



Effects of total flavonoids and flavonol glycosides from *Epimedium koreanum* Nakai on the proliferation and differentiation of primary osteoblasts

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Abstract

In a bioassay-guided drug screening for anti-osteoporosis activity, eight flavonol glycosides were isolated from *Epimedium koreanum* Nakai, which is traditionally widely used in China for the treatment of impotence and osteoporosis. The effects of total flavonoids and flavonol glycosides on the proliferation and differentiation of rat calvarial osteoblast-like cells were evaluated by the MTT method and measuring the activity of alkaline phosphatase (ALP activity). Total flavonoids (1.2×10^{-2} to 6.0×10^{-7} mg/ml) and flavonol glycosides (2.0×10^{-5} to 1.0×10^{-9} mol/l) exhibited a strong inhibition on the proliferation of primary osteoblasts at most concentrations. However, the total flavonoids and icariin significantly promoted the differentiation of primary osteoblasts. The results suggested that flavonoids from *E. koreanum* Nakai may improve the development of osteoblasts by promoting the ALP activity; and icariin might be one of the active constituents facilitating the differentiation of osteoblasts.

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Keywords: *Epimedium koreanum* Nakai; Flavonol glycosides; Osteoblasts; Proliferation; Differentiation

Introduction

Osteoporosis, characterized by low bone mass and microarchitectural deterioration of bone tissue, is a major public health threat among the elderly. Bone is a

mineralized tissue that confers multiple mechanical and metabolic functions to the skeleton. Bone as a mineralized tissue contains two distinct cell types: osteoblasts and osteoclasts. Bone metabolic diseases develop when there is an imbalance between the formation and resorption of bone, which, in turn, depend on the interactions between osteoblasts and osteoclasts. The formation of bone involves a complex series of events, include the proliferation and differentiation of osteoprogenitor cells and result eventually in the formation of a mineralized extracellular matrix. Given the variety and

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importance of the biological processes in which these two cell types participate during development and in postnatal life, there is great interest in understanding their growth, differentiation, and function (Ducy et al., 2000). Estrogen deficiency occurring at menopause plays a major role in the development of osteoporosis in postmenopausal women (Dempster and Lindsay, 1993; Manolagas and Jilka, 1995). In order to prevent adverse effects of estrogen loss, estrogen replacement therapy has been proposed and the use of selective estrogen receptor modulators (SERM) has been developed (Pinkerton and Santen, 1999). Substantial evidence shows that a class of plant-derived substances, the so-called “phytoestrogens,” has estrogenic activities. These include the flavonoid family, comprising isoflavones and flavonols derivatives. Due to their ability to bind estrogen receptor (ER), these naturally occurring compounds could have positive effects against bone loss.

Herba Epimedii (Chinese Pharmacopoeia Commission, 2005), which contains several medically active constituents including flavonoids and phytosteroids, has been widely used in China in the treatment of cardiovascular diseases, infertility, impotence, amnesia, lumbago, arthritis, numbness and weakness of the limbs for thousands of years (Yeung, 1985). Accumulating evidences showed that Herba Epimedii have the potential activity against osteoporosis (An et al., 2000; Nelson et al., 2002; Wu et al., 2003) which has led to the wide use of Herba Epimedii in many Chinese formulas to treat osteoporosis. Recently, several studies have shown that the crude extract, total flavonoids, and main flavonoid constituents from Herba Epimedii (*Epimedium brevicornum* Maxim and *E. koreanum* Nakai) have osteoblastic proliferation-stimulating activity toward osteoblast-like UMR106 cells (Meng et al., 2005a, b; Xie et al., 2005). However, as most of these studies were focused on the crude extract, total flavonoids and main constituents (icariin), the constituents active against anti-osteoporosis are still unknown. To date, no systematic studies have been carried out to evaluate the effects of flavonol glycosides on the proliferation and differentiation of primary osteoblasts. In the present study, we aimed to systematically investigate the effects of total flavonoids and eight flavonol glycosides on the proliferation and differentiation of primary osteoblasts and to find the active constituents in modulating osteoblastic activity.

Materials and methods

Materials

E. koreanum Nakai herb was collected in June 2003–July 2003 in a valley in Xinbin, Liaoning Province and authenticated by Q.-S. Sun, Professor of Pharmacognosy, Shenyang Pharmaceutical University, China. A

voucher specimen (No. 19980816-1) has been deposited in the Herbarium of the Shenzhen Research Center of Traditional Chinese Medicine and Natural Products.

