 3a-*O*-β-glucopyranoside (**5**), (-)-lyoniresinol (**6**), and α-amyrin (**7**). Their structures were determined using spectroscopic and physicochemical techniques. In addition, this paper reports the effects of compounds **2**, **3**, and **5** on osteoblastic MC3T3-E1 cells, which is a well-characterized *in vitro* model for osteoblast differentiation.

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MATERIALS AND METHODS

General methods

The melting points were determined using an Electrothermal IA-9200 without correction. The IR spectra were obtained on a Hitachi 270-30 type spectrometer using KBr discs. The optical rotation was determined on a Jasco DIP-1000 KUY polarimeter. The electrospray ionization (ESI) mass spectra were obtained using an AGILENT 1100 LC-MSD Trap spectrometer. The HRESI-MS spectra

were obtained using a Waters Q/TOF premier equipped with an electrospray ion source. The ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Bruker AM500 FT-NMR spectrometer and TMS was used as the internal standard. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) and YMC RP-18 resins.

Plant material

The leaves of *A. ilicifolius* were collected in Tamdao, Vinh Phuc, Vietnam in January 2006 and identified by Dr. Tran Huy Thai, at the Institute of Ecology and Biological Resources, VAST of Vietnam. An authentic sample (No 20060117) was deposited at the Institute of Natural Products Chemistry, VAST, Vietnam.

Extraction and isolation

The dried leaves of *A. ilicifolius* (3.0 kg) were powdered and extracted three times with hot methanol to give the methanol extract (90.0 g), which was suspended in water and then partitioned with dichloromethane to give the dichloromethane extract (15.0 g) and water layer. The dichloromethane extract (15.0 g) was repeatedly subjected to chromatography on a silica gel column (Φ 50 × L 600 mm), eluted with n-hexane: acetone (gradient from 50:1 to 1:2 v/v) to give six fractions F1 (1.5 g), F2 (1.0 g), F3 (4.5 g), F4 (2.0 g), F5 (3.0 g), and F6 (2.5 g). Compound **7** (white crystals, 22.0 mg) was obtained from fraction F3 (4.5 g) using a silica gel column (Φ 50 × L 700 mm) eluted with *n*-hexane: ethyl acetate (20:1, v/v, 2 L). The fraction F4 (2.0 g) was chromatographed on a silica gel column (Φ 20 × L 600 mm) and eluted with *n*-hexane: ethyl acetate (15:1, v/v, 1.5 L) to give compound **4** (colorless needles, 35.0 mg). Fraction F5 (3.0 g) was chromatographed on a silica gel column (Φ 30 × L 600 mm) using *n*-hexane: ethyl acetate (10:1, v/v, 1.5 L) as a solvent system to give compound **6** (colorless amorphous powder, 55.0 mg). The water layer was chromatographed on a DIANION HP-20 column (Φ 100 × L 600 mm) using H_2O: MeOH [100:0 (2 L), 75:25 (2L), 50:50 (2 L) 25:75 (2 L), and 0:100 (2 L)] as eluent to give fractions FW1 (15.0 g), FW2 (15.5 g), FW3 (25.0 g), FW4 (5.7 g), and FW5 (12.4 g), respectively. Fraction FW3 (25.0 g) was chromatographed on a silica gel column (Φ 60 × L 600 mm) and eluted with $CHCl_3$-MeOH-H_2O (60:10:5, v/v) to give compound **1** (colorless amorphous powder, 13.5 mg), **2** (yellow powder, 28.0 mg), and **3** (yellow powder, 25.0 mg). Fraction FW5 (12.4 g) was chromatographed on a silica gel column (Φ 40 × L 600 mm) using $CHCl_3$-MeOH-H_2O (70:30:4) as eluent to give compound **5** (102.0 mg) as a colorless amorphous powder.

Acancifoliuside (1)

Colorless amorphous powder, mp 235-236°C; $[\alpha]_D^{25}$ -56.7° (MeOH; *c* 1.00); IR (KBr) ν_{max} (cm^{-1}): 3426 (br, OH), 2938, 2866 (CH), 1640 (C=O), 1057 (C-O-C); positive ESI-MS *m/z*: 661.0 [M+Na]$^+$; negative ESI-MS *m/z*: 637.1 [M-H]$^-$; HRESI-MS found *m/z*: 661.1738 (Calc. 661.1739 for $C_{29}H_{34}O_{16}Na$); ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD): see Table I.

