Osteogenic effects of *Phlomis umbrosa* via up-regulation of Runx2 in osteoporosis

JI EUN LEE*, HAESU LEE*, MI HYE KIM and WOONG MO YANG

Department of Convergence Korean Medical Science, College of Korean Medicine, Kyung Hee University, Seoul 02447, Republic of Korea

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Abstract. *Phlomis umbrosa* Turcz (labiatae) has been suggested to promote bone growth. However, the anti-osteoporotic effects of *P. umbrosa* have not yet been elucidated. In the present study, the osteogenic effects of *P. umbrosa* were investigated in an osteoporosis model. ICR female mice were ovariectomized (OVX) to induce osteoporosis for 7 weeks. Treatment with 1, 10 and 100 mg/kg *P. umbrosa* was administrated orally to the OVX mice for 6 weeks. At the end of experiment, the microstructure of the capital femoral epiphysis was investigated. The levels of bone mineral density (BMD), bone mineral content (BMC) and serum osteocalcin concentration were evaluated. In addition, mineralized Saos-2 osteoblast cells were treated with 0.01, 0.1 and 1 μg/ml *P. umbrosa* to analyze the expression of osteoblast differentiation-associated factors. Hyperplasia of the growth plate in the femur was recovered by *P. umbrosa* treatment. BMD and BMC were significantly increased in *P. umbrosa*-treated femurs. Serum calcium concentration was increased following *P. umbrosa* treatment. In addition, the ratio of mineralization was markedly increased in *P. umbrosa*-treated differentiated osteoblasts along with increases in Runx2 levels. *P. umbrosa* conferred its osteogenic effects by upregulating Runx2 in osteoporosis. *P. umbrosa* may be a potential therapeutic material for the treatment of osteoporosis.

Introduction

Osteoporosis is a common bone disease characterized by a decrease in bone strength, an increase in fracture risk and microarchitectural deterioration of the bone tissue (1). A total of >9 million people worldwide suffer from osteoporosis, and its prevalence is increasing within aging societies (2). This skeletal disorder is directly associated with quality of life, as the first symptoms may be osteoporotic fractures in the vertebral column, rib, hip or wrist (3).

Several risk factors for osteoporosis, including estrogen loss, aging, vitamin D deficiency and low dietary calcium, have been widely observed in humans and other mammals (4). Pharmacological treatment for osteoporosis, including bisphosphonates, raloxifene and calcitonin has been suggested to cause side effects including fever, damage to the kidneys, joint pain and osteonecrosis (5). In addition, calcium supplements have occasionally demonstrated adverse effects including abdominal gas, bloating and constipation, although they are used for maintenance of bone remodeling and the prevention of osteoporosis and other bone diseases (6). Clinically, hormone replacement therapy (HRT) has been used to prevent bone loss in postmenopausal women (7). However, it has been revealed that HRT has side effects, including breast cancer, thromboembolic disease, musculoskeletal pain and gastrointestinal intolerance (8). Due to these limitations, the development of alternative anti-osteoporotic treatments is required.

Phlomis umbrosa Turcz (labiatae), a perennial herbaceous plant in Asia, has been traditionally used for treatment of bronchitis, colds, bleeding, arthralgia, rheumatic disease and bone fractures (9). Previous studies have suggested that *P. umbrosa* has anti-inflammatory, anti-nociceptive, anti-allergy and antioxidant activities (9,10). Notably, *P. umbrosa* exhibited beneficial effects on longitudinal bone growth rate in rats (11). However, the anti-osteoporotic effects of *P. umbrosa* have not been investigated yet.

The present study evaluated the therapeutic effects of *P. umbrosa* on osteoporosis in ovariectomized (OVX)-induced mice. In addition, the potential mechanisms of action of *P. umbrosa* extract were investigated in human osteoblasts-like Saos-2 cells.

Materials and methods

Preparation of P. umbrosa. P. umbrosa was purchased from Jungdo Herb, Inc. (Guri, Korea). A total of 100 g *P. umbrosa* was extracted with 1 liter distilled water for 24 h at room temperature (RT) with shaking. Following filtration, the

Correspondence to: Professor Woong Mo Yang, Department of Convergence Korean Medical Science, College of Korean Medicine, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemoon-gu, Seoul 02447, Republic of Korea
E-mail: wmyang@khu.ac.kr

*Contributed equally

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extract was concentrated under decreased pressure with a rotary evaporator and lyophilized (yield=31.44%). The obtained powder was termed 'PU'. A voucher specimen was deposited at the College of Korean Medicine of Kyung Hee University (Seoul, Korea).

