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Effects of Ethyl Acetate Extract of *Poncirus trifoliata* Fruit for Glucocorticoid-Induced Osteoporosis

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Abstract

Poncirus trifoliata fruit (PTF) affects the digestive and cardiovascular systems, and kidney function. The authors studied the effects of ethyl acetate (EtOAc) extract of PTF on the activities of osteoblasts and in an animal model. The main compounds of the EtOAc extract, naringin and poncirin have been confirmed by HPLC and NMR analysis. Effects of osteoblastic differentiation were measured by alkaline phosphatase (ALP) activity, osteopontin (OPN) protein expression and osteoprotegerin (OPG) mRNA expression in MC3T3-E1 cells. Also, osteoclast differentiation was measured by multinucleated cells (MNCs) formation through tartrate resistance acid phosphatase (TRAP)-positive staining. Bone mineral density (BMD) was measured before and after treatment with EtOAc extract of PTF in prednisolone-induced osteoporotic mice. Dexamethasone (DEX) decreased OPN and OPG expression level in MC3T3-E1 cells and ALP activity was decreased by DEX dose-dependently. EtOAc extract of PTF recovered the levels of ALP activity, and the expression of OPN and OPG in MC3T3-E1 cells treated with DEX. In osteoclast differentiation, multinucleated TRAP-positive cell formation was significantly suppressed by the EtOAc extract of PTF. Total body BMD was restored by EtOAc extract of PTF in prednisolone-induced osteoporotic mice. In conclusion, EtOAc extract of PTF recovered DEX-mediated deteriorations in osteoblastic and osteoclastic functions, and increased BMD in glucocorticoid-induced osteoporosis.

Key Words: *Poncirus trifoliata* fruit, Ethyl acetate extract, Osteoblast, Osteoclast, Glucocorticoid-induced osteoporosis

INTRODUCTION

Osteoblasts are cells responsible for bone matrix synthesis during skeletal development, bone formation, and remodeling (Stein *et al.*, 1996). Bone formation is related to important factors, such as osteoblast proliferation, osteoblast differentiation, and life span of mature osteoblasts (Manolagas, 2000). Bone remodeling is characterized by spatial and temporal coupling of bone formation and resorption, and is necessary for skeletal growth and for maintaining normal bone structure. The pathology of osteoporosis is poorly understood, but it is caused by aberrant bone remodeling (Fazzalari, 2008). Osteoporosis is a condition in which bone mass is reduced and bone fragility is increased. Therefore, osteoporosis results in loss of bone strength to such an extent that even modest trauma will cause bone fractures (Kulenović *et al.*, 2006).

Glucocorticoids (GCs) are widely used to treat inflammatory diseases, such as asthma, rheumatoid arthritis, and inflammatory bowel disease (Boling, 2004). However, GCs often produce debilitating side effects such as GC-induced osteoporosis (GIO) (Canalis, 1996). GIO is the most common cause of secondary osteoporosis (Walsh *et al.*, 1996).

Several anti-osteoporotic agents, including bisphosphonates, estrogen, calcitonin, vitamin D, parathyroid hormone, and strontium have been discovered and utilized (Meunier, 2001; Geusens and Reid, 2005; Katagiri, 2006). Recently, several natural extracts having an as anti-osteoporotic effect were reported; the compounds stimulated osteoblast proliferation and differentiation (Suh *et al.*, 2007; Kim *et al.*, 2008).

The dried immature fruit of *Poncirus trifoliata* (L.) $R_{AF.}$ (Rutaceae) (PTF) has been traditionally used for uterine contraction and relaxation, and treating gastrointestinal and cardiovascular diseases in China (Bensky *et al.*, 2004). Previous phytochemical work on the PTF has resulted in the isolation of numerous compounds, including various types of flavonoids, coumarins, and alkaloids (Kim *et al.*, 1999; Liu *et al.*, 2002; Han *et al.*, 2007). The extracts and some isolates of this fruit were found to have diverse biological activities such as anti-

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inflammatory, hypocholesterolemic, and anti-helicobacter pylori activities (Kim *et al.*, 1999; Liu *et al.*, 2002; Shin *et al.*, 2006). Recently, we reported that *n*-hexane fraction of PTF (PTF-Hexane) inhibited the glucocorticoid-induced osteoporosis *in vitro* and *in vivo*, and AnxA6 might play a key role in this effect (Yoon *et al.*, 2011). In addition, poncirin, isolated from this fruit, prevented adipogenesis and enhanced osteoblast differentiation in mesenchymal stem cells (Kim *et al.*, 2011). While the previous studies reported the protective effects of PTF-Hexane on GC-induced osteoporosis, this study aimed to evaluate the effect of ethyl acetate fraction from PTF (PTF-EtOAc) on the differentiation of osteoblast and osteoclast, and consequently elucidate the effect of PTF-EtOAc on the bone metabolism. Moreover, the composition of PTF-Hexane and PTF-EtOAc was quite different. PTF-Hexane contained coumarins (e.g., imperatorin and aurapten) and also lots of oils, while PTF-EtOAc contained poncirin and naringin as the major components and daucosterol-3-β-D-glucose and isosakuranin as the minor components.