Acteoside (2)

Yellow powder, mp 156-157°C; -85.0° (MeOH; *c* 1.00); ^{1}H-NMR (500 MHz, CD_3OD) δ: *aglycone*: 6.72 (d, *J* = 2.0 Hz, H-2), 6.70 (d, *J* = 8.0 Hz, H-5), 6.58 (dd, *J* = 2.0, 8.0 Hz, H-6), 3.74 (m, H_a-α), 4.07 (m, H_b-α), 2.81 (m, H-b), *caffeoyl moiety*: 7.08 (d, *J* = 2.0 Hz, H-2), 6.80 (d, *J* = 8.0 Hz, H-5), 6.97 (dd, *J* = 2.0, 8.0 Hz, H-6), 6.29 (d, *J* = 16.0 Hz, H-β), 7.61 (d, *J* = 16.0 Hz, H-γ), *glucose*: 4.39 (d, *J* = 8.0 Hz, H-1'), 3.41 (dd, *J* = 8.0, 9.0 Hz, H-2'), 3.84 (t, *J* = 9.0 Hz, H-3'), 4.94 (t, *J* = 9.0 Hz, H-4'), 3.53 (m, H-5'), 3.57 (dd, *J* = 3.5, 10.0 Hz, H_a-6'), 3.61 (dd, *J* = 5.5, 10.0 Hz, H_b-6'), *rhamnose*: 5.21 (d, *J* = 1.5 Hz, H-1"), 3.94 (dd, *J* = 1.5, 3.0 Hz, H-2"), 3.60 (m, H-3"), 3.32 (m, H-4"), 3.61 (m, H-5") and 1.11 (d, *J* = 6.5 Hz, H-6"); ^{13}C-NMR (125 MHz, CD_3OD) δ: *aglycone*: 131.48 (C-1), 116.51 (C-2), 144.62 (C-3), 146.08 (C-4), 117.10 (C-5), 121.26 (C-6), 72.21 (C-a), 36.52 (C-b), *caffeoyl moiety*: 127.65 (C-1), 114.70 (C-2), 149.73 (C-3), 146.78 (C-4), 116.31 (C-5), 123.19 (C-6), 168.28 (C-α), 115.24 (C-β), 147.99 (C-γ), *glucose*: 104.16 (C-1'), 76.16 (C-2'), 81.62 (C-3'), 70.38 (C-4'), 75.98 (C-5'), 62.34 (C-6'), *rhamnose*: 102.98 (C-1"), 72.03 (C-2"), 72.31 (C-3"), 73.77 (C-4"), 70.57 (C-5") and 18.42 (C-6").

Isoacteoside (3)

Yellow powder, mp 148-149°C; $[\alpha]_D^{25}$ -52.0° (MeOH; *c* 1.00); ^{1}H-NMR (500 MHz, CD_3OD) δ: *aglycone*: 6.69 (d, *J* = 2.0 Hz, H-2), 6.65 (d, *J* = 8.0 Hz, H-5), 6.54 (dd, *J* = 8.0, 2.0 Hz, H-6), 3.74 (m, H_a-α), 4.00 (m, H_b-α), 2.80 (m, H-β), *caffeoyl moiety*: 7.04 (d, *J* = 2.0 Hz, H-2), 6.77 (d, *J* = 8.0 Hz, H-5), 6.90 (dd, *J* = 2.0, 8.0 Hz, H-6), 6.30 (d, *J* = 16.0 Hz, H-β), 7.58 (d, *J* = 16.0 Hz, H-γ), *glucose*: 4.34 (d, *J* = 7.5 Hz, H-1'), 3.33 (dd, *J* = 7.5, 8.0 Hz, H-2'), 3.57 (dd, *J* = 8.0, 8.0 Hz, H-3'), 3.42 (dd, *J* = 8.0, 8.5 Hz, H-4'), 3.56 (m, H-5'), 4.51 (dd, *J* = 3.0, 12.0 Hz, H_a-6'), 4.37 (dd, *J* = 6.0, 12.0 Hz, H_b-6'), *rhamnose*: 5.20 (d, *J* = 1.0 Hz, H-1"), 3.42 (dd, *J* = 1.0, 3.0 Hz, H-2"), 3.73 (dd, *J* = 3.0, 8.0 Hz, H-3"), 3.42 (dd, *J* = 8.0, 7.5 Hz, H-4"), 4.02 (m, H-5") and 1.26 (d, *J* = 6.0 Hz, H-6"); ^{13}C-NMR (125 MHz, CD_3OD) δ: *aglycone*: 131.39 (C-1), 117.08 (C-2), 144.68 (C-3), 146.15 (C-4), 116.36 (C-5), 121.25 (C-6), 72.35 (C-α), 36.68 (C-β), *caffeoyl moiety*: 127.38 (C-1), 114.92 (C-2), 150.26 (C-3), 147.04 (C-4), 116.57 (C-5), 123.21 (C-6), 169.20 (C-α), 114.55 (C-β), 147.38 (C-γ), *glucose*: 104.39 (C-1'), 75.69 (C-2'), 83.97 (C-3'), 70.04 (C-4'), 75.42 (C-5'), 64.61 (C-6'), *rhamnose*: 102.71 (C-1"), 72.40 (C-2"), 72.26 (C-3"), 74.00 (C-4"), 70.40 (C-5") and 17.86 (C-6").

