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Chemical Constituents from *Artemisia iwayomogi* Increase the Function of Osteoblastic MC3T3-E1 Cells

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Abstract – Chemical investigation of the aerial parts of *Artemisia iwayomogi* has afforded five glycoside compounds. Their chemical structures were characterized by spectroscopic methods to be turpinionoside A (**1**), (*Z*)-3-hexenyl *O*-α-arabinopyranosyl-(1 → 6)-*O*-β-D-glucopyranoside (**2**), (*Z*)-5'-hydroxyjasmone 5'-*O*-β-D-glucopyranoside (**3**), (–)-syringaresinol-4-*O*-β-D-glucopyranoside (**4**), and methyl 3,5-di-*O*-caffeoyl quinate (**5**). All of them were isolated for the first time from *Artemisia* species. The effect of compounds **1** - **5** on the function of osteoblastic MC3T3-E1 cells was examined by checking the cell viability, alkaline phosphatase (ALP) activity, collagen synthesis, and mineralization. Turpinionoside A (**1**) significantly increased the function of osteoblastic MC3T3-E1 cells. Cell viability, ALP activity, collagen synthesis, and mineralization were increased up to 117.2% (2 μM), 110.7% (0.4 μM), 156.0% (0.4 μM), and 143.0 % (2 μM), respectively.

Keywords – *Artemisia iwayomogi,* Glycoside compounds, Osteoblastic MC3T3-E1 cells

Introduction

Osteoporosis associated with estrogen deficiency is the most common cause of age-related bone loss. The progressive decrease in bone mass leads to an increased susceptibility to fractures, which results in substantial morbidity and mortality (Riggs and Melton, 1992). Normal bone remodeling is achieved by a balance of bone formation and bone resorption. These processes are closely regulated and are under the control of both systemic hormones and locally derived cytokines. Osteoblasts, typically located on bone-lining surfaces, are physically positioned to influence bone resorption.

Artemisia iwayomogi, a member of the Compositae, is a perennial herb easily found in Korea and considerable attention has been paid to this medicine because the herbal plant is believed to have a chemopreventive potential (Lee *et al.*, 1998; Lee *et al.*, 1993). Methanol extracts of *A. iwayomogi* inhibited nitric oxide production of lipopolysaccharide-activated macrophages (Ryu *et al.*, 2003) and two sesquiterpenes from *A. iwayomogi* were shown to inhibit the expression of inducible nitric oxide synthase (iNOS) (Ahn *et al.*, 2003). In another study, methanol extracts of *A. iwayomogi* displayed scavenging activity of peroxynitrite ($ONOO^-$), a potent cytotoxic oxidant formed by the reaction between nitric oxide (NO) and superoxide radical (O_2^-) (Kim *et al.*, 2004). AIP1, a water-soluble carbohydrate fraction from *A. iwayomogi*, showed anti-tumor and immunomodulating activities (Koo *et al.*, 1994) and suppressed spontaneous or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced apoptotic death of mouse thymocytes probably by down regulating fas gene expression (Hwang *et al.*, 2005; Ji *et al.*, 2005). In addition, *A. iwayomogi* prevented the immediate-type allergic reaction and inflammatory cytokine secretion in mast cells (Shin *et al.*, 2006).

In our ongoing study to find biologically active compounds from medicinal plants, five glycoside compounds have been isolated from the 70% MeOH extract of the aerial parts of *A. iwayomogi*. The effect of compounds **1** - **5** on the function of osteoblastic MC3T3-E1 cells was examined by checking the cell viability, alkaline phosphatase (ALP) activity, collagen synthesis, and mineralization.

Materials and methods

General experimental procedures – Optical rotations were measured on a JASCO DIP-360 digital polarimeter. NMR spectra were recorded on a Bruker DRX 400 and 500 NMR spectrometers using TMS as an internal

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standard. The electrospray ionization mass spectra were recorded on an AGILENT 1100 LC-MSD trap spectrometer. Column chromatography was conducted using silica gel 60 (40 - 63 and 63 - 200 μm particle size) and RP-18 (40 - 63 μm particle size), which were both obtained from Merck.