PU was identified on the basis of its loganin and sweroside content by high-performance liquid chromatography (HPLC) with diode-array detection. The extract was dissolved in 70% methanol and sonicated for 30 min. Following filtration through a 0.2 μm filter membrane, 10 μl of aliquot was subjected to HPLC Agilent 1100 series (Agilent Technologies, Inc., Santa Clara, CA, USA). Chromatographic separation was achieved using a C18 column (250x4.6 mm, 5 μm; Shiseido, Osaka, Japan). Mobile phase A involved water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The separation temperature was set at 30˚C and a flow rate of 0.45 ml/min. The peak on PU was synchronized with loganin and sweroside. The concentration of loganin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and sweroside (Sigma-Aldrich) in PU was 82.32 and 237.65 μg/ml, respectively.

Animals and treatments. ICR mice were purchased from Raonbio, Inc. (Yongin, Korea). Female 6-week-old ICR mice were housed at 22±1˚C in an atmosphere with 55±10% humidity in a 12 h light: dark cycle with *ad libitum* access to a standard chow diet (Orient Bio., Inc., Seongnam, Korea) and water. The animal experiments were approved by the Institutional Animal Care and Use Committee of Kyung Hee University Laboratory Animal Center [approval no. KHUASP (SE)-15-079].

The mice were randomized into 7 groups (n=7; total=49 mice): Sham-operated mice (Sham group); OVX mice treated orally with vehicle (OVX group); OVX mice injected intraperitoneally with 10 μg/kg 17β-estradiol (E2 group); OVX mice treated orally with 150 mg/kg calcium chloride (Ca group); OVX mice treated orally with 1 mg/kg PU (PU1 group); OVX mice treated orally with 10 mg/kg PU (PU10 group); and OVX mice treated orally with 100 mg/kg PU (PU100 group). E2 and Ca were used as positive controls. All treatments started at 7 weeks following OVX surgery, and lasted for 6 weeks. At 13 weeks after the experiment began, the animals were sacrificed, and blood was collected by cardiac puncture. The right and left femurs were obtained.

Histological analysis. The right femur was fixed in 10% neutralized formalin for 18 h at RT and demineralized with 0.1 M ethylenediaminetetraacetic acid aqueous solution for 1 month. Following demineralization, femur samples were dehydrated by using xylene and consecutive ethanol concentrations (70, 80, 90, 95 and 100%) at 10 min each. Sagittal sections of the paraffin-embedded tissues were sliced at a 7 μm thickness. The slides were stained with hematoxylin for 5 min and eosin solution for 5 sec at RT according to kit instructions (Sigma-Aldrich). Histological changes were monitored using the Leica Microscope DML B2/11888111 equipped with a Leica camera DFC450 (Leica Microsystems, Buffalo Grove, IL, USA) at x100 magnification.

Measurement of bone mineral density (BMD) and bone mineral content (BMC). Following sacrifice, the left femur was collected and cleaned by removing the attached muscles and connective tissues. The sample was stored in 10% neutralized formalin until use. The levels of BMD and BMC in the left femur were determined by dual-energy X-ray absorptiometry with an InAlyzer instrument (Medikors, Seongnam, Korea).

Serum analysis. Samples were prepared from blood collected by cardiac puncture in heparinized tubes. The collected blood was centrifuged at 27,000 x g and 22˚C for 30 min, and then the supernatant was stored at -80˚C until use. The concentration of serum calcium was measured using the Calcium Colorimetric Assay kit (AdipoGen Life Sciences, Shizuoka, Japan) according to the manufacturer's protocol. The concentration of calcium in the serum was measured at 570 nm absorbance using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cell culture. The human osteoblastic Saos-2 cell line (Korean Cell Line Bank, Seoul, Korea) was routinely grown in Dulbecco's modified minimal essential medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% penicillin and 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO_2 and 95% humidity. The culture medium was changed every 3-4 days. To confirm the cytotoxicity of PU, Saos-2 cells were incubated with culture medium containing different concentrations of PU extract (0.01, 0.1 and 1 μg/ml) for 10 days. Subsequently, 2 mg/ml MTT solution was added for 4 h. Dimethyl sulfoxide was then added, and cell viability was measured at an absorbance of 570 nm.

