

MC3T3-E1 조골세포주와 RAW 264.7 파골세포주에서 길경을 함유한 한약재 추출물의 항골다공증 효과

¹한림대학교 식의약품 효능평가 및 기능성소재개발센터, ²동서대학교 식품영양학과
³세명대학교 한의과대학, ⁴세명대학교 화장품과학과, ⁵구운식품 기업부설연구소
정재인¹, 이현숙², 김형준³, 김용민⁴, 김수현⁵, 유동진⁵, 김은지¹

ABSTRACT

Anti-osteoporotic Activity of Mixed Herbal Extract Involving *Platycodon Grandiflorum* Root in Osteoblastic MC3T3-E1 and Osteoclastic RAW 264.7 Cells

Jae-In Jung¹, Hyun-Sook Lee², Hyung-Joon Kim³, Yong-Min Kim⁴,
Soo-Hyun Kim⁵, Dong-Jin Yoo⁵, Eun-Ji Kim¹

¹Center for Efficacy Assessment and Development of Functional Foods
and Drugs, Hallym University

²Dept. of Food Science & Nutrition, Dong-Seo University

³Korean Medicine College, Se-Myung University

⁴Dept. of Cosmetic Sciences, Se-Myung University

⁵Research Institute, Guwoon Food

Objectives: Osteoporosis is considered a serious human disease. We developed an extract of mixed herbs containing root of *Platycodon grandiflorum* (ExMH-PGR), which is expected to be effective in preventing or treating osteoporosis. The aim of this study was to investigate the anti-osteoporotic effect of ExMH-PGR in osteoblastic MC3T3-E1 cells and osteoclastic RAW 264.7 cells.

Methods: To examine the anti-osteoporotic effect of ExMH-PGR, osteoblast and osteoclast differentiation were induced and cultured with various concentrations of ExMH-PGR. Alkaline phosphatase (ALP) activity, collagen synthesis, osteocalcin production, and mineralization in MC3T3-E1 cells were analyzed. Tartrate-resistant acid phosphatase (TRAP) activity and the formation of actin ring in RAW 264.7 cells were analyzed.

Results: ExMH-PGR at concentration up to 25 µg/mL significantly increased ALP activity, collagen synthesis, osteocalcin production, and mineralization in MC3T3-E1 cells. ExMH-PGR at 50 to 200 µg/mL significantly inhibited TRAP activity and the formation of actin ring in RAW 264.7 cells.

Conclusions: These results demonstrate that ExMH-PGR stimulates osteoblastic activities and inhibits osteoclastic activities in *in vitro* systems, suggesting that ExMH-PGR might be considered as an anti-osteoporotic candidate for treatment of osteoporosis disease.

Key Words: Osteoporosis, Osteoblast, Osteoclast, Medicinal Herb, *Platycodon Grandiflorum*

"This study was supported by the Ministry of Trade, Industry and Energy (MOTIE) and Korea Institute for Advanced of Technology (KIAT) through the Research and Promoting Specialized Industry (R0005131), Korea."

Conflict of Interest : The authors declare no potential of interests.

I. Introduction

Osteoporosis is a systemic skeletal disorder characterized by decreased bone density and microstructural degradation. It results in increased bone fragility and fracture risk, consequently causing serious complications¹⁾. Osteoporosis seriously affects human health, and there is no safe and effective method to restore lost bone. Since the number of osteoporosis patients is increasing rapidly as elderly population grows globally, the market for bone disease treatment is expected to grow. Thus, global research institutes and pharmaceutical companies are heavily investing in the development of bone disease treatments. Drugs currently available for prevention and treatment of osteoporosis act by inhibiting osteoclastic bone resorption. Current drugs used for treating osteoporosis include hormone replacement therapy drugs, bisphosphonates, calcitonin, selective estrogen receptor modulators (SERMs), and parathyroid hormone analogs²⁾. Unfortunately, long-term treatment with these drugs can induce serious side effects. Hormone therapy can increase the risk of breast cancer, endothelial carcinoma, heart attack, blood clots, and stroke. The use of bisphosphonates can increase the risk of jaw osteonecrosis, atypical femoral fractures, and atrial fibrillation. Although the use of SERMs reduces the risk of fracture, it increases the risk of thrombosis. The use of parathyroid hormone increases the risk of nausea,

headache, dizziness, hypercalcemia, and hypercalciuria while the use of calcitonin poses oncogenic risk^{3,4)}. However, osteoporosis cannot be treated by only short-term administration of a drug. Long-term administration of the drug is essential. Therefore, there is a need to develop new substance with excellent drug efficacy that has no side effects even with long-term administration.

The root of *Platycodon grandiflorum* (PGR) commonly known as kilkyong or doraji in Korean, kikyo in Japanese, jiegeng in Chinese, and balloon flower in English, is used in both medicinal and edible in Asia. In Korea, PGR has been used for food such as salad, soup, sauces, dried fried vegetables, and mixed vegetables of spices. It is also traditionally used as a treatment for bronchia, asthma, pulmonary tuberculosis, diabetes, and inflammatory diseases⁵⁾. Inflatable flowers are used in both medicinal and edible in Asia.