Plant materials – Aerial parts of *Artemisia iwayomogi* (Compositae) were collected at Jeju island in June 2007 and taxonomically identified by one of us (Young Ho Kim). A voucher specimen (CNU07105) has been deposited at the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Extraction and isolation – The plants (3 kg) were extracted with 70% MeOH at room temperature for overnight (3 × 10 L). The 70% MeOH extract (294 g) was concentrated under vacuum to give a gummy residue, which was then suspended in H_2O (3 L). This solution was extracted with EtOAc (3 × 3 L) to give 45 g of an EtOAc soluble fraction and 220 g of a H_2O soluble fraction. EtOAc soluble fraction (43 g) was chromatographed on a silica gel column eluted with a stepwise gradient of $CHCl_3$ and MeOH, to yield seven fractions (F1 - F7). Fraction F4 was chromatographed on a silica gel column ($CHCl_3$: MeOH : H_2O = 20 : 4 : 0.1) to yield four subfractions (F4A - F4D). Compound **5** (1000 mg) were obtained from F4C (2.5 g) using a RP C_{18} column (MeOH : H_2O = 1 : 1). The H_2O soluble fraction (215 g) was subjected to highly porous synthetic resin column chromatography using a stepwise gradient of H_2O:MeOH (1 : 0 - 3 : 1 - 1 : 1 - 1 : 3 - 0 : 1), to yield five fractions (F8 - F12). Fraction F10 was chromatographed on a silica gel column eluted with $CHCl_3$:MeOH:H_2O stepwise gradient (20 : 1 : 0.1 - 10 : 1 : 0.1 - 5 : 1 : 0.1), to yield six subfractions (F10A - F10F). Compounds **1** (29 mg) and **2** (40 mg) were obtained from F10E using a RP C_{18} column (MeOH : H_2O = 1 : 3). Fraction F11 was chromatographed on a silica gel column eluted with $CHCl_3$: MeOH : H_2O stepwise gradient (16 : 1 : 0.1 - 8 : 1 : 0.1 - 4 : 1 : 0.1), to yield 9 subfractions (F11A - F11I). Compounds **3** (16 mg) and **4** (72 mg) were obtained from F11G using a RP C_{18} column (Acetone : H_2O = 1 : 2).

Turpinionoside A (1) – Colorless oil; $[\alpha]_D^{20}$: –34.6 (*c* 0.30, MeOH); FAB-MS: *m*/*z* = 389 $[M – H]^-$; ^{1}H-NMR (500 MHz, CD_3OD) δ : 0.77 (3H, d, *J* = 6.4 Hz, H-13), 0.86 (3H, s, H-12), 0.93 (3H, s, H-11), 1.19 (3H, d, *J* = 6.4 Hz, H-10), 1.44 (1H, ddd, *J* = 12.0, 12.0, 12.0 Hz, H-4ax), 1.52 (1H, dd, *J* = 12.0, 2.8 Hz, H-2eq), 1.62 (1H, dd, *J* = 12.0, 12.0 Hz, H-2ax), 1.77 (1H, brd, *J* = 12.0 Hz, H-4eq), 1.90 (1H, m, H-5), 3.09 (1H, dd, *J* = 9.2, 8.0 Hz, H-2'), 3.1 - 3.3 (3H, H-3', 4', 5'), 3.61 (1H, dd, *J* = 12.0, 5.2 Hz, H-6'a), 3.82 (1H, dd, *J* = 12.0, 2.0 Hz, H-6'b), 3.90 (1H, m, H-3), 4.21 (1H, d, *J* = 7.6 Hz, H-1'), 4.24 (1H, m, H-9), 5.50 (1H, dd, *J* = 16.0, 1.2 Hz, H-7), 5.68 (1H, dd, *J* = 16.0, 6.4 Hz, H-8); ^{13}C-NMR (125 MHz, CD_3OD) δ : 16.5 (C-13), 24.1 (C-10), 25.1 (C-11), 25.8 (C-12), 35.5 (C-5), 38.1 (C-4), 40.4 (C-1), 42.6 (C-2), 62.8 (C-6'), 69.2 (C-9), 71.7 (C-4'), 75.1 (C-2'), 75.6 (C-3), 77.8 (C-5'), 78.0 (C-3'), 78.3 (C-6), 102.6 (C-1'), 133.7 (C-7), 135.5 (C-8)