Natural extracts have been introduced as novel therapeutic materials for the treatment of bone-related diseases. These drugs were developed by modifying natural products or combining them with other known agents (Turner, 1991; Audran, 2000). In this study, we analyze compounds of EtOAc extract of PTF and investigate the effects of the EtOAc extract in a GC-induced osteoporosis animal model.

MATERIALS AND METHODS

Plant materials

Dried fruits of *Poncirus trifoliata* were purchased from a local Korean herb drug market in Gwangju, Korea, and were authenticated by Professor Eun-Rhan Woo of College of Pharmacy, Chosun University, Korea. Voucher specimens were deposited in the Herbarium of the College of Pharmacy, Chosun University (CSU857-15).

Extraction and isolation

The fruit of *Poncirus trifoliate* (1.2 kg) were extracted with MeOH three times under reflux and 292.0 g of residue were produced. The MeOH extract was suspended in water (H_2O) and partitioned sequentially in *n*-hexane, ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). Only a portion of EtOAc fraction (1.0 g) was chromatographed over a silica gel column using a gradient solvent system of $CHCl_3$:MeOH (20:1→1:1) to give five subfractions, designated as E1-E5. Subfracton E4 (200.0 mg) was subjected to MCI gel column chromatography eluting with a solvent system of MeOH:H_2O (1:1) to yield three subfractions E41-E43. Subfraction E41 (30.8 mg) was purified through silica gel column chromatography eluting with a solvent system of $CHCl_3$:MeOH (5:1) to give compound **1** (naringin, 16.4 mg). Subfraction E42 (81.6 mg) was purified through silica gel column chromatography eluting with a solvent system of $CHCl_3$:MeOH (10:1) to give compound **2** (poncirin , 67.0 mg).

Compound 1 (naringin): mp 160-165°C; $[M]^+$ *m/z* 580.18; molecular formula: $C_{27}H_{32}O_{14}$. ^{1}H-NMR [CD_3OD:$CDCl_3$ (2:1), 300 MHz] : δ 7.41 (2H, d, *J*=8.4 Hz, H-2', H-6'), 6.85 (2 H, d, *J*=8.4 Hz, H-3', H-5'), 6.18 (2H, s, H-6, H-8), 5.36 (1 H, dd, *J*=12.9, 2.7 Hz, H-1'''), 5.26 (1 H, d, *J*=1.5 Hz, H-2), 5.06 (1 H, dd, *J*=7.5, 4.2 Hz, H-1''), 3.44 (1H, m, H-3a), 2.77 (1 H, dd, *J*=17.1, 3 Hz, H-3b),1.3 (3 H, *J*=6 Hz, H-6'''); ^{13}C-NMR [CD_3OD:$CDCl_3$ (2:1), 125 MHz] : δ 196.4 (C-4), 164.5 (C-7), 163.0 (C-5), 162.6 (C-9), 157.0 (C-4'), 128.5 (C-1'), 127.2 (C-2',6'), 114.7 (C-3',5'), 103.1 (C-10), 100.4 (C-1'''), 97.4 (C-1''), 96.1 (C-6), 95.0 (C-8), 78.7 (C-2), 77.2 (C-2''), 77.0 (C-5''), 76.0 (C-3''), 72.0 (C-4'''), 70.2 (C-2'''), 70.1 (C-3'''), 69.2 (C-4''), 68.0 (C-5'''), 60.4 (C-6''), 42.4 (C-3), 16.5 (C-6''').

Compound 2 (poncirin): mp 210-211°C; $[M]^+$ *m/z* 594.19; molecular formula: $C_{28}H_{34}O_{14}$. ^{1}H-NMR [CD_3OD:$CDCl_3$ (2:1), 300 MHz] : δ 7.40 (2 H, d, *J*=8.1 Hz, H-2', H-6'), 6.95 (2 H, d, *J*=7.8 Hz, H-3', H-5'), 6.15 (2 H, s, H-6, H-8), 5.40 (1 H, d, *J*=10.8 ,H-2), 5.25 (1 H, d, *J*=1.0 Hz, H-1'''), 5.06 (1 H, d, *J*=7.5, H-1''), 3.13 (1 H, m, H-3a) 2.78 (1 H, d, *J*=16.8 Hz, H-3b), 1.29 (3 H, d, *J*=5.7 Hz, H-6'''); ^{13}C-NMR [CD_3OD:$CDCl_3$ (2:1), 125 MHz] : δ 196.1 (C-4), 164.5 (C-7), 162.8 (C-9), 162.3 (C-5), 159.3 (C-4'), 129.8 (C-1'), 126.9 (C-2',6'), 113.0 (C-3',5'), 102.9 (C-10), 100.3 (C-1'''), 97.3 (C-1''), 95.9 (C-6), 94.8 (C-8), 78.3 (C-2), 77.0 (C-2''), 76.8 (C-5''), 75.9 (C-3''), 71.8 (C-4'''), 70.1 (C-2'''), 70.0 (C-3'''), 69.0 (C-4''), 67.8 (C-5'''), 60.2 (C-6''), 53.9 (4'-OCH_3), 42.1 (C-3), 16.3 (C-6''').