Acanthaminoside (4)

Colorless needles, mp 213-214°C; $[\alpha]_D^{25}$ +135.5° (MeOH; *c* 1.00); ^{1}H-NMR (500 MHz, CD_3OD) δ: 5.77 (s, H-3), 7.10 (m, H-5), 7.03 (m, H-6, H-7), 6.94 (m, H-8), 4.70 (d, *J* = 8.0 Hz, H-1'), 3.20 (dd, *J* = 8.0, 9.0 Hz, H-2'), 3.38 (dd, *J* = 9.0, 9.0 Hz, H-3'), 3.33 (dd, *J* = 9.0, 9.0 Hz, H-4'), 3.35 (m, H-5'), 3.70 (dd, *J* = 5.0, 12.0 Hz, H_a-6') and 3.87 (dd, *J* = 3.5, 12.0 Hz, H_b-6'); ^{13}C-NMR (125 MHz, CD_3OD) δ: 163.17 (C-2), 96.48 (C-3), 118.99 (C-5), 125.07 (C-6), 124.18 (C-7), 116.83 (C-8), 142.11 (C-9), 127.10 (C-10), 103.91 (C-1'), 74.88 (C-2'), 77.91 (C-3'), 71.01 (C-4'), 78.44 (C-5') and 62.57 (C-6').

(+)-Lyoniresinol 3a-*O*-β-glucopyranoside (5)

Colorless amorphous powder, mp 178-179°C; $[\alpha]_D^{25}$ +22.7° (MeOH; *c* 1.00); ^{1}H-NMR (500 MHz, CD_3OD) δ: 2.63 (dd, *J* = 11.5, 15.0 Hz, H_a-1), 2.74 (dd, *J* = 5.0, 15.0 Hz, H_b-1), 1.72 (m, H-2), 3.67 (dd, *J* = 3.5, 11.0 Hz, H_a-2), 3.56 (dd, *J* = 6.5, 11.0 Hz, H_b-2), 2.10 (m, H-3), 3.47 (dd, *J* = 4.0, 9.5 Hz, H_a-3), 3.91 (dd, *J* = 5.5, 9.5 Hz, H_b-3), 4.44 (d, *J* = 6.5 Hz, H-4), 6.59 (s, H-8), 6.44 (s, H-2', H-6'), 4.30 (d, *J* = 9.0 Hz, H-1"), 3.30 (H-2"), 3.38 (dd, *J* = 9.0, 9.5 Hz, H-3"), 3.31 (H-4"), 3.29 (H-5"), 3.86 (dd, *J* = 3.5, 10.0 Hz, H_a-6"), 3.67 (dd, *J* = 5.5, 10.0 Hz, H_b-6"), 3.37 (s, 5-OMe), 3.87 (s, 7-OMe) and 3.76 (s, 3'-OMe, 5'-OMe); ^{13}C-NMR (125 MHz, CD_3OD) δ: 33.80 (C-1), 40.59 (C-2), 66.21 (C-2a), 46.67 (C-3), 71.46 (C-3a), 42.76 (C-4), 147.56 (C-5), 139.32 (C-6), 148.60 (C-7), 107.83 (C-8), 130.17 (C-9), 126.40 (C-10), 138.88 (C-1'), 106.91 (C-2'), 148.96 (C-3'), 134.46 (C-4'), 148.96 (C-5'), 106.91 (C-6'), 104.08 (C-1"), 75.16 (C-2"), 78.22 (C-3"), 71.65 (C-4"), 77.91 (C-5"), 62.82 (C-6"), 60.15 (5-OMe), 56.59 (7-OMe) and 56.85 (3'-OMe, 5'-OMe).