(*Z*)-3-Hexenyl *O*-*α*-L-arabinopyranosyl-(1 → 6)-*O*-*β*-D-glucopyranoside (2) – A pale yellow gum; $[\alpha]_D^{20}$: –45.6 (*c* 0.20, MeOH); FAB-MS: *m*/*z* = 393 $[M – H]^-$; ^{1}H-NMR (500 MHz, CD_3OD) δ : 0.97 (3H, t, *J* = 7.5 Hz, H-1), 2.08 (2H, quin, *J* = 7.5 Hz, H-2), 2.38 (2H, dd, *J* = 14.0, 7.0 Hz, H-5), 3.18 - 3.53 (6H, m, H-2', 3', 4', 5', 3'', 5''a), 3.54 (1H, m, H-6a), 3.59 (1H, m, H-2''), 3.73 (1H, dd, *J* = 11.5, 5.5 Hz, H-6'a), 3.81 (1H, m, H-4''), 3.84 (1H, dd, *J* = 12.5, 2.5 Hz, H-6b), 3.86 (1H, dd, *J* = 12.5, 2.5 Hz, H-5''b), 4.09 (1H, dd, *J* = 11.5, 2.5 Hz, H-6'b), 4.27 (1H, d, *J* = 8.0 Hz, H-1'), 4.31 (1H, d, *J* = 7.0 Hz, H-1''), 5.37 (1H, m, H-4), 5.46 (1H, m H-3); ^{13}C-NMR (125 MHz, CD_3OD) δ : 14.8 (C-1), 21.7 (C-2), 28.9 (C-5), 66.8 (C-5''), 69.59 (C-4''), 69.61 (C-6'), 70.7 (C-6), 71.7 (C-4'), 72.5 (C-2''), 74.3 (C-3''), 75.2 (C-2'), 77.0 (C-5'), 78.1 (C-3'), 104.5 (C-1'), 105.3 (C-1''), 126.1 (C-4), 134.6 (C-3).

(*Z*)-5'-Hydroxyjasmone 5'-*O*-*β*-D-glucopyranoside (3) – An amorphous powder; m.p.: 144 – 146 ºC; $[\alpha]_D^{20}$: –14.9 (*c* 0.20, MeOH); FAB-MS: *m*/*z* = 341 $[M – H]^-$; ^{1}H-NMR (500 MHz, CD_3OD) δ : 2.11 (3H, s, $-CH_3$), 2.36 (2H, m, H-4), 2.50 (2H, dt, *J* = 6.8, 6.8 Hz, H-9), 2.56 (2H, m, H-5), 2.97 (2H, d, *J* = 7.2 Hz, H-1'), 3.15 - 3.40 (4H, H-2'', 3'', 4'', 5''), 3.58 (1H, dt, *J* = 9.5, 7.0 Hz, H-5'a), 3.67 (1H, dd, *J* = 12.0, 5.0 Hz, H-6''a), 3.87 (1H, dd, *J* = 12.0, 2.0 Hz, H-6''b), 3.91 (1H, dt, *J* = 9.5, 7.0 Hz, H-5'b), 5.34 (1H, m, H-2'), 5.44 (1H, m, H-3'), 4.28 (1H, d, *J* = 8.0 Hz, H-1''); ^{13}C-NMR (125 MHz, CD_3OD) δ : 17.5 ($-CH_3$), 22.1 (C-1'), 29.0 (C-4'), 32.6 (C-5), 35.2 (C-4), 62.8 (C-6''), 70.2 (C-5'), 71.7 (C-4''), 75.1 (C-2''), 77.9 (C-5''), 78.1 (C-3''), 104.3 (C-1''), 127.5 (C-3'), 128.7 (C-2'), 139.8 (C-2), 174.6 (C-1), 212.0 (C-3).