High-performance liquid chromatography (HPLC) analysis

EtOAc extract of PTF was dissolved in methanol. The residue compounds were separated on a Capcell-pak C 18 UG 120 column (4.6×150 mm, 5 μm; Shiseido, Japan) by HPLC equipped with a Waters model 600E system controller, Model 600 HPLC pump and Waters 2487 dual wavelength absorbance detector. The flow rate was 1 ml/min and the detection wavelength was set at 254 nm. The solvent gradient for HPLC was a mixture of acetonitrile (solvent A) in 1% aqueous acetic acid (solvent B): 10% A (30 min), then 40% A (10 min), then 60% A (10 min) and, finally, with 90% A (10 min). In addition, hexane extract of PTF was dissolved in hexane and was separated on a silica column [Waters Nova-pak®Silica, 3.9×150 mm, 4 μm, Waters, Milford, MA, USA.] by the same instrument. The flow rate was 0.8 ml/min and the detection wavelength was set at 260 nm. The solvent system was isocratic condition (Hexane: EtOAc= 9:1).

Cell culture

Osteoblastic MC3T3-E1 cells were cultured in plastic dishes containing alpha-minimum essential medium (α-MEM; Gibco, NJ, USA) plus 10% fetal bovine serum (FBS; Gibco) in a CO_2 incubator (5% CO_2 in air) at 37°C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin-0.02% EDTA in phosphate-buffered saline (PBS; Gibco). Primary bone marrow stromal cells (BMSCs) and bone marrow monocytes (BMMs) were prepared as previously-described (Kawaguchi *et al.*, 1999). Briefly, femurs and tibiae were isolated from 6-week-old male ICR mice. The bone marrow was flushed out with α-MEM and maintained in α-MEM containing 10% FBS. They were then transferred to fresh medium containing 10% FBS and EtOAc extract dissolved in dimethylsulfoxide (DMSO; final concentration ≤0.05% v/v).

Alkaline phosphatase (ALP) activity

Cells were treated with EtOAc extract in the presence or absence of dexamethasone (DEX) for 6 days in 12-well plates. After washing twice with PBS, cells were scraped into 0.1 ml of $NaHCO_3$ buffer (0.25 M, pH 7.5) containing 0.01% Triton×100 on ice and centrifuged. Aliquots of supernatants were assayed for ALP activity by measuring the release of *p*-nitrophenol from

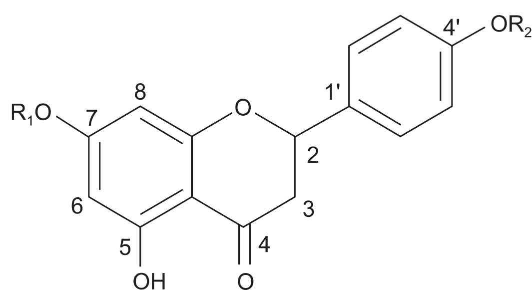
p-nitrophenylphosphate.

Western blot analysis

Cellular proteins were extracted from EtOAc extract-treated and untreated MC3T3-E1 cells in the absence or presence of DEX after 28 days of cell culture. Cells were collected by centrifugation and washed once with PBS. Cell pellets were resuspended in RIPA buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing 1 μg/ml of each of leupeptin, aprotinin, and pepstatin, and incubated for 30 min at 4°C. Cell debris was removed by microcentrifugation at 11,000×g for 30 min at 4°C. Protein concentrations were determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Cell extracts were electroblotted onto polyvinylidene difluoride membranes (Amersham, Buckinghamshire, UK) and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were incubated overnight with blocking solution (5% skim milk) at 4°C and then for 4 hr with primary osteopontin (OPN) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were washed three times with Tween 20/Tris-buffered saline (TTBS), incubated with a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody (Zymed, San Francisco, CA, USA) for 1 hr at room temperature, rewashed three times with TTBS, and developed using an enhanced chemiluminescence detection kit (Amersham).