The major component of PGR is platycodin D, a triterpenoid saponin with various pharmacological activities such as antioxidant activity⁶⁾, anti-inflammatory activity⁷⁾, anti-allergy activity⁸⁾, anti-obesity and hypolipidemic effect^{9,10)}, antiproliferative effect on tumor cells^{11,12)}, immune response augmenting ability¹³⁾, anti-hepato toxicity¹⁴⁾, anti microbial activity¹⁵⁾, and neuroprotective activity¹⁶⁾. Recently, it has been reported that platycodin D can also stimulate osteoblast differentiation and inhibit osteoclastogenesis^{17,18)}. This means that PGR may be useful as an anti-osteoporotic candidate. However,

their bone-related effects have not been fully elucidated yet.

We developed an aqueous extract of mixed herbs containing PGR (ExMH-PGR) during our search for useful ingredient to develop dietary supplement or beverage that could be effective in preventing or treating osteoporosis using natural foods and ingredients. This study was conducted to investigate the anti-osteoporotic effect of ExMH-PGR by measuring its effect on bone metabolism related biomarkers in *in vitro* systems using osteoblastic MC3T3-E1 cells and osteoclastic RAW 264.7 cells.

II. Methods

1. Materials

The reagents and materials in this study were purchased from the indicated suppliers: α -minimal essential medium (α -MEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA), and Alexa Fluor 594 Phalloidin from Thermo Fisher Scientific Inc. (Waltham, MA, USA); Dulbecco's Modified Eagle's Medium (DMEM) from Welgene (Daegu, Korea); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-(4-amidinophenyl)-6-indolecarbamide

dihydrochloride (DAPI), ascorbic acid, β -glycerophosphate, and RANKL from Sigma-Aldrich Co. (St. Louis, MO, USA); TRACP & ALP assay kit and osteocalcin ELISA Kit from Takara Bio Inc. (Kusatsu, Japan); Sirius red collagen detection kit from Chondrex Inc. (Redmond, WA, USA); Osteogenesis assay kit from Millipore (Burlington, MA, USA); PD98059 from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Preparation of ExMH-PGR

PGR and other herbs were purchased from a local herbal medicine drugstore (Wonju, Korea). Compositions and contents of herbs used in ExMH-PGR production are shown in Table 1. All herbs were cut into 5 cm each, washed with water, and dried. Five times water was added to these herbs and the mixture was extracted at 95°C for 6 h using an ultra-high vacuum low temperature extractor (COSMOS-660, Kyungseo machine, Incheon, Korea). The extract was naturally dipped for 2 h and then filtered through a filter paper to separate precipitate and extract. Filtered extract was concentrated under reduced pressure followed by freeze-drying to obtain powder. The resulting powder was used as ExMH-PGR and stored at -20°C.

Table 1. Used to Prepare the Extract of Herbs Mixture [ExMH-PGR*]

Botanical name	Weight (g)
<i>Platycodon grandiflorum</i> (桔梗) root	100
<i>Angelica gigas</i> Nakai (當歸) root	150
<i>Atractylodes macrocephala</i> Koidz (白朮) rhizome	150
<i>Gardenia jasminoides</i> Ellis (梔子) fruit	100
<i>Glycyrrhiza uralensis</i> (甘草) root	100
<i>Menthe arvensis</i> var <i>piperascens</i> (薄荷) leaf	50
<i>Paeonia japonica</i> (白芍藥) root	150
<i>Poria cocos</i> (Schw.) Wolf (茯苓) sclerotium	150
<i>Zingiberis siccatum</i> <i>Rhizoma</i> (乾薑)	50
Total	1,000

*ExMH-PGR : mixed herbs containing root of *Platycodon grandiflorum*

3. Cell culture and differentiation

MC3T3-E1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in α -MEM supplemented with 10% FBS, 100,000 U/L penicillin, and 100 mg/L streptomycin at 37°C in a humidified atmosphere of 5% CO₂. When cells were 90% confluent, they were further incubate in α -MEM supplemented with 10% FBS, 10 mmol/L β -glycerophosphate, and 50 mg/L ascorbicacid (osteoblast differentiation inducing medium, OB-DIM) to induce osteoblast differentiation. Fresh osteoblast differentiation inducing medium was changed every 3 days.

Mouse-derived macrophage RAW 264.7 cells were obtained from the ATCC and cultured in DMEM supplemented with 10% FBS, 100,000 U/L penicillin, and 100 mg/L streptomycin at 37°C in a humidified atmosphere of 5% CO₂. To induce differentiation of RAW 264.7 cells into osteoclast, cells were incubated in DMEM supplemented with 10% FBS, 50 μ g/L RANKL, and 10 μ mol/L PD98059

(osteoclast differentiation inducing medium, OC-DIM) for 5 days. Fresh osteoclast differentiation inducing medium was changed every 2 days.

4. Cell viability assay

MC3T3-E1 cells and RAW 264.7 cells were seeded into a 24-well plate at 5×10^4 cells/well. After incubation for 24 h, cells were treated with various concentrations of ExMH-PGR and incubated for 24 or 48 h. Cell viability was measured by MTT assay, as previously described¹⁹⁾.

5. Measurement of alkaline phosphatase (ALP) activity

MC3T3-E1 cells were plated into 48-well plates at 2×10^4 cells/well and incubated for 24 h. After 24 h of incubation, cells were incubated in OB-DIM containing ExMH-PGR at various concentrations (0, 1, 10, 25 μ g/mL) for 5 days. ALP activity was measured using TRACP & ALP assay kit (Takara Bio Inc.) in accordance with the manufacturer's instructions.