(–)-Syringaresinol-4-*O*-*β*-D-glucopyranoside (4) – Amorphous powder; m.p. 170 - 173 ºC; $[\alpha]_D^{20}$: –21.5 (*c* 0.20, MeOH); FAB-MS: *m*/*z* = 579 $[M – H]^-$; ^{1}H-NMR (500 MHz, CD_3OD) δ : 3.12 (2H, brs, H-8, 8'), 3.20 - 3.50 (4H, H-2'', 3'', 4'', 5''), 3.65 (1H, dd, *J* = 12.0, 5.2 Hz, H-6''b), 3.76 (1H, dd, *J* = 12.0, 2.4 Hz, H-6''a), 3.83 (6H, s, OCH_3 × 2), 3.84 (6H, s, OCH_3 × 2), 3.90 (2H, dd, *J* = 9.2, 3.2 Hz, H-9'), 4.27 (2H, dd, *J* = 15.2, 6.8 Hz, H-9), 4.70 (1H, d, *J* = 4.4 Hz, H-7'), 4.75 (1H, d, *J* = 4.4 Hz, H-7),

4.85 (1H, d, J = 7.8 Hz, H-1''), 6.64 (2H, s, H-2', 6'), 6.71 (2H, s, H-2, 6); ^{13}C-NMR (125 MHz, CD_3OD) δ : 55.5 (C-8'), 55.7 (C-8), 56.8 ($OCH_3 \times 2$), 57.1 ($OCH_3 \times 2$), 62.6 (C-6''), 71.3 (C-4''), 72.8 (C-9'), 72.9 (C-9), 75.7 (C-2''), 77.8 (C-3''), 78.3 (C-5''), 87.2 (C-7), 87.6 (C-7'), 104.5 (C-2', 6'), 104.8 (C-2, 6), 105.3 (C-1''), 133.1 (C-1'), 135.6 (C-4), 136.2 (C-4'), 139.5 (C-1), 149.3 (C-3', 5'), 154.4 (C-3, 5).

Methyl 3,5-di-*O*-caffeoyl quinate (5) – Yellow gum; $[\alpha]^{20}_{D}$: –70.9 (c 0.22, MeOH); FAB-MS: m/z = 529 $[M-H]^-$; ^{1}H-NMR (500 MHz, CD_3OD) δ : 2.10 (1H, m, H-2b), 2.15 (1H, m, H-6b), 2.19 (1H, m, H-6a), 2.21 (1H, m, H-2a), 3.69 (3H, s, OCH_3), 3.89 (1H, dd, J = 6.5, 3.5 Hz, H-4), 5.23 (1H, dd, J = 6.5, 3.5 Hz, H-5), 5.29 (1H, dd, J = 13.0, 3.5 Hz, H-3), 6.12 (1H, d, J = 16.0 Hz, H-2''), 6.24 (1H, d, J = 16.0 Hz, H-2'), 6.69 (2H, d, J = 8.0 Hz, H-8', 8''), 6.78 (2H, dd, J = 8.0, 2.0 Hz, H-9', 9''), 6.97 (2H, d, J = 2.0 Hz, H-5', 5''), 7.45 (1H, d, J = 16.0 Hz, H-3''), 7.52 (1H, d, J = 16.0 Hz, H-3'); ^{13}C-NMR (125 MHz, CD_3OD) δ : 35.6 (C-2), 36.6 (C-6), 53.2 (OCH_3), 69.9 (C-4), 72.0 (C-3), 72.2 (C-5), 74.6 (C-1), 114.8 (C-2''), 115.1 (C-5', 5''), 115.4 (C-2'), 116.4 (C-8''), 116.5 (C-8'), 123.0 (C-9''), 123.1 (C-9'), 127.6 (C-4''), 127.9 (C-4'), 146.7 (C-7''), 146.8 (C-7'), 147.1 (C-3'), 147.4 (C-3''), 149.5 (C-6'), 149.7 (C-6''), 168.0 (C-1''), 168.7 (C-1'), 175.6 (CO).

Cell culture and materials – MC3T3-E1 cells (RCB1126, an osteoblast-like cell line from C57BL/6 mouse calvaria) were obtained from RIKEN Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured at 37 °C in a 5% CO_2 atmosphere in an α-modified minimum essential medium (α-MEM; GIBCO). The medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. 17β-estradiol (Sigma; purity 98 %) was used as the positive control (Kanatani *et al*., 1991).