Animals and experimental procedures

A GC-induced osteoporosis mice model was established as previously-described (Lane *et al.*, 2006). For the GCs study, 6-month-old male ICR mice were obtained from the Central Laboratory Animal (Seoul, Korea). Mice were maintained on a diet of Formular-M07 (Feedlab, Hanam, Korea). Mice were housed at a temperature of 21°C using a 12 hr light/dark cycle. Slow release pellets (Innovative Research of America, Sarasonta, FL, USA) of GC (5 mg/kg 60-day slow-release prednisolone pellets) were administered by subcutaneous implantation. The mice had either sham implantation (sham, n=10) or GC pellet implantation on the same day. The GC implanted mice were randomly divided into three groups: GC-vehicle; GC with PTF-EtOAc (100 mg/kg/d, n=10); GC with strontium chloride ($SrCl_2$; 1,800 mg/kg/d, n=10). Four weeks after GC implantation, mice were orally administered PTF-EtOAc, strontium chloride, or vehicle at once a day. After the 4 weeks of treatment, the mice were used for BMD analysis and euthanized. This animal experiment was approved by the Institutional Animal Care and Use Committee of the Ajou University School of Medicine.



Compound 1 (naringin):
R_1=α-L-rha-(1→2)-β-D-glc, R_2=H
Compound 2 (poncirin):
R_1=α-L-rha-(1→2)-β-D-glc, R_2=CH_3

Fig. 1. Chemical structures of compounds 1 (naringin) and 2 (poncirin) isolated from EtOAc extract of *Poncirus trifoliata* fruit.

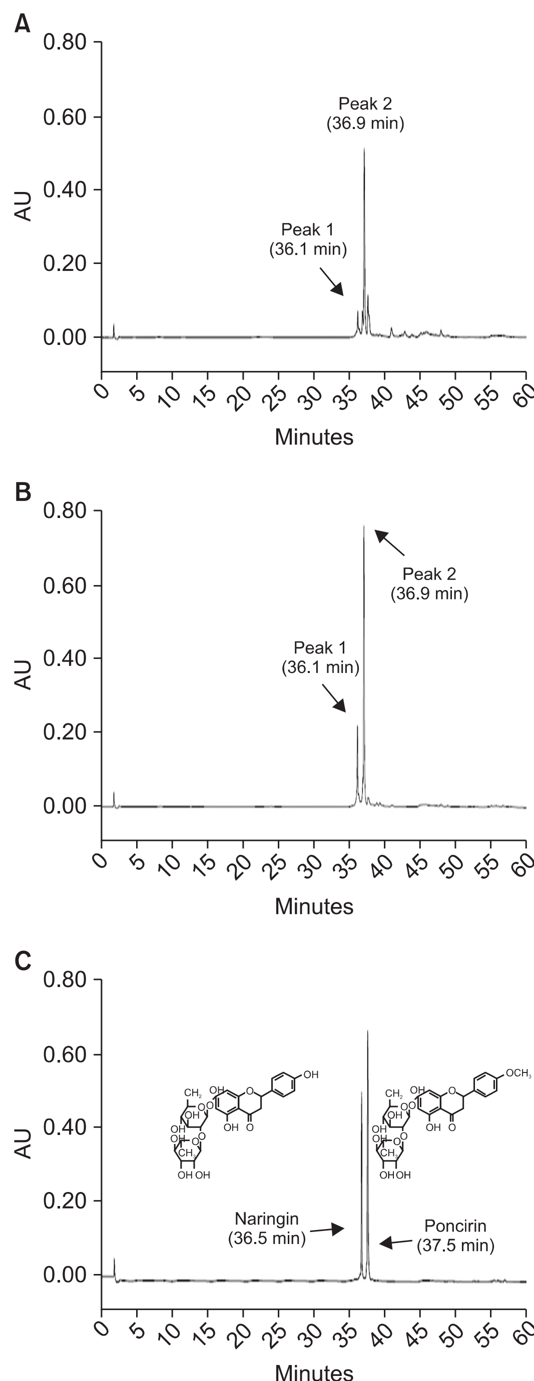


Fig. 2. HPLC chromatograms of isolated compounds from *Poncirus trifoliata* fruit. Chromatograms of EtOAc extract (A), sub-fraction of EtOAc extract (B), and a mixture of standards (C) subjected to HPLC-UV set at 254 nm. Rt of each major peak (peak 1 and peak 2) present in the two samples (A and B) and each flavonoids compound is shown in parenthesis.

Measurement of bone mineral density (BMD)

BMD was measured using a PIXI-mus bone densitometer (GE Lunar, Madison, WI, USA). After anesthetization, mice were placed on the specimen tray. All mice were placed carefully in the same position. BMD of whole body was measured using on-board PIXI-mus software for small animals, and adjusted with body weight of mice.

