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ORIGINAL ARTICLE

Osteoblasts Proliferation and Differentiation Stimulating Activities of the Main Components of *Epimedii folium*

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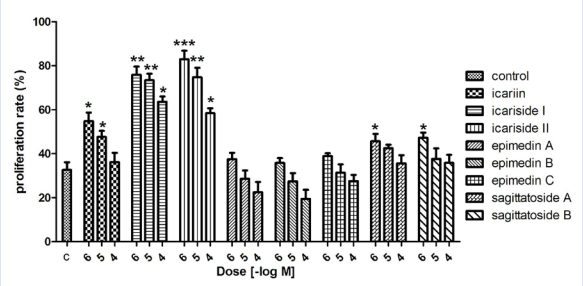
ABSTRACT

Background: Osteoporosis is a disease of bones that leads to an increased risk of fracture. *Epimedii Folium* is commonly used for treating bone fractures and joint diseases for thousands of years in China. **Methods:** This study was aimed to screen active components, which might have the potency to stimulate osteoblasts proliferation and differentiation in *Epimedii Folium*. An HPLC method was established to analyze the main components in *Epimedii Folium*. The MTT and ALP methods were utilized for the assay of osteoblasts proliferation and differentiation activity. Bavachin, a flavonoid compound was treated as the positive control. **Results:** Totally eight compounds have been identified by comparing their retention time with correspondent standard substances. Icariside I and icariside II significantly stimulated cell proliferation and osteoblasts differentiation. All these compounds were found with a characterized flavonoid structure in each of their molecule backbones. **Conclusion:** These results lead to a hypothesis that flavonoid monoglycoside structure might be crucial to exhibit the activity. The structure–effect relationship of these compounds with flavonoid monoglycoside structure in mouse primary calvarial osteoblasts needs to be explored in further research.

Key words: *Epimedii Folium*, flavonoid monoglycoside, osteoblasts, proliferation

SUMMARY

- Eight compounds were identified by comparing their retention time with correspondent standard substances.
- Icariside I and icariside II significantly stimulated cell proliferation and osteoblasts differentiation.
- Flavonoid monoglycoside structure might be crucial to exhibit the osteoblasts proliferation and differentiation activity.



Effects of the main components of *Epimedii Folium* on osteoblasts proliferation after treating 48 h.

Abbreviations used: HPLC: High performance liquid chromatography, MTT: Methylthiazolyldiphenyl - tetrazolium bromide, ALP: Alkaline phosphatase

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INTRODUCTION

Osteoporosis is characterized by low bone-mass density and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture.[1–3] Recent clinical research implied that the cause of osteoporosis is predominantly driven by the disorders of bone metabolism, of which the imbalance between bone formation and bone resorption played a major role. The new bone formation is primarily mediated by osteoblasts, while the bone resorption is mainly depended on the function of the osteoclasts. Therefore, during the process of normal bone remodeling, either increasing the osteoblast proliferation or inducing osteoblast differentiation can enhance bone formation.[4,5] Today, prophylacticotherapeutic measures for osteoporosis involving administration of calcium, vitamin D3, bisphosphonates, or calcitonin, etc., which mainly aim on osteoclast function but ignore the function of osteoblast. However, the effect of these measures are relatively minor, but with some serious side effects. For this reason, screening for anabolic agents to enhance the function of osteoblast is imperative.

Epimedii Folium obtained from aerial parts of Epimedium, which includes many species, such as *Epimedium brevicornu* Maxim, *Epimedium pubescens* Maxim, *Epimedium sagittatum* (Sieb. et Zucc), *Epimedium wushanense* T.S. Ying, and *Epimedium koreanum* Nakai. It is a famous herbal medicine that contains several medically active constituents, including flavonoids and phytosteroids, which are commonly used in China, Japan, and Korea. According to traditional Chinese medicine (TCM) theory, *Epimedii Folium* classified as a "kidney-tonifying" herbal medicine. It has been used extensively to treat osteoporosis, arthritis, and lumbago for thousands of years.[6] A direct relationship between kidney-tonifying functions and bone formation has been confirmed by accumulating evidence from studies demonstrating that *Epimedii Folium* has potential activity against osteoporosis.[7,8]