Cell viability – The cells were suspended in the medium and plated at a density of 5×10^3 cells/well into a 48-well plate (Costar, Cambridge, MA). After 48 h, the medium was replaced with a medium containing 0.3% BSA supplemented with the sample. After 48 h of culture, cell proliferation was measured by MTT {3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide, Sigma, St. Louis, MO, U.S.A.} assay. The medium was removed and 100 μL of 0.5 mg/mL MTT solution was added to each well. The plate was then incubated at 37 °C. After 4 h, the medium was removed, and 100 μL DMSO was added and mixed well to dissolve the formazan crystals. MTT reduction in living cells was quantified at 570 nm on a microplate reader; wells with untreated cells served as controls.

Alkaline phosphatase (ALP) activity – The cells were treated, at 90% confluence, with a culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid, to initiate differentiation. The medium was changed every 2 - 3 days. After 8 days, the cells were cultured with a medium containing 0.3% BSA and samples individually for 2 days. On harvesting, the medium was removed and the cell monolayer was gently washed twice with PBS. The cells were lysed with 0.2% Triton X-100, with the lysate centrifuged at 14,000 × g for 5 min. The clear supernatant was used to measure the ALP activity, which was determined using an ALP activity assay kit (Asan Co. Korea).

Collagen contents – The cells were treated, at 90 % confluence, with a culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid. The medium was changed every 2 - 3 days. After 8 days, the cells were cultured with a medium containing 0.3% BSA and samples individually for 2 days. On harvesting, the medium was removed and the cell monolayer was gently washed twice with PBS. The collagen content was quantified by a Sirius Red-based colorimetric assay. The cultured osteoblasts were washed with PBS, followed by fixation with Bouin's fluid for 1 h. After fixation, 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 by Sirius Red dye reagent for 1 h under mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove any non-bound dye. The stained material was dissolved in 0.1 N NaOH. And the absorbance was measured at 550 nm.

Calcium deposition assay – The cells were treated, at 90 % confluence, with a culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid. After 12 days, the cells were cultured with a medium containing 0.3% BSA and samples individually for 2 days. Staining with Alizarin Red S is a standard method for the visualization of nodular patterns and calcium depositions of osteoblast cultures *in vitro*. At harvest, the cells were fixed with 70% ethanol for 1 h and stained with 40 mM Alizarin Red S for 10 min with gentle shaking. To quantify the bound dye, the stain was solubilized with 1 mL 10% (w/v) cetylpyridinum chloride for 15 min while shaking shielded from light. The absorbance of the solubilized stain was measured at 561 nm.

Statistics – Statistical analysis was performed using one-way ANOVA with SAS statistical software. Data are presented as mean ± SEM. (n = 3), and significance was accepted at $P < 0.05$.