Statistical analyses

The results are presented as mean ± S.D. for all parameters measured. In animal studies, statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA). Differences between the groups were examined using a one-way analysis of variance (ANOVA). *in vitro* studies were analyzed by Student's *t*-test. Statistical tests were used for comparisons between groups and statistical significance was established at $p<0.05$.

RESULTS

Extraction and Isolation

The EtOAc extract of PTF was chromatographed on columns of silica gel and MCI gel afforded two compounds **1**, and **2** (Fig. 1). Compounds **1** and 2 were identified as a naringin and poncirin, respectively, by comparing the NMR spectral data with those of reported in literature (Cho *et al.*, 2000).

Identifying compounds of EtOAc extract using HPLC

HPLC chromatogram of EtOAc extract showed two major peaks (Fig. 2A, 2B). Peak 1 and 2 had a retention time of 36.1 and 36.9 min, respectively, which were identified as naringin and poncirin based on the comparative studies with standards (Fig. 2A-C). Quantitative analysis showed that naringin and poncirin made up 7.9% and 68.0% of the total EtOAc extract of PTF, suggesting that the biological activity of EtOAc extract of PTF may be derived from those major compounds. On the contrary to the EtOAc extract, the nonpolar hexane fraction

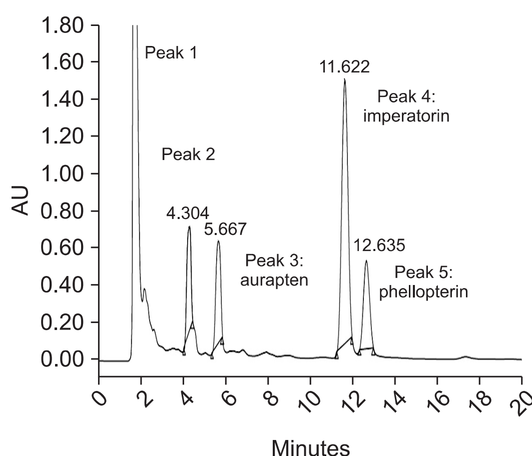


Fig. 3. HPLC chromatogram of hexane fraction of *Poncirus trifoliata* fruit. The hexane fraction of PTF dissolved in hexane, was separated on a silica column [Waters Nova-pak®Silica (3.9×150 mm, 4.0 μm)] by HPLC equipped with a Waters 600E solvent delivery system and a Waters 2487 UV detector (190-400 nm). The flow rate was 0.8 ml/min, and the detection wavelength was set at 260 nm. The solvent system was isocratic condition (Hexane:EtOAc=9:1). R_t of three major peaks (peak 3, 4, and 5) were identified as the aurapten, imperatorin, and phellopterin, respectively, by the comparative studies.