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The aim of the present study was to screen and identify compounds with activity to stimulate osteoblasts proliferation and differentiation from *Epimedii Folium*. An HPLC method was established to identify the main components. In this experiment, the effect of the components on osteoblasts was investigated by using primary osteoblastic cells that isolated from newborn Wistar rats. The MTT and ALP assay methods were utilized to measure the activity of osteoblasts proliferation and differentiation correspondently.

MATERIALS AND METHODS

Reagents and materials

The herb material of *Epimedii Folium* was purchased from Nanjing Haiyuan Prepared Slices of Chinese Crude Drugs Co. Ltd (Nanjing, China), and the authentication of samples was conducted by Fei Ding of Nanjing Haiyuan Prepared Slices of Chinese Crude Drugs Co. Ltd. Voucher specimens (No. NJHYCM-20141102) were deposited at the herbarium of Nanjing Haiyuan Prepared Slices of Chinese Crude Drugs Co. Ltd (Nanjing, China)

Extraction and identification

Dried *Epimedii Folium* were grind and the fine powder was collected by using a 60 mesh sieve. Take 2 g of the fine powder, and then reflux it with 100 mL of ethanol for 60 min. The extract solution was filtered into a round bottom flask for vacuum evaporation and concentration. The residual was dissolved with mobile phase and transferred into a 5 mL volumetric flask and diluted to the exact volume as sample solution. The HPLC equipment used is Shimadzu HPLC system including quaternary pump, a diode array detector (DAD) and a column oven (Tokyo, Japan). Analysis was achieved on a YMC-Pack C18 column (250 × 4.6 mm i.d., 5 μm particle size).The chromatographic conditions was recorded as following: injection volume was 10 μL; column temperature was maintained at 35°C; the detection wavelength was set at 254 nm; the mobile phase was composed of acetonitrile (A) and 0.1% formic acid (B) with gradient elution system (0–25 min, 5–15% A; 25–60 min, 15–35% A; 60-95 min, 35–55% A; 95–115 min, 55–70% A; 115–120 min, 70–5% A) at a flow rate of 1.0 mL/min.

Standard substances

Reference standards used in the experiments were isolated and purified in our own laboratory. The structure was characterized on the basis of NMR, MS, and UV spectral analysis by the authors. The purity of all of the standards was over 98%. Stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma, Steinheim, Germany) and stored at-20°C. Acetonitrile was HPLC grade (Tedia, USA).

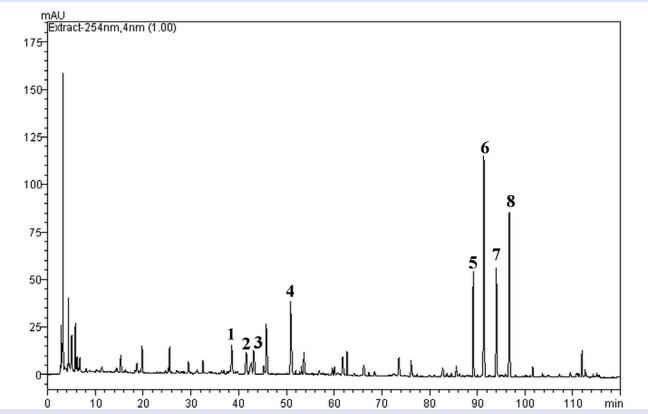

Figure 1: HPLC chromatogram for ethanol extract of Epimedii Folium.

Note: (1) Epimedin A, (2) epimedin B, (3) epimedin C, (4) icariin, (5) sagittatoside A, (6) sagittatoside B, (7) icariside I, (8) icariside II.