6. Measurement of collagen synthesis

The degree of collagen synthesis was measured by staining cells using Sirius Red, an anionic dye that strongly binds to collagen molecules. MC3T3-E1 cells were plated and treated with ExMH-PGR as described above. Cells were incubated for 8 days. Collagen synthesis was measured using Sirius Red collagen detection kit (Chondrex) according to the manufacturer's instructions.

7. Measurement of mineralization

Alizarin Red S, an anthraquinonoid derivative, was used to measure the amount of calcium deposited in osteoblasts to investigate mineralization of osteoblasts. MC3T3-E1 cells were plated and treated with ExMH-PGR as described above. Cells were incubated for 14 days to induce mineral deposition in cells. Mineral deposition of cells was measured by the method described in osteogenesis assay kit (Millipore).

8. Measurement of osteocalcin production

MC3T3-E1 cells were plated and treated with ExMH-PGR as described above. Cells were incubated for 14 days. Media conditioned for 24 h were collected. Levels of osteoblast-secreted osteocalcin in 24 h-conditioned media were measured using osteocalcin ELISA Kit (Takara Bio Inc.) according to the manufacturer's instructions.

9. Measurement of tartrate-resistant acid phosphatase (TRAP) activity

RAW 264.7 cells were seeded into 24-well

plates at 5×10^4 cells/well and incubated for 24 h. Following 24 h, cells were incubated for 5 days in OC-DIM containing ExMH-PGR at various concentrations (0, 50, 100, 200 $\mu\text{g}/\text{mL}$). After 5 days, TRAP activity of the cells treated with various concentration of ExMH-PGR was measured using TRACP & ALP assay kit (Takara Bio Inc.) in accordance with the manufacturer's instructions.

10. Morphological observation of actin ring in RAW 264.7 cells

RAW 264.7 cells were seeded into 24-well plates containing cover glass at 1×10^4 cells/well and treated with ExMH-PGR as described above. After 5 days, cells were washed with PBS, fixed with 4% paraformaldehyde containing 0.1% Triton X-100, and stained with Alexa Fluor 594 Phalloidin and DAPI to stain actin and nuclei, respectively. Morphological changes of actin ring in cells were observed via microscopy. Photographs were taken with a Carl Zeiss AxioImager microscope (Carl Zeiss, Jena, Germany).

11. Statistical analysis

All statistical analysis was performed with SAS software version 9.4 (SAS Institute, Cary, NC, USA). Data are expressed as mean \pm SE. Statistical significance was determined using ANOVA followed by Duncan's multiple range test to express the difference between experimental groups. Statistical significance was set at $P < 0.05$.

III. Results

1. Effect of ExMH-PGR on cell viability in MC3T3-E1 and RAW 264.7 cells

In order to determine the concentration of ExMH-PGR that could affect the viability of MC3T3-E1 and RAW 264.7 cells, we incubated cells for 48 h in culture media containing 0~800 $\mu\text{g}/\text{mL}$ ExMH-PGR followed by MTT assay. No remarkable cytotoxicity was observed when MC3T3-E1 cells were exposed to ExMH-PGR at concentration up to 800 $\mu\text{g}/\text{mL}$ for 48 h. In RAW 264.7 cells, no notable cytotoxicity was observed after exposure to ExMH-PGR at concentration of 0 to 200 $\mu\text{g}/\text{mL}$. However, cell viability was significantly decreased when the concentrations of ExMH-PGR was above 200 $\mu\text{g}/\text{mL}$ (data not shown). To exclude cytotoxic effects, we used ExMH-PGR at concentrations below 200 $\mu\text{g}/\text{mL}$ in subsequent experiments.

2. ExMH-PGR promotes early osteoblast differentiation in MC3T3-E1 cells

Based on preliminary experiments, the appropriate concentration range of ExMH-PGR to treat MC3T3-E1 cells was chosen to be 0~5 $\mu\text{g}/\text{mL}$. We first examined the effect of ExMH-PGR on ALP activity as an early-stage marker of osteoblast differentiation in MC3T3-E1 cells. The activity of ALP was dramatically increased by OB-DIM. Treatment of cells with 1 $\mu\text{g}/\text{mL}$ ExMH-PGR did not significantly affect OB-DIM-induced ALP activity.

Treatment of cells with 10 or 25 $\mu\text{g}/\text{mL}$ ExMH-PGR significantly increased OB-DIM-induced ALP activity. However, there was no significant difference in ALP activity between 10 and 25 $\mu\text{g}/\text{mL}$ ExMH-PGR treatment groups (Fig. 1).

Collagen synthesis was measured to investigate the effect of ExMH-PGR on osteoblast differentiation because collagen is another early-stage marker of osteoblast differentiation. Similar to ALP activity, collagen synthesis was greatly increased by OB-DIM. OB-DIM-induced collagen synthesis was not significantly affected by treatment with 1 $\mu\text{g}/\text{mL}$ ExMH-PGR. However, treatment of cells with 10 or 25 $\mu\text{g}/\text{mL}$ ExMH-PGR significantly increased OB-DIM-induced collagen synthesis (Fig. 2). These results indicate that ExMH-PGR can promote early stage osteoblast differentiation of MC3TC-E1 cells.