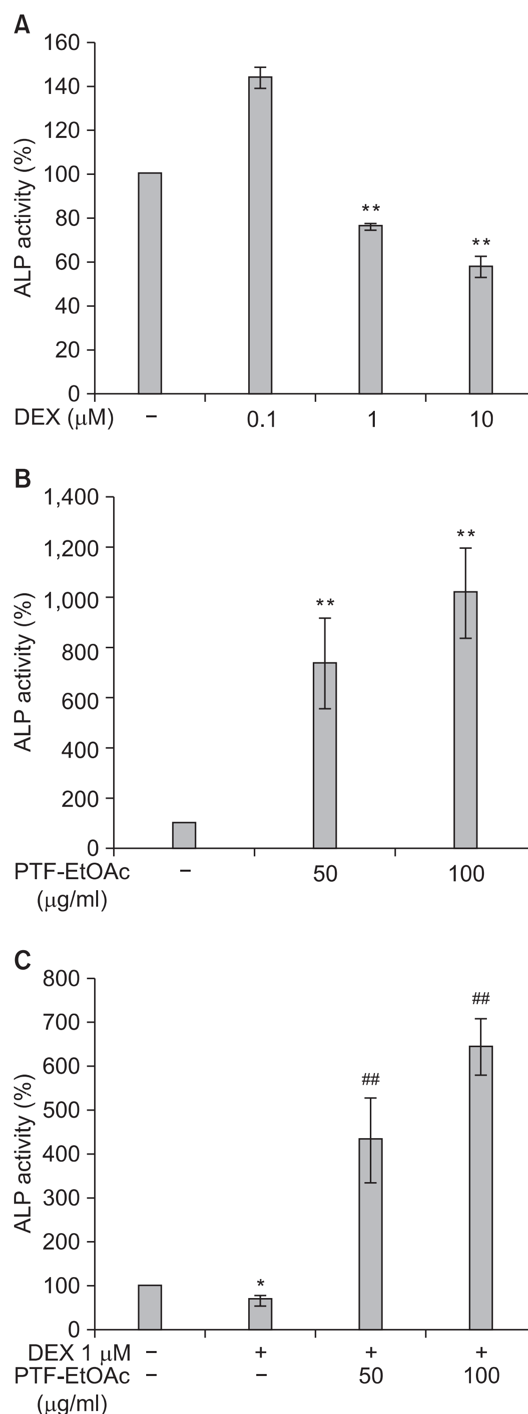


Fig. 4. Effect of EtOAc extract of *Poncirus trifoliata* fruit on DEX and ALP activity. (A) Concentration-dependent effect of DEX on ALP activity. ALP activity was measured in MC3T3-E1 cells incubated with DEX for 6 days. ALP activities were measured in MC3T3-E1 cells incubated with EtOAc extract in the absence (B) or presence (C) of DEX for 6 days. Results are expressed as percentage of control (A, B, and C). $^{**}p<0.01$ vs. control, $^{*}p<0.05$ vs. control, $^{\#\#}p<0.01$ vs. DEX.

was separated on a silica column [Waters Nova-pak®Silica, 3.9×150 mm, 4 μm, Milford, MA, USA]. As shown in Fig. 3, the HPLC chromatogram of hexane fraction showed five peaks (Fig. 3). Peaks 3, 4, and 5 were identified as aurapten, imperatorin, and phellopterin based on the comparative studies with standards. The remaining peak 1, and 2 assumed to be essential oils, and coumarin derivative, respectively.

Effect of EtOAc extract on ALP activity in MC3T3-E1 cells

To define the effect of DEX on ALP activity, MC3T3-E1 cells were treated with various concentrations of DEX. DEX concentrations of 1 and 10 μM inhibited ALP activity (Fig. 4A). EtOAc extract significantly increased ALP activity in cultured MC3T3-E1cells (Fig. 4B). The EtOAc extract increased ALP activity about 10-fold at 100 μg/ml. ALP activity was decreased by DEX, but the EtOAc extract recovered ALP activity (Fig. 4C).

Effect of EtOAc extract on OPN protein expression in MC3T3-E1 cells

OPN protein expression in MC3T3-E1 cells was increased by EtOAc extract (e.g., 31% by 50 μg/ml of EtOAc extract) (Fig. 5A). DEX significantly reduced OPN protein expression, 53% compared with the control group, and this reduction was recovered by the EtOAc extract (85% compared with the DEX group; Fig 5B).

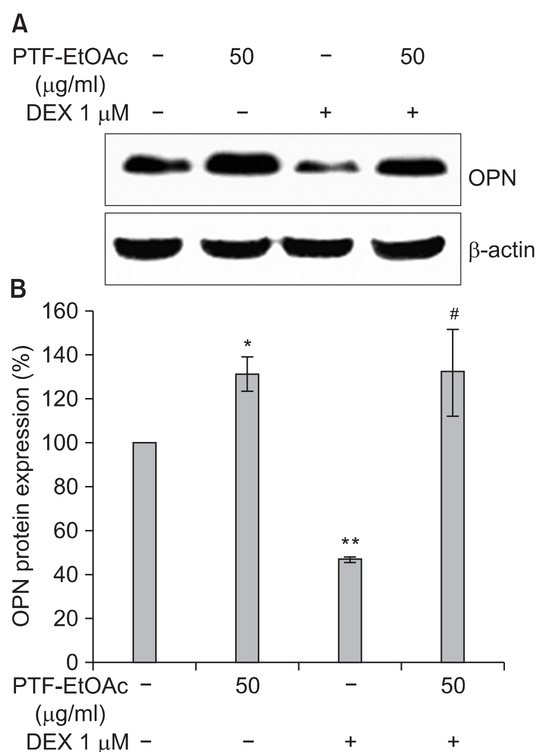


Fig. 5. Effect of EtOAc extract of *Poncirus trifoliata* fruit on osteopontin (OPN) expression in MC3T3-E1 cells. Effect of EtOAc extract on OPN expression was determined after 28 days. The blot at the top shows that OPN level increase with EtOAc extract in the absence or presence of DEX. The blot at the bottom shows the expression of the β-actin (A). β-actin level was used as loading controls. The Immunoblots were quantified by densitometer (B). Results are expressed as percentage of control. $^{**}p$<0.01 vs. control, $^{*}p$<0.05 vs. control, $^{\#}p$<0.05 vs. DEX.

Effect of EtOAc extract on BMD in prednisolone treated mice

Prednisolone-treated mice revealed a reduction in BMD versus sham-operated mice. Strontium was used as a positive control, and significantly increased BMD as compared with the prednisolone group. EtOAc extract treatment prevented the BMD loss induced by prednisolone (Fig. 6).