Mineralized matrix formation assay. The cells were seeded in 6-well plates at density of $0.8x10^5$ cells/well and stabilized for 24 h. To induce osteoblast differentiation, 50 μg/ml L-ascorbic acid (AA; Thermo Fisher Scientific, Inc.) and 10 mM β-glycerophosphate (β-GP; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were added into osteogenic culture medium for 10 days. The culture medium was changed every 3-4 days. Then, the cells were fixed in 10% formalin for 10 min and stained with the 40 mM Alizarin Red-S (pH 4.2; Sigma-Aldrich; Merck KGaA) for 15 min, all at RT. The plates were observed under the Leica Microscope DML B2/11888111 equipped with Leica camera DFC450 at x100 magnification. For quantification of Alizarin red S, 500 μl citrate solution containing 20% methanol and 10% acetic acid was added for 20 min at RT, and the absorbance of supernatants was measured at 570 nm using an ELISA reader (Molecular Devices, LLC., Downingtown, PA, USA).

Western blot analysis. The Saos-2 cells were lysed with radioimmunoprecipitation assay lysis buffer (BioPrince, Seoul, Korea) containing protease inhibitors (Sigma-Aldrich). The Bradford method was used for quantification of total protein. Subsequently, 20 μg of each sample was resolved using 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% bovine serum albumin (Sigma-Aldrich) for 1 h at RT and then incubated with primary antibodies against runt-related transcription

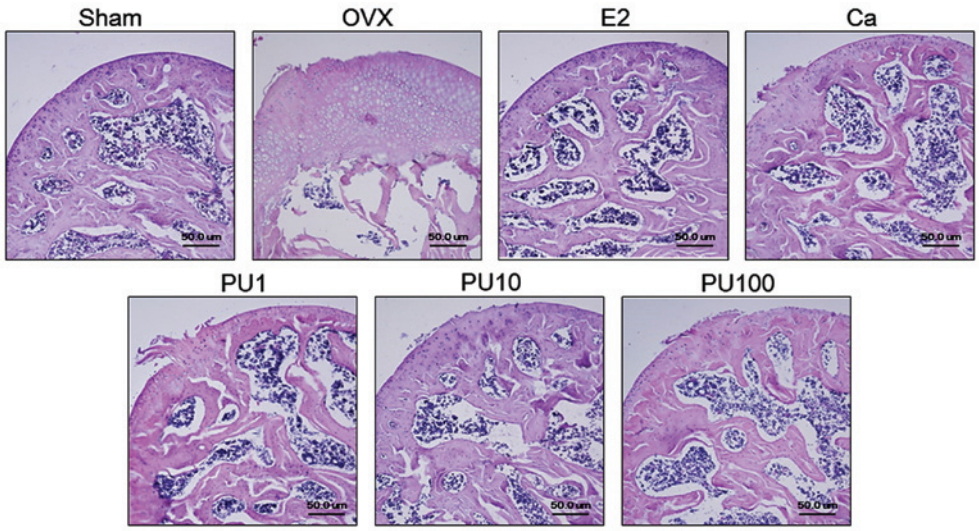

Figure 1. Effect of PU on the growth plate thickness of the epiphyseal plate in femurs. The sagittal sections were stained with hematoxylin and eosin. Magnification, x100. OVX, ovariectomized mice; E2, 17β-estradiol; Ca, calcium chloride; PU, *Phlomis umbrosa* powder.