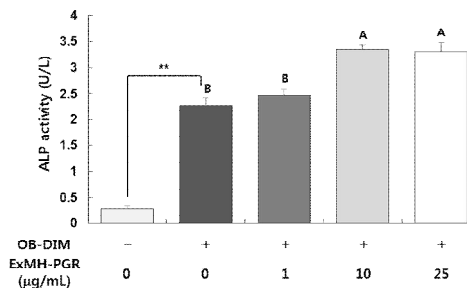


Fig. 1. Effect of ExMH-PGR on ALP activity in MC3T3-E1 cells.

MC3T3-E1 cells were plated at 2×10^4 cells/well and incubated for 24 h. After 24 h of incubation, cells were incubated for 5 days in OB-DIM containing various concentrations of ExMH-PGR. ALP activity was measured. Each bar represents the mean \pm SE. ** $P < 0.01$ significantly different from OB-DIM untreated group. Means without a common letter differ significantly at $P < 0.05$.

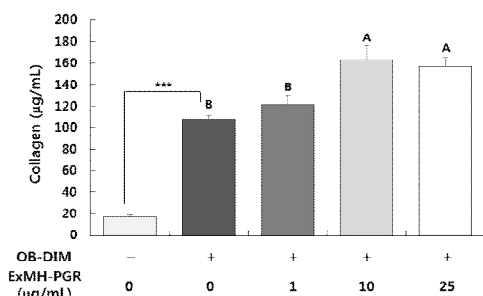


Fig. 2. Effect of ExMH-PGR on collagen synthesis in MC3T3-E1 cells.

MC3T3-E1 cells were plated at 2×10^4 cells/well and incubated for 24 h. After 24 h of incubation, cells were incubated for 8 days in OB-DIM containing various concentrations of ExMH-PGR. Collagen synthesis was measured. Each bar represents the mean \pm SE. *** $P < 0.001$ significantly different from OB-DIM untreated group. Means without a common letter differ significantly at $P < 0.05$.

3. ExMH-PGR encourages late osteoblast differentiation in MC3T3-E1 cells

To investigate whether ExMH-PGR affects late process of osteoblast differentiation, we measured the production of osteocalcin and the degree of mineralization as late-stage markers of osteoblast differentiation in MC3T3-E1 cells. As shown in Fig. 3, OB-DIM markedly stimulated the production of osteocalcin and ExMH-PGR significantly promoted OB-DIM-induced osteocalcin production in MC3T3-E1 cells.

Alizarin Red has been used in biochemical assay to quantitatively determine the presence of calcific deposition by cells of osteogenic lineage by colorimetry. The amount of calcified is proportional to the degree of Alizarin Red staining²⁰). Therefore, Alizarin red staining was performed in this study to determine the effect of ExMH-PGR on mineralization in MC3T3-E1 cells. As demonstrated in Fig. 4, the degree of mineralization in MC3T3-E1 cells was

increased with induction of differentiation by OB-DIM. OB-DIM induced mineralization was effectively promoted by treatment with 1, 10, and 25 $\mu\text{g/mL}$ ExMH-PGR. However, there was no significant difference in mineralization among ExMH-PGR treatment groups at different concentrations (1, 10, and 25 $\mu\text{g/mL}$).

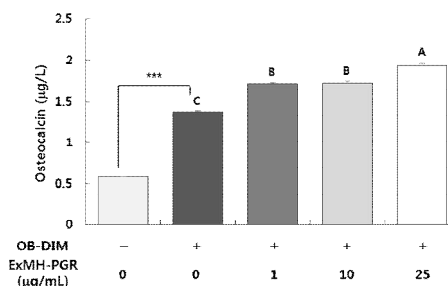


Fig. 3. Effect of ExMH-PGR on osteocalcin production in MC3T3-E1 cells.

MC3T3-E1 cells were plated at 2×10^4 cells/well and incubated for 24 h. After 24 h of incubation, cells were incubated for 14 days in OB-DIM containing various concentrations of ExMH-PGR. Media conditioned for 24 h were collected. Levels of osteoblast-secreted osteocalcin in 24 h-conditioned media were measured. Each bar represents the mean \pm SE. *** $P < 0.001$ significantly different from OB-DIM untreated group. Means without a common letter differ significantly at $P < 0.05$.

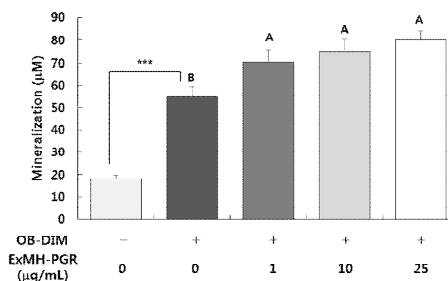


Fig. 4. Effect of ExMH-PGR on mineralization in MC3T3-E1 cells.

MC3T3-E1 cells were plated at 2×10^4 cells/well and incubated for 24 h. After 24 h of incubation, cells were incubated for 14 days in OB-DIM containing various concentrations of ExMH-PGR. The degree of mineralization was measured. Each bar represents the mean \pm SE. *** $P < 0.001$ significantly different from OB-DIM untreated group. Means without a common letter differ significantly at $P < 0.05$.