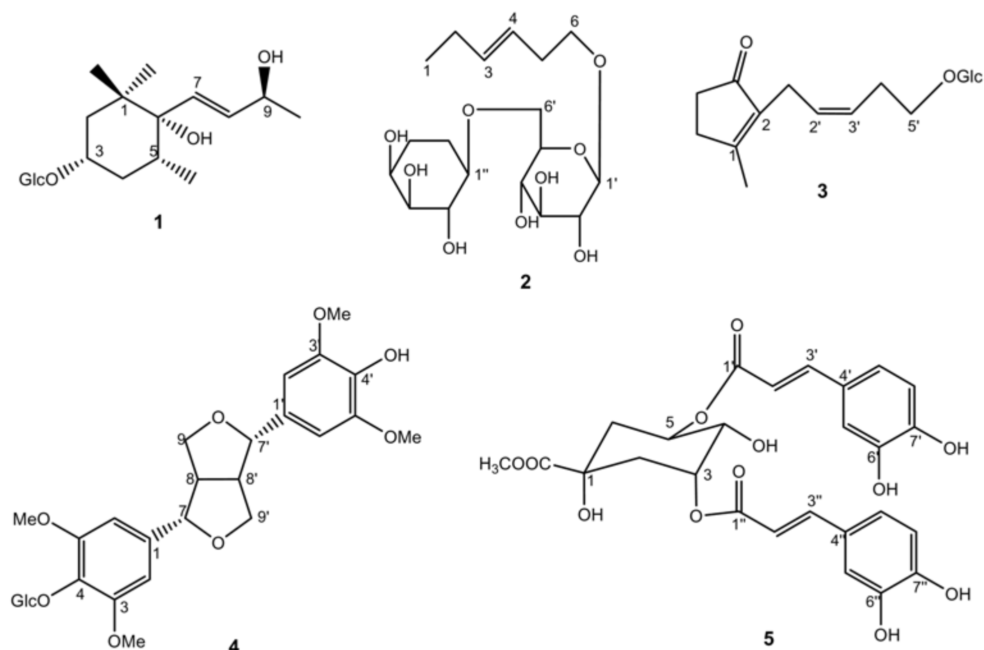

Fig. 1. Structures of compounds **1** - **5**.

Results and discussion

Compounds **1** - **5** were identified by comparing the ^{1}H, ^{13}C-NMR, and MS spectral data with the literature values to be turpinionoside A (**1**) (Yu *et al.*, 2002), (*Z*)-3-hexenyl *O*-*α*-L-arabinopyranosyl-(1 → 6)-*O*-*β*-D-glucopyranoside (**2**) (Kishida *et al.* 2005), (*Z*)-5'-hydroxyjasmone 5'-*O*-*β*-D-glucopyranoside (**3**) (Kitajima *et al.*, 2004), (–)-syringaresinol-4-*O*-*β*-D-glucopyranoside (**4**) (Jung *et al.*, 2004), and methyl 3,5-di-*O*-caffeoyl quinate (**5**) (Choi *et al.*, 2003). All of them were isolated for the first time from *Artemisia* species.

To investigate the effect of the isolated compounds on bone metabolism, a cell culture system was employed in our study. MC3T3-E1 cells, derived from newborn mouse calvarias, display osteoblast-like characteristics (Stein and Lian, 1993). ALP, collagen and mineralization are the most widely recognized biochemical markers for osteoblastic activity. Therefore, we examined the effect of isolated compounds on the cell viability, ALP activity, collagen synthesis and mineralization of osteoblastic MC3T3-E1 cells.

Osteoblastic MC3T3-E1 cells, at confluence, were cultured with a differentiation-inducing medium and incubated in a medium containing the individual isolated compounds. Firstly, each compound (0.4 - 10 μM) could increase the cell viability of the MC3T3-E1 cells as shown in Fig. 2. Among these, compounds **1** and **3,** as shown in Fig. 3, significantly increased the ALP activity up to 110.7% at a concentration of 0.4 μM and 110.4% at a concentration of 2 μM, respectively. Furthermore, their effects on collagen synthesis were evaluated using a Sirius Red-based colorimetric assay. As the results shown in Fig. 4, compounds **1** and **4** significantly increased the collagen synthesis up to 156.1 and 156.0% at a concentration of 0.4 μM, respectively, compare to that of the control and stronger than the positive control, 17*β*-estradiol. Compounds **2**, **3**, and **5** also showed good activity to increase collagen synthesis up to 126.0, 124.9, and 135.1 % at a concentration of 0.4 μM, respectively, compare to that of the control and stronger than the positive control. The effect of mineralization, another important process in differentiation, was then examined by measuring the calcium deposition by Alizarin Red staining. As the results shown in Fig. 5, compounds **1** and **5** significantly increased the mineralization up to 143.0% at a concentration of 2 μM and 132.9% at a concentration of 0.4 μM, respectively, compare to that of the control and stronger than the positive control. Compounds **2** and **4** also showed good activity to increase mineralization up to 127.1 and 123.9%, respectively, compare to that of the control and stronger than the positive control. At a concentration of 0.4 μM, compound **3** showed comparatively weaker effects to increase mineralization up to 109.3% compare to that of the control.