Effect of EtOAc extract on OPG expression in MC3T3-E1 and tartrate resistance acid phosphate (TRAP)-positive stain in primary bone marrow monocytes (BMMs)

OPG mRNA expression was measured by reverse transcription-polymerase chain reaction. Cells treated for 24 h with different concentration of EtOAc extracts or with cells treated with DEX. OPG expression was increased by EtOAc extract. DEX suppressed OPG expression, but EtOAc recovered OPG expression in the presence of DEX (Fig. 7A). Fig. 7B shows the effect of EtOAc extract on the formation of TRAP-positive multinucleated cells (MNCs) in the absence or presence of DEX for 6 days. The addition of DEX enhanced TRAP-positive MNCs. However, EtOAc extract was significantly decreased DEX-enhanced TRAP-positive cells.

DISCUSSION

In the present study, GCs were used to produce model of osteoporosis *in vitro* and *in vivo*. In general, GCs, like DEX and prednisolone, are frequently used to treat inflammatory and immune disorders. However, prolonged GC therapy can induce bone loss (Schäcke *et al.*, 2002). GCs also inhibit osteoblast proliferation and differentiation by inhibiting DNA synthesis and ALP colonies formation (Chen, 2004). A previous *in vivo* study reported that GCs reduced bone formation and increased bone resorption in mice (Weinstein *et al.*, 2002). O'Brien *et al.* (2004) reported that prednisolone-treated mice

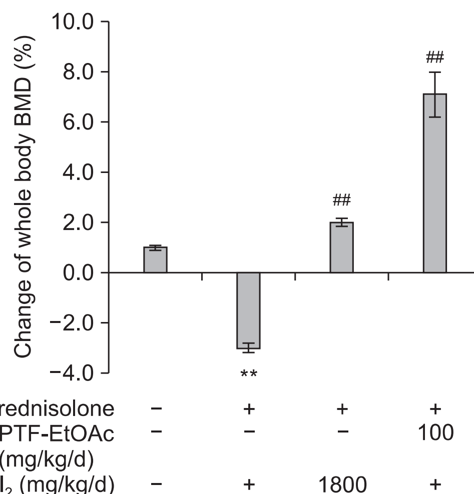


Fig. 6. Effect of EtOAc extract of *Poncirus trifoliata* fruit on change of BMD in prednisolone treated-mice. BMD was measured 4 weeks after the implantation in sham-operated mice, prednisolone-treated mice, and prednisolone treated mice with 1.8 g/kg/day of strontium chloride ($SrCl_2$), 100 mg/kg/day of EtOAc extract. Results are expressed as percentage of change of whole body BMD. $^{**}p$<0.01 vs. sham group, $^{\#\#}p$<0.01 vs. prednisolone group.