4. ExMH-PGR inhibits osteoclast differentiation in RAW 264.7 cells

To investigate the effect of ExMH-PGR on osteoclast differentiation, we incubated RAW 264.7 cells in OC-DIM to induce osteoclast differentiation and treated cells with various concentrations of ExMH-PGR. We then measured TRAP activity as osteoclast differentiation marker. OC-DIM markedly increased TRAP activity in RAW 264.7 cells. OC-DIM-induced TRAP activity was significantly decreased with increasing ExMH-PGR concentration (Fig. 5).

Next, we performed experiments to determine the role of ExMH-PGR in the formation of actin ring by immunofluorescence analyses. Actin ring structure on osteoclast surface is essential for bone resorption process of osteoclast, while osteoclast can absorb bone matrix through this structure. When osteoclast is differentiated, its size becomes large and the surface forms the actin ring, which is involved in the bone resorption process. Thus, the formation of actin rings in osteoclasts is an important indicator

of the ability of cells to absorb bone²¹⁾. In ExMH-PGR untreated control group, actin rings were the most vivid and cells were dense. These actin rings were markedly decreased with the concentration of ExMH-PGR used for treatment was increased (Fig. 6). This result shows that treatment with ExMH-PGR can inhibit the differentiation of osteoclast to a stage where bone resorption is possible.

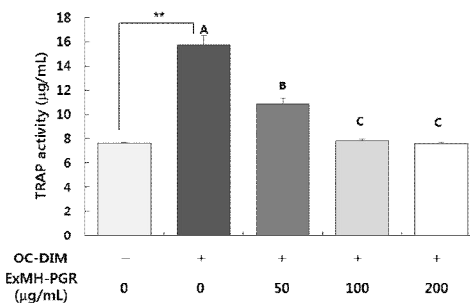


Fig. 5. Effect of ExMH-PGR on TRAP activity in RAW 264.7 cells.

RAW 264.7 cells were plated at 5×10^4 cells/well and incubated for 24 h. After 24 h of incubation, cells were incubated for 5 days in OC-DIM containing various concentrations of ExMH-PGR. TRAP activity was measured. Each bar represents the mean \pm SE. ** $P < 0.01$ significantly different from OC-DIM untreated group. Means without a common letter differ significantly at $P < 0.05$.

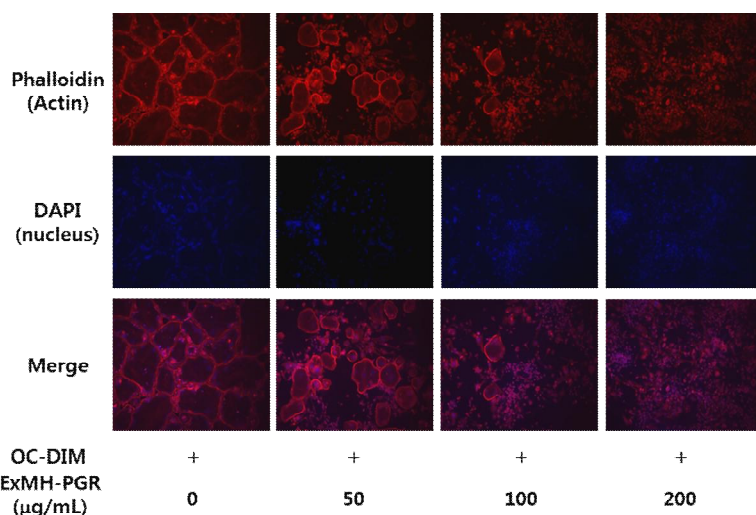


Fig. 6. Effect of ExMH-PGR on actin ring formation in RAW 264.7 cells. RAW 264.7 cells were seeded into 24-well plates containing cover glass at 1×10^4 cells/well and incubated for 24 h. After 24 h of incubation, cells were incubated for 5 days in OC-DIM containing various concentrations of ExMH-PGR. After 5 days, cells were fixed with 4% paraformaldehyde containing 0.1% Triton X-100 and stained with Alexa Fluor 594 Phalloidin and DAPI to stain actin and nuclei, respectively. Morphological changes of actin ring in cells were observed via microscopy (n=3; magnification, 200 \times).

IV. Discussion

Phytochemicals, or phytonutrients, are plant-based compounds that have a low possibility of side effects and a fairly high effectiveness in preventing and treating chronic diseases such as cardiovascular diseases, cancer, and osteoporosis²². PGR contains various phytochemicals such as polysaccharides, flavonoids, polyphenolic, and polyacetylene as well as saponin. Triterpenoid saponins isolated from PGR have been reported to be the major class of its bioactive compounds⁵. Most of the PGR saponin is Platycosin D, which is known to have a variety of physiological effects such as obesity, inflammation and cancer prevention. It was recently reported

that platycodin D can increase bone mineral density²³. Administration of PGR reduced ovariectomy-induced bone loss and alleviated the reduction of plasma levels of alkaline phosphatase, calcium, and phosphorus observed in ovariectomized mice. This nutritional approach to the treatment of osteoporosis could be seen as a feasible alternative to pharmaceutical ones¹⁸.