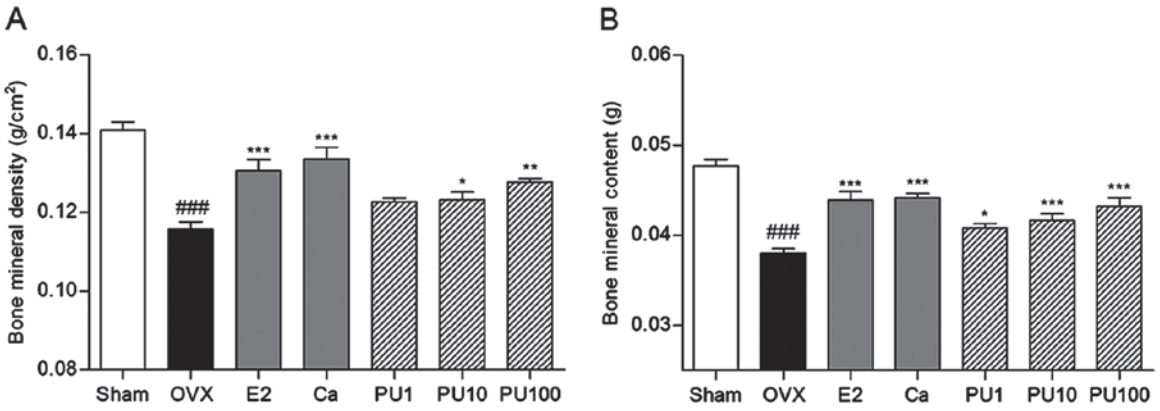

Figure 2. Effects of PU extract on the (A) bone mineral density and (B) bone mineral content in OVX mice. Results are presented as mean ± standard error of the mean. ###P<0.001 vs. Sham group; *P<0.05, **P<0.01 and ***P<0.001 vs. OVX group. OVX, ovariectomized mice; E2, 17β-estradiol; Ca, calcium chloride; PU, *Phlomis umbrosa* powder.

factor 2 (Runx2; 1:700 dilution; cat. no. 12556; Cell Signaling Technology, Inc., Danvers, MA, USA), transcription factor Sp7 (osterix; 1:1,000 dilution; cat. no. ab22552; Abcam, Cambridge, MA, USA) and β-actin (1:1,000 dilution; cat. no. sc-69879; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted in TBS containing 0.1% Tween-20 (TBS-T) overnight at 4˚C. The membrane was washed and incubated with m-IgG BP-HRP (1:2,000 dilution; cat. no. sc-516102; Santa Cruz Biotechnology, Inc.) and mouse anti-rabbit immunoglobulin G-horseradish peroxidase (1:2,000 dilution; cat. no. sc-2357, Santa Cruz Biotechnology, Inc.) diluted in TBS-T for 2 h at RT. Following washing, the bands were visualized with enhanced chemiluminescence (ECL) reagent (Amersham; GE Healthcare, Chicago, IL, USA). β-actin was used as an internal loading control for Runx2 and osterix. The band intensity was quantified using ImageJ software version 1.38e (National Institutes of Health, Bethesda, MD, USA). All experiments were performed in triplicate.

Statistical analysis. Significance was determined by one-way analysis of variance and followed by Dunnett's post-hoc test, using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. All values are expressed as the mean ± standard error of the mean.

Results

Effect of PU on the growth plate thickness of femur. The thickness of the epiphyseal plate was significantly increased in the OVX group compared with the Sham group. Administration of E2 and Ca decreased the growth plate thickness compared with OVX group. Similarly, PU-treated mice (1, 10 and 100 mg/kg) exhibited an amelioration of growth plate hyperplasia compared with the OVX-induced osteoporotic mice (Fig. 1).

Effects of PU on BMD and BMC. The BMD level of the OVX group (0.115±0.004 g/cm^2) was significantly decreased by 0.025 g/cm^2 compared with the Sham group (0.140±0.006 g/cm^2). E2 injection and Ca administration as positive controls significantly increased the level of BMD

(0.130±0.005 and 0.133±0.006 g/cm^2, respectively). Similar to the results from the positive controls, there were significant increases in BMC level in 10 and 100 mg/kg PU-treated femurs (0.123±0.005 and 0.127±0.002 g/cm^2, respectively; Fig. 2A) compared with in the OVX group.

The BMC level of the OVX mice (0.038±0.001 g) was 19.15% decreased compared with the Sham group (0.047±0.002 g). E2 injection and Ca administration as positive controls significantly increased BMC (0.043±0.001 and 0.044±0.001 g, respectively). PU treatment with 1, 10 and 100 mg/kg induced significant increases in BMC levels at all concentrations to 0.040±0.001; 0.041±0.002 and 0.043±0.002 g, respectively, compared with in OVX mice (Fig. 2B).