Cell culture

Rat calvarial osteoblasts were isolated from calvaria of new-born Wistar rats. After sacrifice of the rat, the calvarium (frontal and parietal bone) was taken immediately and all adhering soft tissues were removed. The cleared calvaria was subjected to 0.05% trypsin for digestion 30 min at 37°C and then washed by PBS for three times. Then it was cut into pieces and transferred to 0.1% collagenase II for twice digestion, 60 min each. The released cells were collected by centrifugation, and washed with phosphate-buffered saline. The suspension was cultured in α-MEM with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were seeded in a 100 mm culture dish at a density of 5000 cells/cm^3 and incubated at 37°C with 5% of CO_2. After osteoblast cells reaching 80% confluence, they were harvested with 0.25% trypsin-EDTA solution and seeded in different tissue culture plates for the following assays.

Cell proliferation assay

Cell proliferation was measured by the 3-[4, 5-dimethylthiazol]-2, 5-diphenylterazolium bromide assay (MTT assay). In this assay, osteoblasts were plated in 96-well plates at a density of 5×10^3 cells/well. After cultured for 24 h, the cells in various wells were treated with each compound at concentrations of 0, 1, 10, 100 μM. After 24 and 48 h incubation, 20 μL MTT (0.5 mg/mL) was added to each well and incubated at 37°C for another 4 h. Then, the medium of each well was removed, formazan salts were dissolved in freshly added 100 mL dimethylsulfoxide (DMSO), and the plate was read at 490 nm by a microplate reader (Bio-Rad, model 550). All tests were performed in triplication independently.

Proliferation rate (%) = (sample OD − blank OD)/(control OD − blank OD) × 100%

Measurement of alkaline phosphatase (ALP) activity

ALP is one of the early markers of osteoblast differentiation. High ALP secretion represents strong cell-differentiation activities. Primary osteoblasts were seeded at a density of 5 × 10^3 cells/well in 96-well plates with 10% FBS. After incubation for 24 h, the cells in various wells were treated with each compound at concentrations of 0, 1, 10, 100 μM. In this assay, ALP activity was measured by using an ALP assay kit. After 24, 48, and 72 h incubation, 20 μL of supernatant was transferred to a new 96-well plate. ALP activity of the supernatant was measured in accordance with the instruction of the kit. The plate was read at 405 nm by a microplate reader.

Statistical analysis

All data were expressed as means ± standard deviation (SD) and all tests were conducted in triplication independently. Statistical analysis were carried out with the SPSS 16.0 software. Statistical analysis was performed by one-way analysis of variance followed by the Student's t-test. *P* value less than 0.05 was considered statistically significant.

Results and discussion

Identification of the main components in Epimedii Folium

HPLC method was established to characterize the main components in *Epimedii Folium*. Eight main components have been found and identified by comparing the retention time with correspondent standard substance. HPLC chromatogram of *Epimedii Folium* was shown in Figure 1 and the structures of eight compounds are shown in Figure 2.

Observation of the osteoblast morphology

Osteoblasts were spherical, which suspended in the medium, and gradually settled to the bottom of the dish and then growth adherently. The cells in logarithmic growth phase grew with a population doubles after 24 h culture. Matured osteoblasts showed a fibroblastic morphology, with many projections after completely growth. The cells were generally triangular or polygonal monocytes, containing 1–3 nucleoli each. Overlapped cell growth causes accumulation of collagen and deposition of calcium, and finally leads to the formation of mineralized nodules. After 12 days of incubation, the cells were utilized to perform ALP staining assay in accordance with the instruction of the kit.

Effect of the main components on osteoblast proliferation

In present work, the proliferation activity of each compound was assessed by using MTT assay on rat calvarial osteoblasts. Bavachin was used as the positive control. The results for each compound were recorded as the proliferation rates. All compounds did not have significant effects on cell proliferation after 24 h incubation. The osteoblasts proliferation activities of the main components of *Epimedii Folium*, along with positive control bavachin were presented in Figure 3. Epimedin A, epimedin B and epimedin C showed similar cell proliferation activity compared to control. Icariside I and icariside II showed significant cell proliferation activity compared to control and in a dose-dependent manner after 48 h treatment [Figure 3].