Newborn NIH mice were obtained from the Guangzhou University of Traditional Medicine. Trypsin and fetal bovine serum were purchased from Gibco. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), benzylpenicillin, Dulbecco's Modified Eagle's Medium (DMEM, without phenol red), streptomycin, collagen II were from Sigma. An alkaline phosphatase (ALP) activity kit was obtained from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China), and a micro-protein assay kit was purchased from Beyotime Biotechnology (Haimen, China). Preparative high performance liquid chromatography was obtained from Shimadzu (Japan). Silica gel (Qingdao Haiyang Chemical Co., Ltd, China), Sephadex LH-20 (Amersham Biosciences, USA), and ODS (Fuji Silysia, Japan) were used in column chromatography. All other chemicals were of analytical grade.

Extraction and isolation

The dried and powdered aerial parts of *E. koreanum* Nakai (55000 g) were refluxed for 2 h with water (550 000 ml, 2 times). Then the extract was concentrated and subjected to macroporous adsorptive resins eluted using 0%, 30%, 50%, 95% alcohol (V/V) which represented a yield of 5.5%, 1.7%, 1.2%, 0.2%, respectively. The total flavonoids were concentrated in the 50% fraction identified by coupling the HCl–Mg reaction and UV spectra (data not shown). Followed by animal studies, 50% alcoholic fraction showed a strong effect on bone mineral density (BMD) in total and cortical bones (Xie et al., 2005). Thus, a part (130 g) of 50% alcoholic fraction was subjected to column chromatography on silica gel. The column was subjected to gradient elution with CHCl₃:MeOH by a stepwise manner (99:1→1:1) and collected into 16 fractions. Eight flavonol glycosides were isolated from fraction 4, 5, 7, 8 and 10 by the combination of Sephadex LH-20 column chromatography, ODS reversed-phase column chromatography, and preparative high-pressure liquid chromatography.

Eight compounds were icariin (I, 1.5 g), baohuoside-I (II, 44.2 mg), sagittatoside B (III, 31.4 mg), baohuoside-II (IV, 7.9 mg), astragalin (V, 7.0 mg), 3,5,7-trihydroxy-4'-methoxy-8-prenylflavone-3-O- α -L-rhamnopyranosyl-(1→2)- α -L-rhamnopyranoside (VI, 34.8 mg), epimedin C (VII, 23.7 mg) and epimedin B (VIII, 85.8 mg) (Fig. 1). The purity of these compounds was more than 99.0% authenticated by RP-HPLC with DAD, and their structures were identified by comparison of their physical properties and IR and NMR spectral data with literature values (Mizuno et al., 1988; Sun et al., 1998).

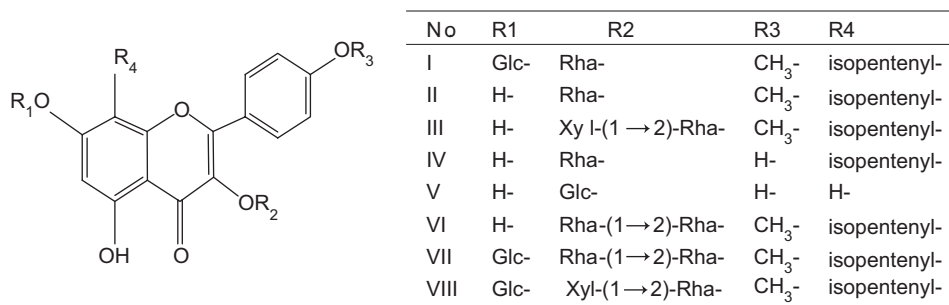


Fig. 1. Chemical structures of eight flavonoids from *E. koreanum* Nakai.

Preparation of test samples

Total flavonoids and flavonol glycosides were dissolved in dimethylsulfoxide (DMSO) at a concentration of 6 mg/ml and 100 mmol/l, respectively, and diluted in culture medium to the working solution before use. Assuming that the average molecular weight of flavonol glycosides was 600, the concentration of total flavonoids, for example, 1.2×10^{-2} and 6.0×10^{-3} mg/ml, were equal to 2.0×10^{-5} and 1.0×10^{-5} mol/l. NaF was dissolved in PBS to yield concentrations of 10 mmol/l and diluted in culture medium as positive control. To avoid DMSO toxicity, the concentration of the solvent was less than 0.1% (V/V). For effects of steroids on growth or differentiation, culture media were charcoal stripped and without phenol red.