Effect of PU on serum calcium levels. The serum calcium level was significantly decreased in OVX group (8.96±0.24 mg/dl) compared with the Sham group (10.70±0.52 mg/dl). E2 and Ca treatment significantly increased the calcium level compared with the OVX group (9.83±0.14 and 9.80±0.63 mg/dl, respectively). Similarly, administration of 1, 10 and 100 mg/kg PU markedly increased the calcium levels to 9.77±0.49, 9.78±0.50 and 9.84±0.55 mg/dl, respectively (Fig. 3).

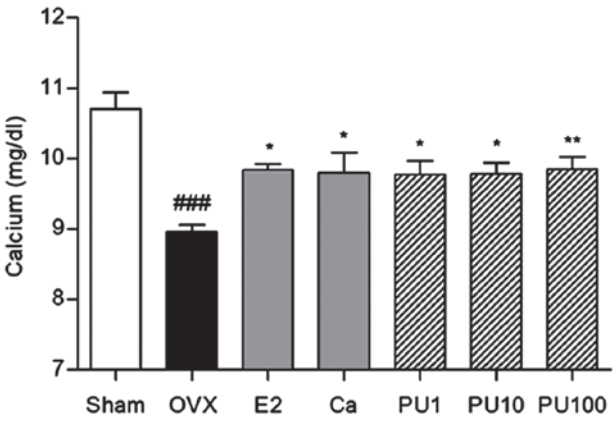

Figure 3. Effect of PU on serum calcium concentrations in OVX mice. Results are presented as mean ± standard error of the mean. ###P<0.001 vs. Sham group; *P<0.05 and **P<0.01 vs. OVX group. OVX, ovariectomized mice; E2, 17β-estradiol; Ca, calcium chloride; PU, *Phlomis umbrosa* powder.

Effect of PU on matrix mineralization of Saos-2 osteoblast cells. The cells in the presence of AA and β-GP exhibited intense red coloring. Addition of 0.1 and 1 μg/ml PU markedly increased the intensities of Alizarin Red S staining compared with control cells in the absence of PU. The percent of calcification was 12.32±3.80 and 25.62±0.68%, respectively (Fig. 4). No cytotoxic effect was observed in Saos-2 cells at any concentration of PU.

Effects of PU on Runx2 and osterix expression levels. Runx2 expression levels were significantly increased by 0.01, 0.1 and 1 μg/ml PU treatment as compared with differentiated Saos-2 osteoblast cells (39.04±13.03, 46.73±7.32 and 63.06±6.92%, respectively; Fig. 5A). However, osterix expression was not significantly altered compared with the differentiated cells. To confirm the osteogenic effects of PU on Runx2 expression, cells were treated with PU in the absence of differentiation medium for 10 days. Consistent with the result from differentiated osteoblasts, Runx2 expression was significantly increased by 0.1 and 1 µg/ml PU treatment (41.62±14.31 and 69.81±11.00%, respectively; Fig. 5B) in undifferentiated Saos-2 osteoblast cells. By contrast, osterix expression was not increased in PU-treated cells.

Discussion

Osteoporotic bone exhibits high incidence rates of fracture risk factors, including loss of bone mass, deterioration of bone structure and hyperplasia of epiphyseal growth plate (12,13). BMD and BMC measurements are the primary parameters used for the diagnosis of osteoporosis (14). In the present study, the thickness of epiphyseal growth plate was markedly increased in OVX mice. Also, the bone fragility parameters BMD and BMC were decreased in OVX mice, as expected, and administration of PU recovered the hyperplasia of growth plate and the loss of bone mass. In addition, serum calcium level is positively associated with activity of bone formation and maintenance of bone integrity (15). The results of the present study demonstrated that treatment with PU significantly increased serum calcium levels. Therefore, it appears that PU treatment ameliorates the destruction of bone structure and bone minerals in osteoporosis.

Imbalance between bone resorption and bone deposition is a crucial pathogenic event in osteoporosis (16), as development and maintenance of bone tissue requires a continuous process of bone resorption and bone deposition (3). Osteoblasts, differentiated from bone marrow mesenchymal stem cells, are responsible for bone formation (17). As calcium deposition is accompanied by bone mineralization in the process of bone formation, calcium content in mature osteoblasts differentiated from osteoblast like Saos-2 cells was observed in the present study. PU treatment notably increased the calcium content of the mineralized matrix. Therefore, these results demonstrate that PU treatments have the capability to promote bone matrix mineralization in osteoblasts.