Effect of the main components on osteoblast differentiation

The effects of each compound on osteoblasts differentiation were determined by ALP activity detection. The ALP activity reflects the level of differentiation in osteoblasts. Measuring ALP activity in osteoblasts culture medium is a simple, fast, and accurate assay method. Result manifested that most of the test compounds did not have significant effects on ALP activity of osteoblasts at the concentrations of 1–100 μM in primary osteoblasts after 24–48 h treated compared to control. Among them, icariside I and icariside II exhibited higher ALP activity than other compounds. They showed stronger osteoblasts differentiation activity comparing with the positive control bavachin. The osteoblasts proliferation activities of the main components and the positive control are shown in Figure 4.

Osteoporosis threatens human health seriously and causes huge cost annually. Currently, more and more researches have been demonstrated that compounds obtained from herbs providing a promising way for the prevention and treatment of osteoporosis. In traditional Chinese medicine, osteoporosis was considered a disorder caused by insufficiency of the Yang of kidney. *Epimedii Folium* is an herb that thought can warm kidney and activate Yang, thus frequently been prescribed to treat bone fractures and joint diseases by TCM practitioners. In previous studies, various activities of the extract as well as individual compounds, which were isolated from *Epimedii Folium* have been clarified.

To the best knowledge of the authors, the main components of *Epimedii Folium* were first utilized to investigate their osteoblasts stimulating activities in this research. HPLC method was developed to analyze the ethanol extract of *Epimedii Folium* and 8 chromatographic peaks were assigned. The osteoblast proliferation and differentiation activity were measured by MTT and ALP assay, respectively. The result exhibited that icariside I and icariside II had significant cell-proliferation stimulation activity at a dose dependent manner. Moreover, icariside I and icariside II showed more potent than bavachin in promoting osteoblast proliferation and differentiation. Both icariside I and icariside II are belonging to flavonoid monoglycosides. The difference is icariside I has a glycoside at C-7, while icariside II at C-3. Currently, a number of flavonoids with different number of glycosides isolated from natural plants have been proved to enhance osteoblasts function, such as apigenin and puerarin.[9–11] However, to the present, the structure–activity relationship of flavonoid glycosides on osteoblast proliferation has never been reported. In future study, the molecular mechanism of icariside I and icariside II on bone metabolism needs to be explored.