(-)-Lyoniresinol (6)

Colorless amorphous powder, mp 195-196°C; $[\alpha]_D^{25}$ - 47.7° (MeOH; *c* 1.00); ^{1}H-NMR (500 MHz, CD_3OD) δ: 2.66 (dd, *J* = 11.5, 15.0 Hz, H_a-1'), 2.97 (dd, *J* = 5.0, 15.0 Hz, H_b-1'), 1.65 (m, H-2), 3.54 (dd, *J* = 3.0, 10.5 Hz, H_a-2a), 3.62 (dd, *J* = 5.0, 10.5 Hz, H_b-2a), 1.99 (m, H-3), 4.32 (d, *J* = 4.0 Hz, H-4), 6.60 (s, H-8), 3.52 (m, H-3a), 3.40 (s, 5-OMe), 3.88 (s, 7-OMe) and 3.75 (s, 3'-OMe, 5'-OMe); ^{13}C-NMR (125 MHz, CD_3OD) δ: 33.35 (C-1), 40.92 (C-2), 66.81 (C-2a), 48.83 (C-3), 64.24 (C-3a), 42.30 (C-4), 147.70 (C-5), 139.30 (C-6), 148.67 (C-7), 107.82 (C-8), 130.20 (C-9), 126.25 (C-10), 134.57 (C-1'), 106.94 (C-2', C-6'), 149.08

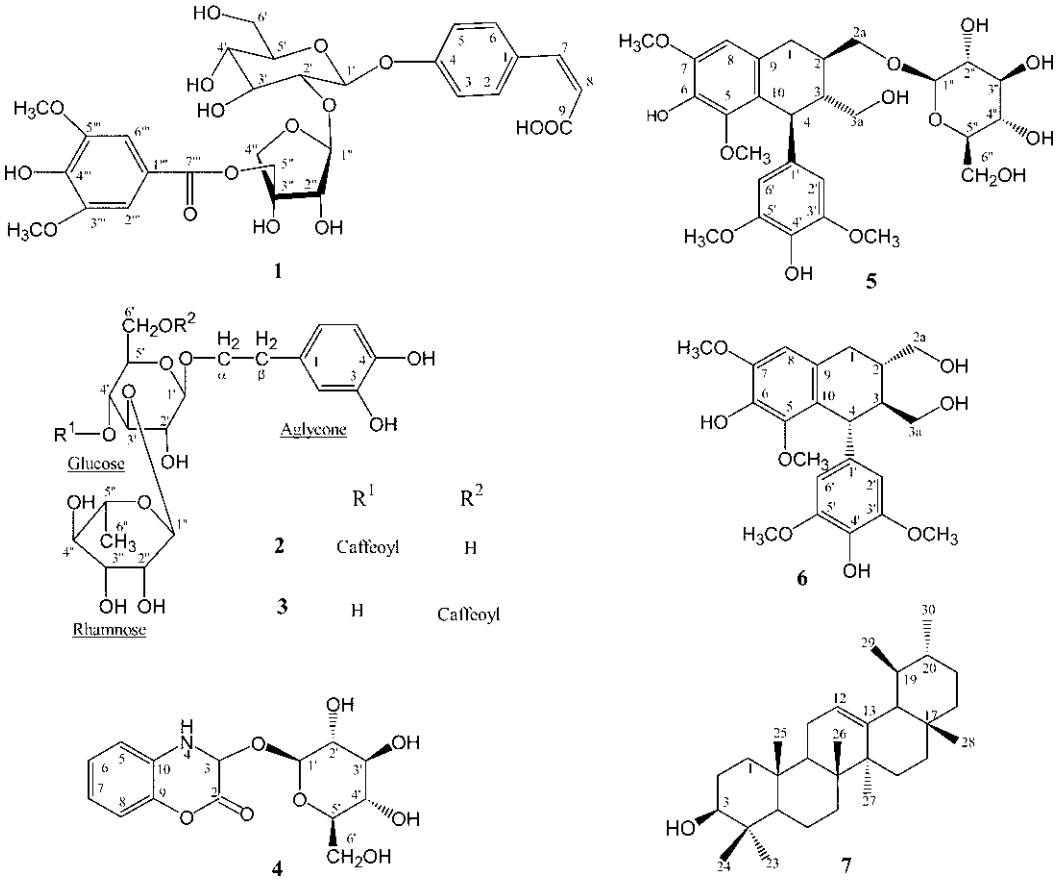

Fig. 1. Structures of compounds **1**-**7**

(C-3', C-5'), 138.89 (C-4'), 60.17 (5-OMe), 56.63 (7-OMe) and 56.80 (3'-OMe, 5'-OMe).