To clarify this osteogenic effect of PU, the expression of bone differentiation-associated markers including Runx2 and osterix were analyzed in Saos-2 cells. The mineralization of osteoblasts is regulated by several osteogenic factors including Runx2, osterix, bone morphogenic protein (BMP), mothers against decapentaplegic homolog 1, insulin-like growth factor (IGF)-1, β-catenin and transforming growth factor-β (18,19). In particular, Runx2 and osterix serve key roles in the differentiation and proliferation of the osteoblast lineage (20,21). Osteoblast-specific transcription factors are also involved in the process of newly-formed matrix mineralization, which leads to osteogenesis (22). In the present study, the expression of Runx2 during osteogenic differentiation was improved by PU treatment in Saos-2 cells, while osterix expression was not increased by PU. These data suggest that PU may induce osteoblast differentiation and mineralization by stimulating Runx2.

Lee *et al* (11) identified that the longitudinal bone growth rate of adolescent rats was increased by *P. umbrosa* administration via upregulation of IGF-1 and BMP-2. Also, a herbal-based formula including *P. umbrosa* was demonstrated to exhibit ameliorative effects on pre-, peri and

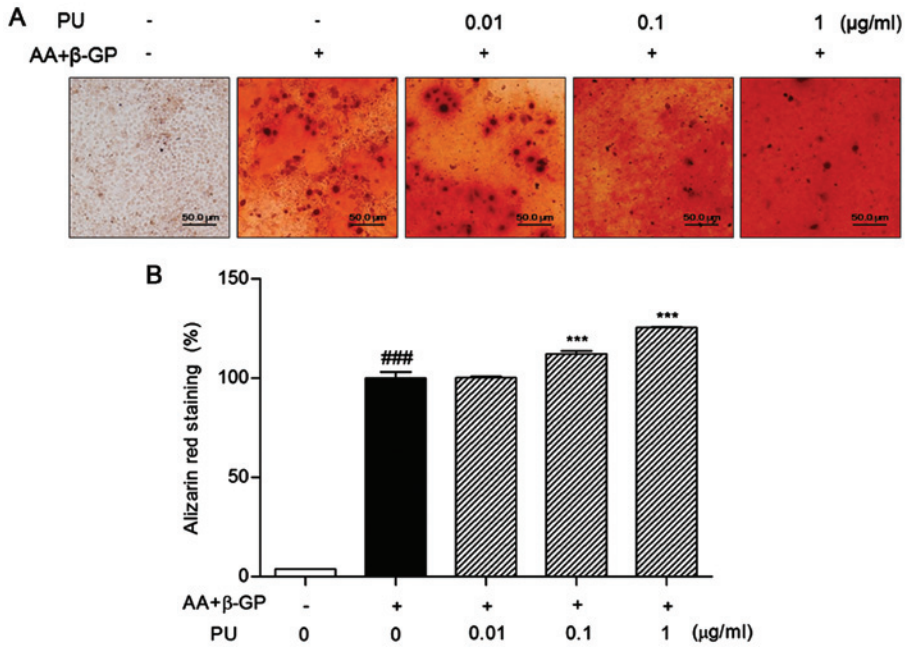

Figure 4. Effect of PU on matrix mineralization in Saos-2 osteoblast cells. (A) Differentiation of the Saos-2 cells was induced by AA+β-GP in the presence or absence of PU for 10 days. The mineralized matrix was stained with alizarin red and observed under a microscope. Magnification, x40. (B) Following staining, the dye was extracted and quantified. ###P<0.001 vs. non-treated cells. ***P<0.001 vs. AA+β-GP-induced differentiated cells. PU, *Phlomis umbrosa* powder; AA, L-ascorbic acid; β-GP, β-glycerophosphate.