According to the review article of Ann et al.²⁴, there were *Hominis Placenta* (紫河車), *Phlomis Radix* (續斷), *Eucommiae Cortex* (杜冲), *Psoraleae Fructus* (補骨脂), *Allii Tuberosi Semen* (韭子), *Cervi Pantotrichum Cornu* (鹿茸), *Cervi Cornu* (鹿角), *Nokgyonggol* (鹿脛骨), *Morindae Radix* (巴戟天), *Trigonellae Semen* (葫蘆

巴), *Drynariae Rhizoma* (骨碎補), *Cynomorii Herba* (鎖陽), *Dendrobii Herba* (石斛), *Pelodiscis Carapax* (鱉甲), *Lycii Fructus* (枸杞子), *Ganodermae Polyporus* (靈芝), *Ginseng Radix* (人蔘), *Dioscoreae Rhizoma* (山藥), *Ginseng Radix Rubra* (紅蔘), *Rehmanniae Radix Preparata* (熟地黃), *Polygoni Multiflori Radix* (何首烏), *Carthami Fructus* (紅花子), *Sambuci Lignum* (接骨木), *Caraganae Radix* (骨擔草), *Corni Fructus* (山茱萸), *Mori Folium* (桑), *Loranthi Ramulus* (寄生), and *Platycodon grandiflorum* (桔梗) as medicinal herbs used in the trial of osteoporosis efficacy. The extract of PG have been reported to be effective in improving bone metabolism in ovariectomized rats²⁵). An example of the use of PGR as an oriental gynecologist has been reported to be effective for the apoptosis of uterine leiomyoma cells as a material of *Hyulbuchukeo-tang* (血府逐瘀湯)²⁶).

We prepared herbal extract from a mixture of ordinary medicinal herbs with various concentrations of PGR. We conducted preliminary experiments to determine the most effective concentrations of PGR that could activate osteoblasts and inactivate osteoclasts. Compositions of ExMH-PGR are shown in Table 1. Ingredients used with PGR for the preparation of ExMH-PGR are: *Angelica gigas* Nakai (root), *Atractylodes macrocephala* Koidz (rhizome), *Gardenia jasminoides* Ellis (fruit), *Glycyrrhiza uralensis* (root), *Menthe arvensis* var *piperascens* (leaf), *Paeonia japonica* (root), *Poria cocos* Wolf (sclerotium), and *Zingiberis siccatum*

Rhizoma. They have tonic, digestive, diuretic, antihydrotic, antiphlogistic, laxative, choleric, hemostatic, antiphlogistic, mucolytic, expectorant, analgesic, stomachic, antiemetic, antidiarrheal, antiasthmatic, hemostatic, and cardiotonic effects. They have traditionally been used in Korea and China to treat gynecological disorders such as menopausal disorders, dysmenorrhea, digestive and respiratory diseases, and herbal toxicity detoxification. In other words, these components of ExMH-PGR are not related to osteoporosis except for PGR^{27,28}). In this *in vitro* study, we investigated the efficacy of ExMH-PGR as a dietary supplement for prevention and treatment of osteoporosis.

In our present study, we analyzed ALP activity, collagen synthesis, mineralization, and osteocalcin concentration as biomarkers related to bone formation using MC3T3-E1 cells as an osteoblast cell line. ALP is present in almost all tissues, especially in the bone, kidney, intestinal mucosa, placenta, liver and bile duct. ALP level in bone tissue increases when active bone formation is occurring, because ALP is a byproduct of osteoblast activity²⁹). Collagen is an important protein distributed in each connective tissue. It is present in various parts of the body. Up to now, 28 kinds of collagen have been identified. However, over 90% of human collagen is type 1 collagen which is abundant in the skin, bone and ligaments. It is mainly synthesized by osteoblasts³⁰). The ability to form bone calcification is an important

biomarker for osteoblast differentiation³¹⁾. Osteocalcin is a protein secreted by osteoblasts. It is involved in bone mineralization and calcium ion homeostasis as well as bone formation. Since osteocalcin is produced by osteoblasts, it is often used as an index of the bone formation process. It has been reported that higher serum-osteocalcin levels are relatively strongly correlated with increases in bone mineral density while using anabolic bone formation drugs to treat osteoporosis. In many studies, osteocalcin is used as a primary biomarker to determine the effect of the drug to help bone formation³²⁾. In this study, we showed that PGR has the ability to increase bone formation in osteoblasts cells. ExMH-PGR treatment increased ALP activity, collagen synthesis, mineralization, and osteocalcin production. Numerous studies have reported that anti-osteoporotic agents can increase osteoblast differentiation and suppress osteoclast differentiation at the same time³³⁻⁵⁾. PGR-derived saponins appear to stimulate osteoblast differentiation by regulating RUNX2, the principle osteogenic master gene for bone formation¹⁷⁾.

According to cellular and molecular-level osteoporosis studies, the mechanism of bone loss is usually caused by osteoclast differentiation and bone resorption beyond osteoblast bone formation³⁶⁾. To investigate the effect of ExMH-PGR on the function of osteoclast, we analyzed TRAP activity and actin ring formation in RAW 264.7 cells. Osteoclasts are the cells involved