α-Amyrin (7)

White crystals, mp 184-185°C; $[\alpha]_D^{25}$ +85.0° (MeOH; *c* 1.00); ^{13}C-NMR (125 MHz, CD_3OD) δ: 38.12 (C-1), 27.91 (C-2), 79.72 (C-3), 39.84 (C-4), 54.40 (C-5), 19.48 (C-6), 34.35 (C-7), 40.80 (C-8), 49.17 (C-9), 38.12 (C-10), 24.36 (C-11), 126.87 (C-12), 139.68 (C-13), 43.26 (C-14), 29.23 (C-15), 25.35 (C-16), 34.35 (C-17), 56.76 (C-18), 40.42 (C-19, C-20), 31.79 (C-21), 40.01 (C-22), 28.77 (C-23), 16.01 (C-24), 16.37 (C-25), 17.64 (C-26), 24.09 (C-27), 28.77 (C-28), 17.83 (C-29) and 21.56 (C-30).

Acid hydrolysis of 1

Compound **1** (8.0 mg) was treated with a mixture of 1 : 1 2 M HCl and 1,4-dioxane (2 mL) at 100°C for 3 h. The reaction mixture was then neutralized by the addition of Ag_2CO_3 and filtered. The aqueous layer containing monosaccharides was concentrated and applied to a silica gel column eluted with EtOAc-MeOH-H_2O (4 : 1 : 0.1) to afford D-glucose (2.5 mg, R*f* 0.16, $[\alpha]_D^{25}$ +50°) and D-apiose (1.5 mg, R*f* 0.29, $[\alpha]_D^{25}$ +20°), and compared with authentic samples.

Cell culture

The MC3T3-E1 cells (RCB1126, an osteoblast-like cell line from C57BL/6 mouse calvaria) were obtained from the RIKEN Cell Bank (Tsukuba, Japan), and cultured at 37°C in a 5% CO_2 atmosphere and α-modified minimal essential medium (α-MEM; GIBCO). Unless specified otherwise, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/mL streptomycin.

Cell growth

A MTT test was used to determine the growth of MC3T3-E1 cells. The cells were seeded at a density of 5×10^3 cells per well in 48-well plates. The cell viability was determined 2 days after treatment with the compounds using a colorimetric assay based on the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide salt (MTT) by the viable cells. Briefly, the MTT solution (0.5 mg/mL) was added to the cells and incubated at 37°C to allow cleavage of the tetrazolium ring by mitochondrial dehydrogenases and the formation of blue formazan crystals. After 2 h, the residual MTT was removed carefully and the crystals were dissolved by incubation with DMSO for 20 min. The plates were shaken for 5 min and the absorbance at 570 nm was measured using a spectrophotometer.

Alkaline phosphatase activity

When the cells reached 90% confluence, they were treated with the culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid to initiate *in vitro* mineralization. The medium was changed every 2–3 days. After 6 days, the cells were cultured with the medium containing 0.3% bovine serum albumin (BSA) and isolated compounds individually for 2 days. Upon harvesting, the medium was removed and the cell monolayer was washed gently twice with phosphate buffered saline (PBS). The cells were lysed with 0.2% Triton X-100. The lysate was centrifuged at 14,000×*g* for 5 min. The clear supernatant was used to measure the alkaline phosphatase (ALP) activity, which was determined using an ALP activity assay kit (Asan Co. Korea).

Measurement of collagen content

The collagen content was determined using a Sirius Red-based colorimetric assay (Tullberg-Reinert and Jundt, 1999). The cultured osteoblasts were washed with PBS, which was followed by fixation with Bouins fluid for 1 h. Subsequently, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained with a Sirius Red dye reagent for 1 h under mild shaking on a shaker. The solution was then removed and the cultures were washed with 0.01 N HCl to remove the non-bound dye. The stained material was dissolved in 0.1 N NaOH and the absorbance was measured at 550 nm and referenced to 0.1 N NaOH.

Calcium deposition assay

When the cells reached 90% confluence, they were treated with the culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid, to initiate *in vitro* mineralization. After 10 days, the cells were cultured with the medium containing 0.3% BSA and individually with the isolated compounds for 2 days. Upon harvesting, the cells were fixed with 70% ethanol for 1 h, and then stained with 40 mM Alizarin Red S for 10 min with gentle shaking. The bound dye was quantified by dissolving the stain in 10% cetylpyridinum chloride with constant shaking for 15 min in the dark. The absorbance of the dissolved stain was measured at 561 nm.