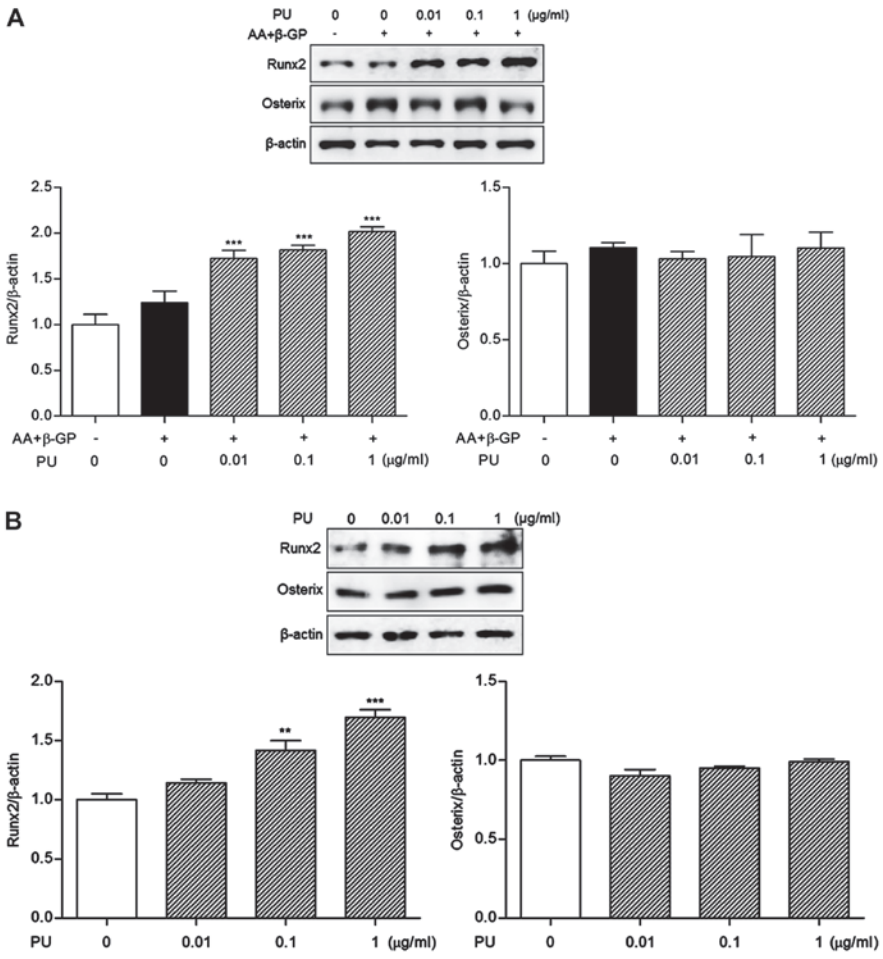

Figure 5. Effects of PU on Runx2 and osterix expression levels in differentiated Saos-2 cells and undifferentiated cells. (A) Differentiation of Saos-2 cells was induced by AA+β-GP treatment in the presence or absence of PU for 10 days. (B) Undifferentiated Saos-2 cells were treated with PU for 10 days in the absence of AA+β-GP. Results are presented as mean ± standard error of the mean. **P<0.01 and ***P<0.001 vs. non-treated cells. PU, *Phlomis umbrosa* powder; Runx2, runt-related transcription factor 2; osterix, transcription factor Sp7; AA, L-ascorbic acid; β-GP, β-glycerophosphate.

post-menopausal symptoms in a randomized, double-blind, placebo-controlled trial involving 72 subjects (ISRCTN 959534) (23). In the present study, PU exhibited osteogenic effects in OVX-induced osteoporosis mice, which was consistent with data from previous studies (11,23). Considering the data from previous studies and the experimental results of the present study, *P. umbrosa* may possess the potential to be used in post-menopausal osteoporosis.

P. umbrosa ameliorates osteoporosis through its osteogenic effects. *P. umbrosa* recovered bone mineral loss and the structure of osteoporotic bone. *P. umbrosa* promoted matrix formation in osteoblasts by regulating Runx2. Accordingly, *P. umbrosa* may represent a novel anti-osteoporotic herbal candidate for the treatment of osteoporosis as a bone-forming agent.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

All authors participated in the study design, interpretation and analysis of the data and review of the manuscript; JEL, MHK and WMY contributed to the analysis design, JEL, HL and MHK analyzed the data; JEL and WMY drafted the manuscript; and WMY provided supervision of the study.

Ethics approval and consent to participate

Experimental protocols involving animals were approved by the Institutional Animal Ethics Committee of Kyung Hee University, Seoul, Korea [approval no. KHUASP (SE)-15-079].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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