Isolation of rat primary osteoblasts

Primary osteoblasts were prepared from 3-day-old NIH mice calvarias following the sequential enzymatic digestion method described previously with a little modification (Chen and Fry, 1999). Briefly, skull (frontal and parietal bones) were dissected; then the endosteum and periosteum were stripped off, and the bone was cut into approximately 1–2 mm² pieces and digested sequentially using trypsin (2.5 g/l) for 30 min and collagenase II (1.0 g/l) twice for 1 h each time. The cells were collected and cultured in Dulbecco's Modified Eagle Medium (DMEM without phenol red) supplemented with 10% heat-inactivated and charcoal-stripped fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, for 24 h in a humidified atmosphere of 5% CO₂ in air at 37 °C, then media were changed.

Generating a standard curve between cell number and absorbance

The relationship between cell number and absorbance was examined to validate the cell proliferation assay. Counted with a hemocytometer, cells were diluted using

DMEM without phenol red into eight concentrations before seeding into 96-well culture plates. They were allowed to attach for 8 h at 37 °C and 5% CO₂, and MTT was added and cultured for another 4 h. At the end of this experiment, absorbance at 570 nm was determined.

Proliferation assay

Rat primary osteoblasts were treated with various concentrations of flavonoids for 48 h, and osteoblast viability was determined by testing mitochondrial enzyme function according to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method as described previously (Carmichael et al., 1987). Briefly, 20,000 rat primary osteoblasts were subcultured into a 96-well tissue culture plate, and incubated 24 h prior to addition of tested compounds; then, cultured for another 48 h. Cells were treated with MTT (20 µl, 5 mg/ml) 4 h prior the end of the experiment. At the end of this experiment, the supernatant was removed and DMSO (100 µl) was added to dissolve formazan, and absorbance was measured on a microplate spectrophotometer (Bio-rad Model 680, USA) at 570 nm with a reference at 655 nm.

Alkaline phosphatase activity assay

Rat primary osteoblasts (20,000 cells per well) were seeded in 48-well culture plates and cultured overnight at 37 °C in a 5% CO₂ humidified incubator. Total flavonoids and flavonol glycosides were added to culture medium at final concentrations as shown in Table 1, and cultured for a further 2 days. The plates were washed twice with ice-cold D-Hank's and lysed by two freezing and thaw cycles. Aliquots of supernatants were subjected to alkaline phosphatase activity and protein content measurement using an alkaline phosphatase activity kit and a micro-Bradford assay kit; all results were normalized by total protein content.

Statistical analysis

Data were expressed as the mean \pm standard deviation (SD). Statistical significances were analyzed using the ANOVA test. A value of $p < 0.05$ was considered significant. Linear regression analysis was performed by the correlation coefficient. Growth inhibition ratios (GIR) were calculated according to the following equation: $GIR\% = (A_{\text{sample}} - A_{\text{blank}}) / A_{\text{blank}} \times 100$, where A is the average absorbance of four experiments with 6 replicates.

Results

Standard curve between cell number and absorbance

The result indicated a strong linear correlation in the range of 2.4×10^3 – 3.8×10^4 cell/ml between absorbance (Y) and cell number (X): $Y = 0.081 + 5 \times 10^{-5}X$ ($r^2 = 0.9919$).

Proliferation assay

As shown in Table 1, NaF stimulated the proliferation of rat primary osteoblasts about 18%, and the inhibitory effect was stable and persistent through 1.0×10^{-5} – 1.0×10^{-7} mol/l. On the contrary, all of the tested samples inhibited the proliferation of rat primary osteoblasts at all concentrations (1.2×10^{-2} mg/ml or 2.0×10^{-5} mol/l to 6.0×10^{-7} mg/ml or 1.0×10^{-9} mol/l). The maximum inhibitory ratio (%) of total flavonoids (TF), I, II, III, IV, V, VI, VII, VIII was 35.8%, 10.6%, 19.8%, 26.5%, 22.5%, 9.5%, 30.7%, 16.7%, 15.6% at concentrations of 6.0×10^{-6} mg/ml, 1.0×10^{-7} (and 1.0×10^{-9}) mol/l, 2.0×10^{-5} , 2.0×10^{-5} , 1.0×10^{-9} , 1.0×10^{-8} , 1.0×10^{-8} , 1.0×10^{-6} , 1.0×10^{-5} mol/l, respectively.