in bone matrix resorption and bone and calcium homeostasis. Since TRAP is a specific marker of osteoclast and its secretion increases during bone resorption, TRAP activity has been used to confirm osteoclast differentiation³⁷⁾. An actin ring is a unique actin structure essential for bone resorption by osteoclasts. Activation of osteoclasts begins with recognition of the matrix, adhesion to the bone surface, and rearrangement of the cytoskeleton. The osteoclast cytoskeleton can polarize the osteoclast to create a ruffled border with a separate environment of the resorptive microenvironment of the bone-cell interface and form a gasket-like structure. This is called actin ring or sealing zone. Actin rings separate the resorptive microenvironment from the normal extracellular space^{21,38)}. Osteoclasts synthesize and secrete lytic enzymes in the cytoplasm, which acidifies the lacunae underneath osteoclasts by exocytosis. Osteoclasts then bring in the degraded material through endocytosis. These exocytic and endocytic processes are essential sequences for effective bone resorption by osteoclasts^{21,39)}. In this study, RAW 264.7 cells treated with ExMH-PGR showed decreased TRAP activity and actin ring dose-dependently (Fig. 5, 6). The inhibitory effect of PGR and its major component, platycodin D, on osteoclast activity have also been reported in other studies. Lee *et al.*⁴⁰⁾ have reported that oral administration of platycodin D inhibits osteoclast formation and blocks bone destruction caused by

breast cancer. Choi *et al.*¹⁸⁾ have also reported that platycodin D inhibits ERK/p38 MAPK signaling pathway as well as NF- κ B signaling and thus inhibits osteoclast differentiation.

These results suggest that ExMH-PGR promotes osteoblast activity and inhibits osteoclast activity. However, in our study, PGR was not as a single medicinal product, but was a part of a combination. Thus, it cannot be guaranteed that these results were entirely caused by PGR. Further research on many components of PGR and their osteoporosis-related mechanisms needs conducting.

V. Conclusion

In this study, we conducted an *in vitro*

study to determine whether ExMH-PGR, an extract of mixed herbs containing root of *Platycodon grandiflorum*, was effective in preventing or treating osteoporosis. ExMH-PGR treatment significantly increased ALP activity, collagen synthesis, mineralization, and osteocalcin production in osteoblasts and inhibited TRAP activity and actin ring formation in osteoclast. These results indicate that ExMH-PGR can be regarded as an anti-osteoporotic candidate for treatment of osteoporosis disease. In the future, it will be necessary to confirm its effect through follow-up study in animal and human and clarify its action mechanism.

- Received : Sep 19, 2018
- Revised : Sep 24, 2018
- Accepted : Nov 26, 2018

국문초록

목적: 본 연구는 길경(*Platycodon grandiflorum* root)를 함유한 복합 한약재 추출물(ExMH-PGR)의 골다공증 예방 및 치료효과를 알아보기 위해 MC3T3-E1 조골세포주와 RAW 264.7 파골세포주를 이용하여 *in vitro* 수준에서 검증하였다.

방법: 배양액에 다양한 농도의 ExMH-PGR를 첨가하여 MC3T3-E1 세포와 RAW 264.7 세포의 세포증식을 비교하였다. MC3T3-E1 세포에서 Alkaline phosphatase (ALP) 활성, 콜라겐 합성, 오스테올칼신 생성, 무기질 축적을 분석하였다. RAW 264.7 세포에서 Tartrate-resistant acid phosphatase (TRAP) 활성과 actin ring 형성 정도를 분석하였다.

결과: ExMH-PGR는 25 µg/mL 농도까지 유의한 수준으로 ALP 활성, 콜라겐 합성, 그리고 오스테올칼신 형성을 증가시켰다. 50~200 µg/mL의 ExMH-PGR은 TRAP 활성과 actin ring 형성을 유의하게 억제했다.

결론: ExMH-PGR은 조골세포의 활성을 촉진하고 파골세포의 활성을 억제하는 효과가 있어 골다공증 치료에 유용할 가능성이 있다.

중심단어: 골다공증, 조골세포, 파골세포, 약초, 길경

References

1. van den Bergh JP, van Geel TA, Geusens PP. Osteoporosis, frailty and fracture: implications for case finding and therapy. *Nat Rev Rheumatol.* 2012;8(3):163-72.
2. He JB, Chen MH, Lin DK. New insights into the tonifying kidney-yin herbs and formulas for the treatment of osteoporosis. *Arch Osteoporosis.* 2017;12(1):14.
3. Rossouw JE, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's health initiative randomized controlled trial. *JAMA* 2002;288(3):321-33.
4. O'Ryan FS, Lo JC. Bisphosphonate-related osteonecrosis of the jaw in patients with oral bisphosphonate exposure: clinical course and outcomes. *J Oral and Maxillofac Surg* 2012;70(8):1844-53.
5. Zhang L, et al. *Platycodon grandiflorus* - an ethnopharmacological, phytochemical and pharmacological review. *J Ethnopharmacol.* 2015;164:147-61.
6. Ryu CS, et al. Evaluation of the total oxidant scavenging capacity of saponins isolated from *Platycodon grandiflorum*. *Food Chem.* 2012;132(1):333-7.
7. Ashok BT, Ali R. The aging paradox: free radical theory of aging. *Exp Gerontol.* 1999;34(3):293-303.
8. Halliwell B. Oxidative stress and neurodegeneration: where are we now? *J Neurochem.* 2006;97(6):1634-58.
9. Zhao HL, et al. Hypocholesterolemic