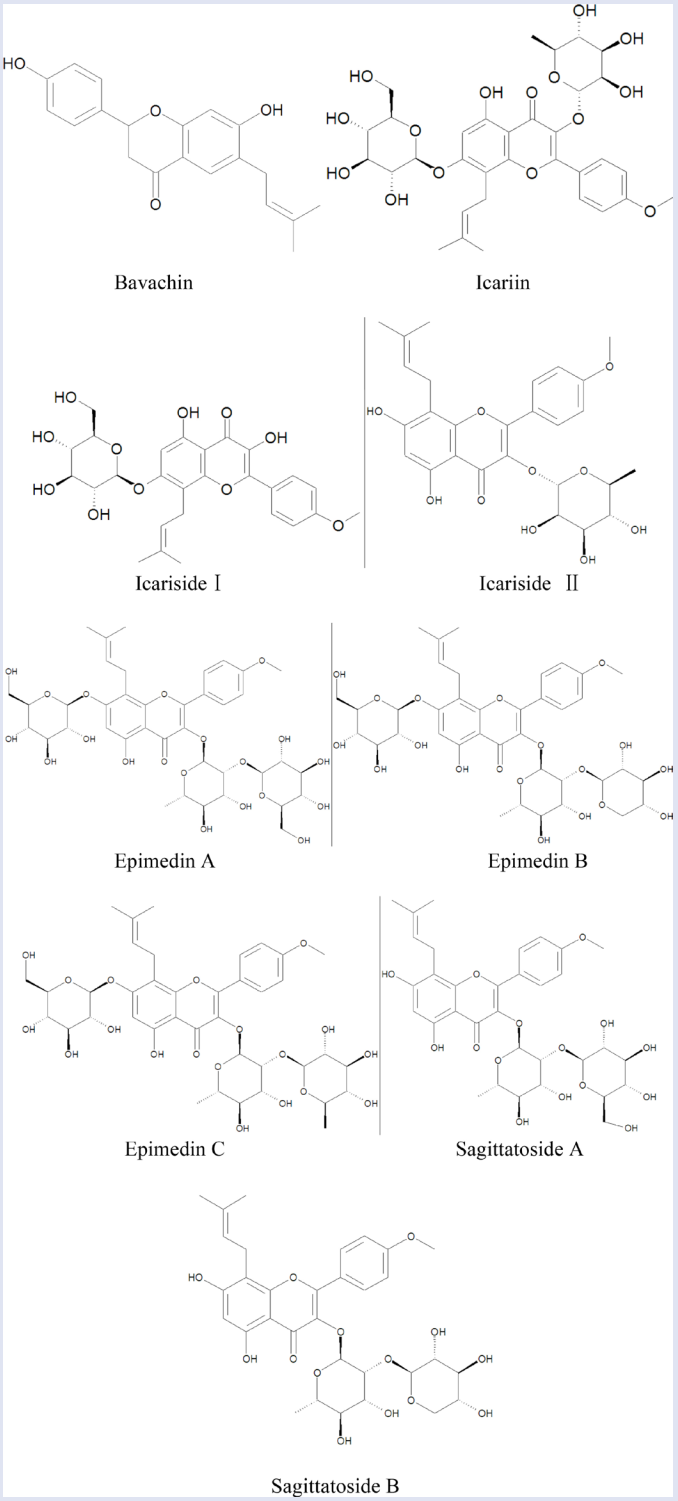

Figure 2: Chemical structures of the investigated compounds.