Statistics

The results are expressed as the mean±SEM. Statistical analysis was carried out using a one-way ANOVA (P<0.05) on the SAS statistical software.

RESULTS AND DISCUSSION

Repeated column chromatography of the methanolic

extract of the leaves of *A. ilicifolius* on silica gel and YMC RP-18 yielded a new compound (**1**) and six known (**2**-**7**) compounds. The IR spectrum of compound **1** showed the presence of OH, C=O and C–O–C groups at 3426, 1640 and 1057 cm^{-1}, respectively. The ^{1}H-NMR spectrum of compound **1** exhibited the signals of a *para*-substituted benzene ring at δ 6.90 (2H, d, *J* = 8.0 Hz) and 7.40 (2H, d, *J* = 8.0 Hz), two protons of a tetra-substituted benzene ring at δ 7.28 (2H, s), two protons of a double bond with *cis* configuration at δ 6.23 and 5.93 (*J* = 13.0 Hz), two methoxyl groups at 3.87 (s, 6H), an anomeric proton of a glucopyranose at δ 4.95 (*J* = 7.0 Hz), and another anomeric proton at δ 5.51 (*J* = 1.0 Hz). The signals at δ_H 5.51 (*J* = 1.0 Hz), 3.41 (*J* = 1.0 Hz), 3.92 (*J* = 9.5 Hz)/4.26 (*J* = 9.5 Hz), 4.39 (*J* = 11.5 Hz)/4.27 (*J* = 11.5 Hz) together with signals at δ_C 110.60, 77.99, 79.25, 75.29, and 68.07 confirmed the appearance of a β-D-apiofuranosyl unit, and the signals at δ_C 100.50, 78.76, 78.65, 71.41, 78.69, and 62.39 suggested a β-D-glucopyranosyl group linking by ether linkage to a β-D-apiofuranosyl unit at C-2 (Zhong *et al*., 1998). The hydrolysis of compound **1** with 2 M HCl in 1,4-dioxane gave D-apiose and D-glucose. The ^{13}C-NMR spectrum of compound **1** confirmed the presence of a carboxyl group at δ 176.50 and a carboxylate group at δ 167.64. Comparison the NMR data of compound **1** with those of seguinoside F (Zhong *et al*., 1998) and (*Z*)-4-coumaric acid 4-β-D-apiofuranosyl-(1"→2")-*O*-β-D-glucopyranoside from *A. ilicifolius* (Wu *et al*., 2003a) confirmed that structure of compound **1** included 4-hydroxy-3,5-dimethoxybenzoate, β-D-apiofuranosyl, and β-D-glucopyranosyl units. The higher carbon chemical shift of C-5" (δ 68.07) compared with that of (*Z*)-4-coumaric acid 4-β-D-apiofuranosyl-(1"→2")-*O*-β-D-glucopyranoside (δ 64.2) (Wu *et al*., 2003a) suggested the linking of the 4-hydroxy-3,5-dimethoxybenzoate group at C-5" by an ether linkage. In the HMBC spectrum, H-8 (δ 5.93) showed a correlated with C-1 (δ 132.01)/C-7 (δ 130.59)/C-9 (δ 176.50), as well as H-7 (δ 6.23) showed a correlated with C-2 (δ 131.19)/C-9 (δ 176.50) confirming that the carboxyl group attached to the double bond, which was linked to the *para* position of the benzene ring. In addition, the HMBC spectrum revealed cross peaks between H-1' (δ 4.95) and C-4 (δ 158.14), H-1" (δ 5.51) to C-2' (δ 78.76) and between H-5" (δ 4.39/4.27) to C3" (δ 79.25)/C-4" (δ 75.29)/C-7"' (δ 167.64). The evidence confirmed that structure of compound **1** resembled that of seguinoside F (Zhong *et al*., 1998) with the addition of 4-hydroxy-3,5-dimethoxybenzoate (Wu *et al*., 2003a). This was further defined by the observation of quasi ion peaks at *m/z* 661.0 $[M+Na]^+$ in the positive ESI-MS and *m/z* 637.1 $[M-H]^-$ in the negative ESI-MS spectra, corresponding to the molecular formula, $C_{29}H_{34}O_{16}$. In addition, this molecular formula was further determined by the HREI-MS spectrum (Found *m/z*: 661.1738 $[M+Na]^+$, Calc. 661.17395 for $C_{29}H_{34}O_{16}Na$). The structure of compound **1** was proposed to be a new natural product called acancifoliuside based on the above data and the NMR results summarized in Table I.