- and anti-obesity effects of saponins from *Platycodon grandiflorum* in hamsters fed atherogenic diets. *J Food Sci.* 2008; 73(8):H195-200.
10. Wu J, et al. Cholesterol metabolism regulation and antioxidant effect of platycodin D on hyperlipidemic emulsion-reduced rats. *Afr J Pharm Pharmacol.* 2011;5(22):2444-53.
 11. Choi YH, et al. Antiproliferative effects of saponins from the roots of *Platycodon grandiflorum* on cultured human tumor cells. *J Nat Prod.* 2010;73(11):1863-7.
 12. Zhang Z, et al. Platycodin D, a triterpenoid saponin from *Platycodon grandiflorum*, suppresses the growth and invasion of human oral squamous cell carcinoma cells via the NF- κ B pathway. *J Biochem Mol Toxicol.* 2017;31(9) e21934:1-7.
 13. Halliwell B. Oxidative stress and cancer: have we moved forward? *Biochem J.* 2007;401(1):1-11.
 14. Evans P, Halliwell B. Micronutrients: oxidant/antioxidant status. *Br J Nutr.* 2001;85(S2):S67-S74.
 15. Zhang L, et al. Anti-fungal and anti-bacterial activities of ethanol extracts of selected traditional Chinese medicinal herbs. *Asian Pac J Trop Med.* 2013; 6(9):673-81.
 16. Ahn KS, et al. Platycodin D-induced apoptosis through nuclear factor- κ B activation in immortalized keratinocytes. *Eur J Pharmacol.* 2006;537(1-3):1-11.
 17. Jeong HM, et al. Saponins from the roots of *Platycodon grandiflorum* stimulate osteoblast differentiation via p38 MAPK- and ERK-dependent RUNX2 activation. *Food Chem Toxicol.* 2010; 48(12):3362-8.
 18. Choi JH, et al. Platycodin D inhibits osteoclastogenesis by repressing the NFATc1 and MAPK signaling pathway. *J Cellular Biochem.* 2017;118(4):860-8.
 19. Kim EJ, et al. Trans-10,cis-12-conjugated linoleic acid inhibits Caco-2 colon cancer cell growth. *Am J Physiol Gastrointest Liver Physiol.* 2001;283(2):357-67.
 20. Puchtler H, Meloan SN, Terry MS. On the history and mechanism of Alizarin red S stains for calcium. *J Histochem Cytochem.* 1969;17(2):110-24.
 21. Soya NS, Alles N. Osteoclast function and bone-resorbing activity: An overview. *Biochem Biophys Res Commun.* 2016; 476(3):115-20.
 22. Scalbert A, et al. Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr.* 2005;45(4):287-306.
 23. Hwang YP, et al. Saponins, especially platycodin D, from *platycodon grandiflorum* modulate hepatic lipogenesis in high-fat diet-fed rats and high glucose-exposed HepG2 cells. *Toxicol Appl Pharmacol.* 2013;267(2):174-83.
 24. Ann JY, et al. A review of anti-osteoporosis effects of herbal extracts in Korean journals. *The Korean Journal of Oriental Medical Prescription.* 2008;16(2):47-69.
 25. Kim M. Effect of *Platycodon Grandiflorum* A. extract in bone metabolism in ovariectomized rats. *Korean J. Oriental*

- Physiology & Pathology. 2008;22(1):183-8.
26. Moon NY, Kim DC, Baek SH. Effect of Hyulbuchukeotang on inhibition of proliferation of uterine leiomyoma cells and cell apoptosis. *The Journal of Oriental Obstetrics and Gynecology*. 2006;19(2):186-98.
 27. Tang W, Eisenbrand G. Chinese drugs of plant origin. Heidelberg:Springer. 1992:113-25, 199-201, 539-43, 567-88, 703-10, 1011-5.
 28. Gou X, He G, Guo X. *Poria cocos* (Schw.) Wolf 茯苓 (Fuling, Indian Bread). *Dietary Chinese Herbs*. 2015;87:781-8.
 29. Sharma U, Pal D, Prasad R. Alkaline phosphatase: an overview. *Indian J Clin Biochem*. 2014;29(3):269-78.
 30. Daneault A. Hydrolyzed collagen contributes to osteoblast differentiation in vitro and subsequent bone health in vivo. *Osteoarthritis and Cartilage*. 2014;22:S131.
 31. Lian JB, Stein GS. Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. *Crit Rev Oral Biol Med*. 1992;3(3):269-305.
 32. Lee NK, et al. Endocrine regulation of energy metabolism by the skeleton. *Cell*. 2007;130(3):456-69.
 33. Lee HW, et al. Berberine promotes osteoblast differentiation by Runx2 activation with p38 MAPK. *J Bone Miner Res*. 2008;23(8):1227-37.
 34. Satue M, et al. Quercitrin and taxifolin stimulate osteoblast differentiation in MC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7 cells. *Biochem Pharmacol*. 2013;86(10):1476-86.
 35. Zhou L, et al. Berberine sulfate attenuates osteoclast differentiation through RANKL induced NF-kappaB and NFAT pathways. *Int J Mol Sci*. 2015;16(11):27087-96.
 36. Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000;289(5484):1504-8.
 37. Hayman AR. Tartrate-resistant acid phosphatase (TRAP) and the osteoclast/immune cell dichotomy. *Autoimmunity*. 2008;41(3):218-23.
 38. Teitelbaum SL. The osteoclast and its unique cytoskeleton. *Ann. N.Y. Acad. Sci*. 2011;1240(1):14-7.
 39. Schwayer C, et al. Actin rings of power. *Dev Cell*. 2016;37(6):493-506.
 40. Lee SK, et al. Platycodin D blocks breast cancer-induced bone destruction by inhibiting osteoclastogenesis and the growth of breast cancer cells. *Cell Physiol Biochem*. 2015;36(5):1809-20.