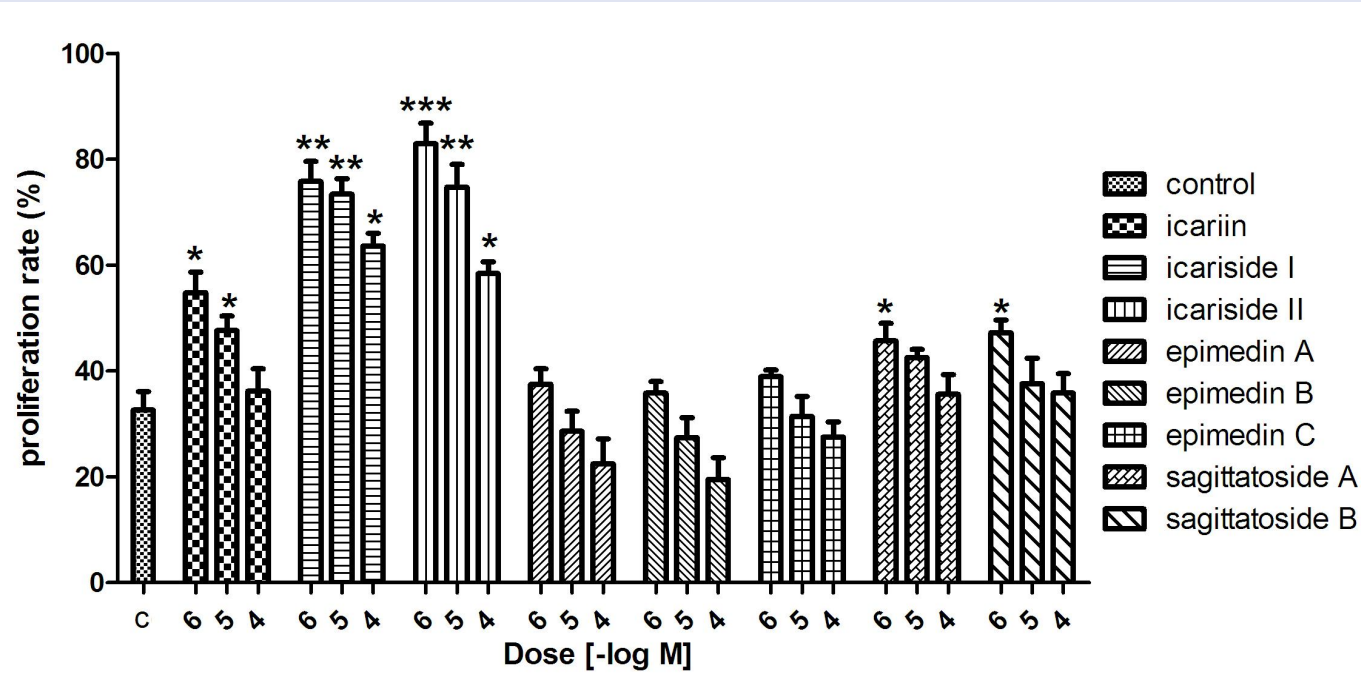

Figure 3: Effects of the main components of *Epimedii Folium* on osteoblasts proliferation after treating 48 h.

Note: Osteoblast proliferation activity was represented as the proliferation rate. Data are presented as means ± SD in three independent experiments. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control.

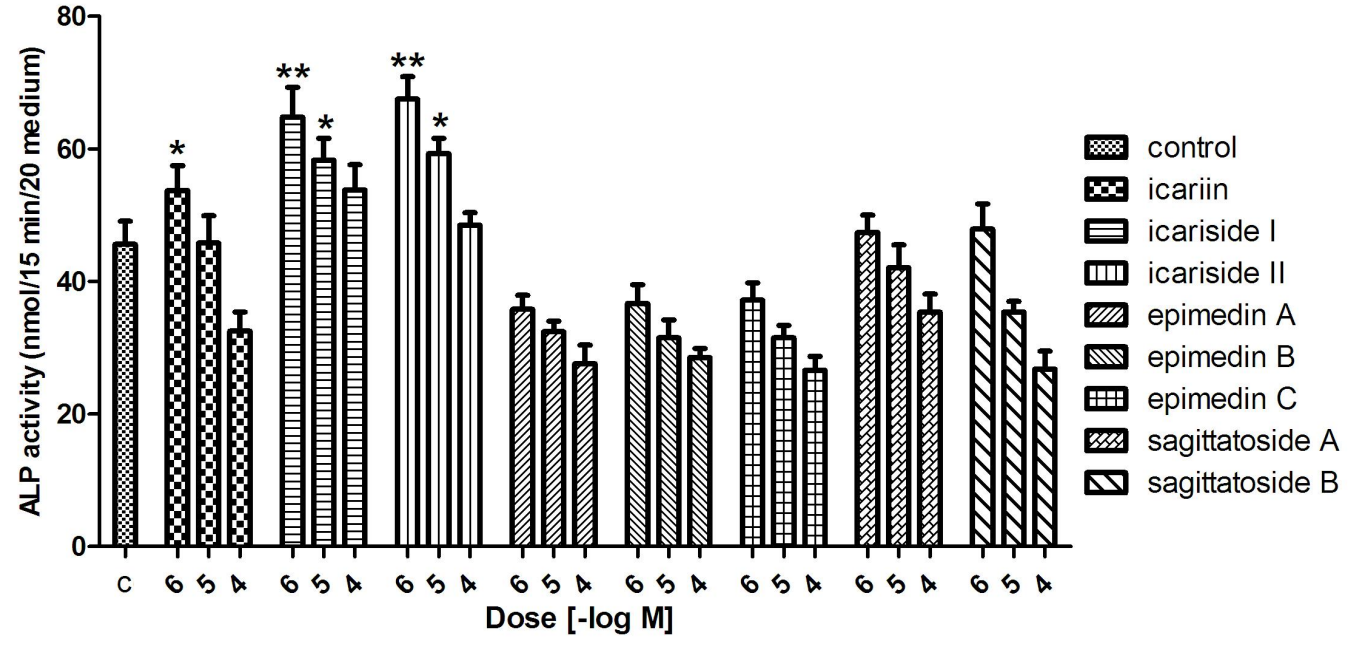

Figure 4: Effects of the main components of *Epimedii Folium* on osteoblasts ALP activity.