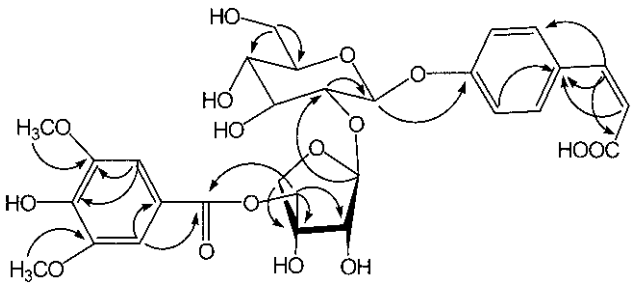

Fig. 2. Selected HMBC correlations of compound **1**

Table I. ^{1}H- and ^{13}C-NMR data for compound **1**

C	δ_C #	**1**		
		δ_C [a,b]	DEPT	δ_H [a,c] (*J*, Hz)
1	131.4	132.01		
2	130.2	131.19	CH	7.40 d (8.0)
3	116.5	116.76	CH	6.90 d (8.0)
4	156.5	158.14		
5	116.5	116.76	CH	6.90 d (8.0)
6	130.2	131.19	CH	7.40 d (8.0)
7	130.3	130.59	CH	6.23 d (13.0)
8	126.3	127.00	CH	5.93 d (13.0)
9	177.8	176.50		
1'	99.2	100.50	CH	4.95 d (7.0)
2'	79.0	78.76	CH	3.63 dd (7.0, 8.0)
3'	76.5	78.65	CH	3.65 dd (8.0, 8.0)
4'	69.7	71.41	CH	3.41 dd (8.0, 8.0)
5v	76.2	78.69	CH	3.72 m
6'	61.0	62.39	CH_2	3.71 dd (3.5, 10.0)
1"	109.8	110.60	CH	5.51 d (1.0)
2"	77.4	77.99	CH	3.41 d (1.0)
3"	79.9	79.25		
4"	74.2	75.29	CH_2	3.92 d (9.5)
5"	64.2	68.07	CH_2	4.39 d (11.5)
1"'		121.08		
2"'		108.46	CH	7.28 s
3"'		148.90		
4"'		142.10		
5"'		148.90		
6"'		108.46	CH	7.28 s
7"'		167.64		
2×OCH_3		56.94	CH_3	3.87 s

#δ_C of (Z)-4-coumaric acid 4-β-D-apiofuranosyl-(1→2)-O-β-D-glucopyranoside (Wu *et al*., 2003a), [a]Measured in CD_3OD, [b]125 MHz, [c]500 MHz.

Compound **2**-**7** were identified as acteoside (Sasaki *et al*., 1989), isoacteoside (Miyase *et al*., 1982), acanthaminoside (Amer *et al*., 2004), (+)-lyoniresinol 3a-*O*-β-glucopyranoside (Kanchanapoom *et al*., 2001b), (-)-lyoniresinol (Ohashi *et al*., 1994), and α-amyrin (Ngounou *et al*., 1988), respectively. Compounds **4**-**7** were isolated for the first time from *A. ilicifolius.*

This study examined the effects of compounds **2**, **3**, and **5** on the function of osteoblasts. The MC3T3-E1 cells were incubated with the compounds (0.3~30 μM) and the level of cell growth was measured (Fig. 3). There was significant MC3T3-E1 cell growth after exposure to the compounds (P<0.05). At a concentration of 30 μM, compound **3** increased the level of cell growth to 145.7% compared with the control. Based on this preliminary observation, the differentiation-inducing activities of the compounds on MC3T3-E1 cells were evaluated by assessing the intracellular ALP activity, collagen content, and calcium deposition. The effect of the compounds on osteoblast differentiation was first assessed by measuring the ALP activity, which is one of the major osteoblast differentiation markers. Compounds **2**, **3**, and **5** increased the ALP activity significantly at concentrations of 0.3~30 μM (Table II). At 30 μM, compound **3** increased the activity of ALP to 141.6% compared with that of control. These compounds significantly increased the activity of ALP in osteoblastic MC3T3-E1 cells. Therefore, the effect of the compounds on collagen synthesis was investigated further using a Sirius Red-based colorimetric assay. As shown in Table II, compounds **2**, **3**, and **5** at concentrations of 0.3 μM significantly increased the level of collagen synthesis. The effects of the compounds on mineralization, another important process in differentiation, were measured by measuring the level of calcium deposition by Alizarin Red staining. Compounds **2**, **3**, and **5** had a significant stimulatory effect on mineralization. At 3 μM, there was an approximately about 117.2% increase in mineralization observed after treatment with compounds **3** compared with the control.