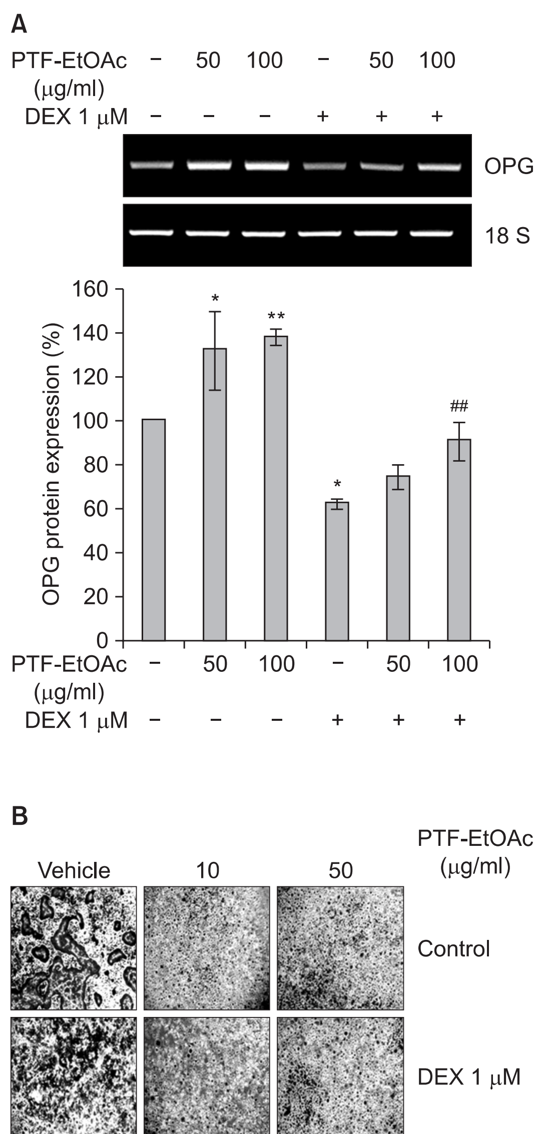


Fig. 7. Effect of EtOAc extract of *Poncirus trifoliata* fruit on OPG mRNA expression and MNCs formation in MC3T3-E1 cells and BMMs. (A) Total RNA extracted from confluent MC3T3-E1 cells treated EtOAc extracts in the absence or presence of DEX. OPG mRNA levels were determined by RT-PCR. (B) BMMs were incubated for 5 days with EtOAc extract in the absence or presence of DEX. TRAP staining shows formation of multinucleated TRAP-positive cells. Results are expressed as percentage of control. $^{**}p<0.01$ vs. control, $^{*}p<0.05$ vs. control, $^{\#\#}p<0.01$ vs. DEX.

had lower bone strength because of decreased total bone volume and architecture. Therefore, trabecular bone structural changes and bone fragility by inhibition of osteoblast proliferation and differentiation were important elements in GC-induced osteoporosis.

Recently, developments of natural extracts were applied in various fields of industry, including health functional foods. Traditionally, PTF is used to treat ailments of the digestive system, such as dyspepsia, constipation, and abdominal distension (Yeung, 1985). However, PTF was recently reported to have HMG-CoA reductase inhibition activity (Liu *et al.*, 2002), which is known to be related with increased BMD (Mundy, 2001).

In this study, we determined compounds of EtOAc extract of PTF through HPLC and NMR, and observed that EtOAc extract of PTF increased ALP activity, OPN and OPG expression, and so, osteoblast differentiation. Also, EtOAc extract suppressed TRAP-positive MNC formation during osteoclast differentiation. DEX, as an osteoporosis inducer, decreased ALP activity, OPN and OPG expression. These reductions were recovered by EtOAc extract. OPN and OPG, as synthesized by osteoblast cells, are important mediators of bone development (Khosla, 2001). Specially, OPG inhibits bone resorption through competitive interaction with receptor on the surface of osteoclast (Sato *et al.*, 1994; Khosla, 2001). In our study, although TRAP-positive staining was increased by DEX, MNC formation was not observed. Kim *et al.* (2006) reported that DEX regulates osteoclast precursor proliferation, but does not influence in functional differentiation as bone resorption through MNCs formation. Therefore, our data suggest that EtOAc extract increases osteoblast differentiation through increases ALP activity and modulation of osteoclastic activity via OPN, OPG and MNC formation. Recently, we reported that PTF-Hexane inhibited the glucocorticoid-induced osteoporosis *in vitro* and *in vivo*, and AnxA6 might play a key role in this effect (Yoon *et al.*, 2011). While the previous studies reported the protective effects of PTF-Hexane on GC-induced osteoporosis, this study aimed to evaluate the effect of PTF-EtOAc on the differentiation of osteoblast and osteoclast, and consequently elucidate the effect of PTF-EtOAc on the bone metabolism. The major difference between PTF-Hexane and PTF-EtOAc is that their chemical compositions are totally different. By using the repeated column chromatography, we identified coumarins such as imperatorin and aurapten and also lots of oils from PTF-Hexane. Furthermore, HPLC chromatogram of the PTF-Hexane showed the differences of chemical composition between PTF-hexane and PTF-EtOAc. On the other hand, from PTF-EtOAc, poncirin and naringin were identified as the major compounds and daucosterol-3-β-D-glucose and isosakuranin as the minor components. Particularly, poncirin and naringin were detected only from PTF-EtOAc and were not detectible from PTF-Hexane at all.

Poncirin and naringin are flavanone rhamonoglucosides that are regarded as the main components of PTF. Among the components of PTF, naringin was reported to influence proliferation and differentiation in osteoblastic cells and bone mesenchymal stem cells (Ding *et al.*, 2009; Zhang *et al.*, 2009). Wei *et al.* (2007) also reported that *in vivo* naringin improved length and the diameter of the bone in the retinoic acid-induced osteoporosis model. However, the role of poncirin in bone metabolism and osteoporosis has not been reported.

In the present study, the subcutaneous prednisolone pellet implantation method was used to induce osteoporosis in a mouse model (Weinstein *et al.*, 1998). EtOAc extract prevented prednisolone-induced bone loss in the mice. Strontium, as an anabolic agent, increases bone mass by stimulating osteoblastic proliferation and differentiation (Malluche *et al.*, 2006; Neuprez *et al.*, 2008). Presently, BMD was increased more by EtOAc extract compared with strontium-treated group in the GC-induced osteoporotic model.

In summary, our results suggest that EtOAc extract of PTF stimulates the differentiation of osteoblasts. Furthermore, this extract can prevent bone loss in a GC-induced mouse model

of osteoporosis.

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