Note: Data are presented as means ± SD in three independent experiments. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control.

CONCLUSION

In prophase study, icariside I and icariside II exhibited significant effect to enhance osteoblasts differentiation than other components. So far, a number of flavonoid glycosides were identified in *Epimedii Folium*.[12] Furthermore, a variety of studies indicated that flavonoid monoglycoside enhances biological activities due to their interactions with target proteins.[13] All these results led to a hypothesis that a flavonoid monoglycoside may be crucial to target osteoblasts. It is worthwhile to explore the structure–activity relationship of the flavonoid monoglycoside on calvarial osteoblasts in future study.

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Nil

Conflicts of interest

There are no conflicts of interest

REFERENCES

1. Kim TH, Jung JW, Ha BG, Hong JM, Park EK, Kim HJ. The effects of luteolin on osteoclast differentiation, function *in vitro* and ovariectomy-induced bone loss. J Nutr Biochem 2011;22:8-15.
2. Li N, Jiang Y, Wooley PH, Xu Z, Yang SY. Naringin promotes osteoblast differentiation and effectively reverses ovariectomy-associated osteoporosis. J Orthop Sci 2013;18:478-85.
3. Sinningen K, Albus E, Thiele S, Grossklaus S, Kurth T, Udey MC. Loss of milk fat globule-epidermal growth factor 8 (MFG-E8) in mice leads to low bone mass and accelerates ovariectomy-associated bone loss by increasing osteoclastogenesis. Bone 2015;76:107-14.
4. Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science 2000;289:1501-4.
5. Lane NE, Kelman A. A review of anabolic therapies for osteoporosis. Arthritis Res Ther 2003;5:214-22.
6. An SJ, Li T, Li E. Effect of kidney-tonifying herbs on ovary function and bone mass in postmenopausal women. Journal of Osteoporosis 2000;6:55-9.
7. Ma HP, Jia ZP, Ge X, He XY, Chen KM, Bai MH. Studies on the therapeutic effect of total flavonoids of Herba Epimedii on experimental osteoporosis in rats. West China J Pharma Sci 2002;17:163-7.
8. Zhang DW, Cheng Y, Wang NL, Zhang JC, Yang MS, Yao XS. Effects of total flavonoids and flavonol glycosides from *Epimedium koreanum* Nakai on the proliferation and differentiation of primary osteoblasts. Phytomedicine 2008;15:55-61.
9. Li B, Liu H, Jia S. Puerarin enhances bone mass by promoting osteoblastogenesis and slightly lowering bone marrow adiposity in ovariectomized rats. Biol Pharm Bull 2014;37:1919-25.
10. Zhang X, Zhou C, Zha X, Xu Z, Li L, Liu Y. Apigenin promotes osteogenic differentiation of human mesenchymal stem cells through JNK and p38 MAPK pathways. Mol Cell Biochem 2015;407:41-50.
11. Ma HP, Ming LG, Ge BF, Icariin is more potent than genistein in promoting osteoblast differentiation and mineralization *in vitro*. J Cell Biochem 2011;112:916-23.
12. Chen XJ, Tang ZH, Li XW, Xie CX, Lu JJ, Wang YT. Chemical constituents, quality control, and bioactivity of *Epimedii Folium* (Yinyanghuo). Am J Chin Med 2015;43:783-34.
13. Simons R, Vincken JP, Bakx EJ, Verbruggen MA, Gruppen H. A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography electrospray ionisation mass spectrometry PM_437_15R2.pdf in licorice root extracts. Rapid Commun Mass Spectrom 2009;23:3083-93.