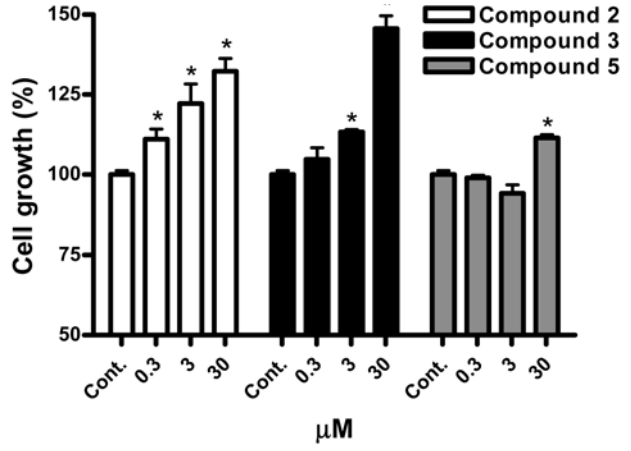

Fig. 3. Effect of compounds **2**, **3**, and **5** on the growth of MC3T3-E1 cells. The data shown are the mean±SEM, which is expressed as a percentage of the control. The control value for the MTT assay was 0.433±0.005 OD. * P<0.05 vs. control

Osteoblasts are the bone-forming cells of the skeleton; they synthesize and regulate the deposition and mineralization of the extra-cellular matrix of the bone. MC3T3-E1 cells, an osteoblast-like cell line, have been reported to retain their capacity to differentiate into osteoblasts, and may provide very useful information on the effects of phytochemicals on the differentiation of osteoblasts (Kodama *et al*., 1981). This study demonstrated that compounds **2**, **3**, and **5** can increase the cell growth, ALP activity, collagen content, and calcium deposition of osteoblastic MC3T3-E1 cells, indicating their anabolic effect. Papoutsi *et al*. (2006) reported that acteoside isolated from *Verbascum macrurum* was an antiestrogen in breast cancer cells but has no effect on endometrial cells. In addition, acteoside caused a significant increase in the ALP activity of the KS483 osteoblast cell line. Moreover,

Table II. Effect of compounds **2**, **3**, and **5** on the differentiation of MC3T3-E1 cells

	μM	ALP activity (%)	Collagen content (%)	Mineralization (%)
Compound **2**	0.3	125.1±4.446*	113.5±3.737*	104.4±1.870
	3	122.5±3.018*	101.7±0.659	108.7±2.195*
	30	113.6±3.507*	108.4±2.820	110.1±1.516*
Compound **3**	0.3	115.1±2.748*	126.4±5.127*	110.6±1.823*
	3	114.3±2.027*	120.4±3.300*	117.2±2.847*
	30	141.6±5.738*	110.0±3.304*	110.8±0.559*
Compound **5**	0.3	118.9±5.575*	122.2±7.179*	108.1±2.222*
	3	110.9±2.664*	121.2±2.455*	106.7±1.118*
	30	116.8±2.601*	110.7±3.893	107.0±1.005*

The data shown are the mean±SEM, expressed as a percentage of the control. The control values for ALP activity, collagen content, and mineralization were 0.977±0.018 Unit/10^6 cells, 14.54±0.166 mg, and 0.391±0.006 OD, respectively. * P<0.05 vs. control.

treatment with ICI 182780 in the presence of acteoside abolished its positive effect on the activity of ALP. Acteoside possesses the necessary phenolic rings as well as a hydroxyl group in the molecule, which are prerequisites that allow recognition of the estrogen receptor (ER) and favor the activity of the ER-ligand (Fang *et al*., 2001). This suggests that acteoside enhances the activity of ALP, in part, *via* an ER-dependent pathway. In this study, acteoside (**2**), isoacteoside (**3**), and (+)-lyoniresinol 3a-*O*-β-glucopyranoside (**5**) of *A. ilicifolius* leaves had direct stimulatory effects on bone formation in cultured MC3T3-E1 osteoblast cells. Accordingly, *A. ilicifolius* leaves might be useful as a pharmacological agent for the treatment of osteoporosis.

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