Osteoblasts are the bone-forming cells of the skeleton

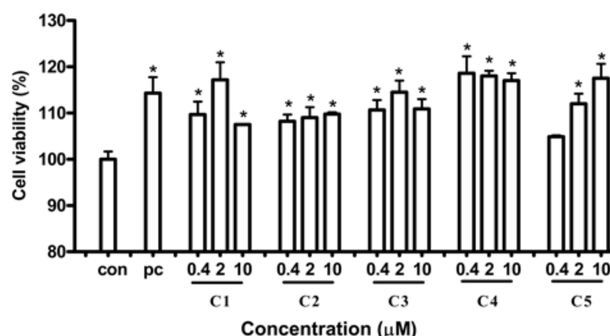

Fig. 2. Effect of compounds **1** - **5** on the cell viability of MC3T3-E1 Cells. Data were expressed as a percentage of control (0.383 ± 0.006 OD). * $P < 0.05$ vs. control. pc = positive control (17*β*-estradiol, 0.4 μM).

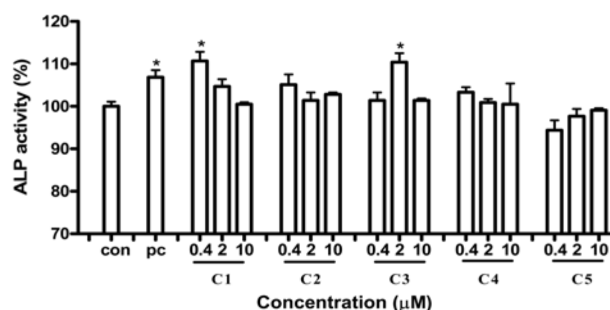

Fig. 3. Effect of compounds **1** - **5** on the ALP activity of MC3T3-E1 Cells. Data were expressed as a percentage of control (0.510 ± 0.010 Unit/10^6 cells). * $P < 0.05$ vs. control. pc = positive control (17*β*-estradiol, 0.4 μM).

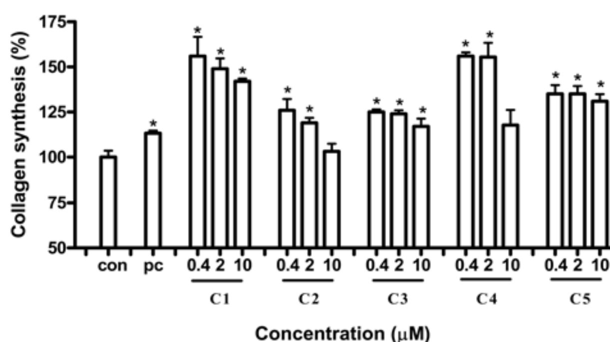

Fig. 4. Effect of compounds **1** - **5** on the collagen synthesis of MC3T3-E1 Cells. Data were expressed as a percentage of control (8.542 ± 0.364 μg/10^6 cells). * $P < 0.05$ vs. control. pc = positive control (17*β*-estradiol, 0.4 μM).

synthesizing and regulating 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 about the effects of phytochemicals on the differentiation

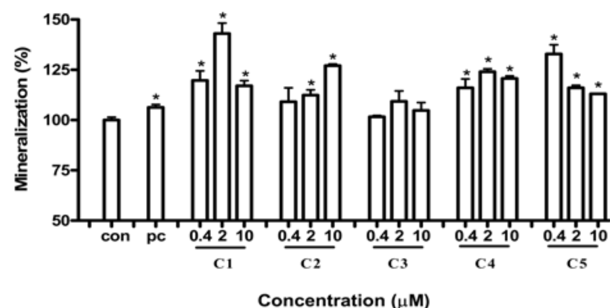

Fig. 5. Effect of compounds **1** - **5** on the mineralization of MC3T3-E1 Cells. Data were expressed as a percentage of control (0.344 ± 0.012 OD). * $P < 0.05$ vs. control. pc = positive control (17*β*-estradiol, 0.4 μM).

of osteoblasts (Sueyoshi *et al.*, 2006). These results indicated that compounds **1** - **5**, which could stimulate osteoblastic bone formation, may be useful for the prevention and treatment of osteoporosis.

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