Alkaline phosphatase activity assay

To study the effects of flavonoids on the differentiation of rat primary osteoblasts, icariin was selected as a representative constituent and compared with total flavonoids. Although icariin and total flavonoids at all concentrations inhibited the proliferation of rat primary osteoblasts, they significantly increased the alkaline phosphatase activity of osteoblasts. In a similar dosage range, icariin and total flavonoids exhibited a facilitative effect similar to the alkaline phosphatase activity, with a large fluctuation and not dose-dependently (Fig. 2).

Discussion

Bone, a highly dynamic tissue, undergoes continual processes of remodeling. Bone remodeling is a very complex process of tightly coordinated action by the bone resorbing osteoclasts and the bone-forming osteoblasts. Derived from a mesenchymal cell lineage, osteoblasts are responsible for forming new bone matrix in their differentiated state (Wlodarski, 1990). It has been postulated that bone loss associated with aging is caused by a defect in the osteoblast cell lineage (Katzburg et al., 1999; Rodriguez et al., 1999). Either the mesenchymal precursor population is insufficient or has lost the capacity to proliferate and/or differentiate into sufficient numbers of functioning osteoblasts. Osteoblast differentiation is a complex process orchestrated by the timely activation of specific transcription factors that regulate the expression of certain genes and thus define the osteoblast phenotype. These are agents that stimulate bone formation, including fluoride and anabolic steroids (Harada and Rodan, 2003). Among these, estrogen replacement therapy (ERT) used to be a popular regime for prevention and treatment of postmenopausal osteoporosis. As recent evidence suggests that ERT is associated with increased risk of breast, ovarian and endometrial cancer (Davison and Davis, 2003; Nelson et al., 2002), selective estrogen receptor modulators are being developed and phytoestrogens have become more and more popular (Pinkerton and Santen, 1999).

Several traditional Chinese herbs have been reported to have therapeutic effects on osteoporosis and bone fracture in animal studies (Chen et al., 2004; Hidaka et al., 1997; Huang and You, 1997; Xie et al., 2005). Recently, several studies show that total flavonoids and icariin from *Herba Epimedii* stimulated the proliferation and differentiation of primary osteoblasts (Han et al., 2003; Li et al., 2002; Wang et al., 2002) and osteoblast-like UMR106 cells (Meng et al., 2005a,b; Xie et al., 2005). However, there are studies showing different results. For example, the water extract of a traditional Chinese herb (*Drunaria fortunei*) inhibited the proliferation and differentiation of rat osteoblasts (Liu et al., 2001). Furthermore, the flavonoid extract from *Epimedium sagittatum* has been found to be effective in preventing osteoporosis induced by ovariectomy in rats, although no appreciable effect was observed when primary osteoblasts were exposed to the flavonoid extract in vitro (Chen et al., 2004). Quercetin, one of the major flavonoids in certain plants, inhibited the proliferation, differentiation, and mineralization of osteoblasts in vitro (Notoya et al., 2004). In our experiment, total flavonoids and eight flavonoids all inhibited the proliferation of primary osteoblasts. Our results were contrary to those from several studies based on the UMR 106 cell line, which is a clonal derivative of

Table 1. Effects of total flavonoids and flavonol glycosides on osteoblast proliferation

Samples	Concentration	$A_{\text{blank}} (\bar{x} \pm \text{SD})$	$A_{595}/A_{655 \text{ nm}} (\bar{x} \pm \text{SD})$	Inhibition ratio (%)
NaF (mol/l)	1.0×10^{-5}	0.300 ± 0.011	0.350 ± 0.019	16.5 ^{**,#}
	1.0×10^{-6}		0.358 ± 0.015	19.0 ^{**,#}
	1.0×10^{-7}		0.355 ± 0.017	18.4 ^{**,#}
TF (mg/ml)	1.2×10^{-2}	0.285 ± 0.009	0.208 ± 0.018	27.0 ^{***}
	6.0×10^{-3}		0.214 ± 0.021	24.9 ^{**}
	6.0×10^{-4}		0.206 ± 0.018	27.7 ^{***}
	6.0×10^{-5}		0.186 ± 0.012	34.7 ^{***}
	6.0×10^{-6}		0.183 ± 0.009	35.8 ^{***}
	6.0×10^{-7}		0.191 ± 0.018	33.0 ^{***}
I (mol/l)	2.0×10^{-5}	0.254 ± 0.009	0.232 ± 0.002	8.7 ^{**}
	1.0×10^{-5}		0.239 ± 0.007	5.9 [*]
	1.0×10^{-6}		0.245 ± 0.012	3.5
	1.0×10^{-7}		0.227 ± 0.020	10.6 ^{**}
	1.0×10^{-8}		0.241 ± 0.007	5.1 [*]
	1.0×10^{-9}		0.227 ± 0.009	10.6 ^{**}
II (mol/l)	2.0×10^{-5}	0.257 ± 0.014	0.206 ± 0.012	19.8 ^{***}
	1.0×10^{-5}		0.222 ± 0.012	13.6 ^{**}
	1.0×10^{-6}		0.240 ± 0.011	6.6 [*]
	1.0×10^{-7}		0.245 ± 0.014	4.7
	1.0×10^{-8}		0.243 ± 0.007	5.4 ^{**}
	1.0×10^{-9}		0.245 ± 0.007	4.7 ^{**}
III (mol/l)	2.0×10^{-5}	0.257 ± 0.014	0.189 ± 0.014	26.5 ^{***}
	1.0×10^{-5}		0.209 ± 0.012	18.7 ^{***}
	1.0×10^{-6}		0.229 ± 0.016	10.9 ^{**}
	1.0×10^{-7}		0.238 ± 0.020	7.4
	1.0×10^{-8}		0.231 ± 0.020	10.1 [*]
	1.0×10^{-9}		0.250 ± 0.009	2.7
IV (mol/l)	2.0×10^{-5}	0.262 ± 0.019	0.216 ± 0.018	17.6 ^{**}
	1.0×10^{-5}		0.225 ± 0.021	14.1 ^{**}
	1.0×10^{-6}		0.237 ± 0.021	9.5 [*]
	1.0×10^{-7}		0.211 ± 0.019	19.5 ^{***}
	1.0×10^{-8}		0.212 ± 0.020	19.1 ^{***}
	1.0×10^{-9}		0.203 ± 0.013	22.5 ^{***}
V (mol/l)	2.0×10^{-5}	0.262 ± 0.019	0.260 ± 0.010	0.76
	1.0×10^{-5}		0.266 ± 0.016	1.5 [#]
	1.0×10^{-6}		0.256 ± 0.014	4.2
	1.0×10^{-7}		0.248 ± 0.022	5.3
	1.0×10^{-8}		0.237 ± 0.015	9.5 [*]
	1.0×10^{-9}		0.239 ± 0.005	8.8 [*]
VI (mol/l)	2.0×10^{-5}	0.254 ± 0.014	0.205 ± 0.017	19.3 ^{***}
	1.0×10^{-5}		0.190 ± 0.010	25.2 ^{***}
	1.0×10^{-6}		0.189 ± 0.011	25.6 ^{***}
	1.0×10^{-7}		0.178 ± 0.018	29.9 ^{***}
	1.0×10^{-8}		0.176 ± 0.012	30.7 ^{***}
	1.0×10^{-9}		0.177 ± 0.019	30.3 ^{***}
VII (mol/l)	2.0×10^{-5}	0.251 ± 0.017	0.242 ± 0.022	3.6
	1.0×10^{-5}		0.226 ± 0.021	10.0 ^{**}
	1.0×10^{-6}		0.209 ± 0.008	16.7 ^{***}
	1.0×10^{-7}		0.211 ± 0.017	15.9 ^{***}
	1.0×10^{-8}		0.217 ± 0.021	13.5 ^{***}
	1.0×10^{-9}		0.222 ± 0.011	11.6 ^{***}

Table 1. (continued)

Samples	Concentration	$A_{\text{blank}} (\bar{x} \pm \text{SD})$	$A_{595}/A_{655 \text{ nm}} (\bar{x} \pm \text{SD})$	Inhibition ratio (%)
VIII (mol/l)	2.0×10^{-5}	0.263 ± 0.009	0.225 ± 0.010	14.4 ^{***}
	1.0×10^{-5}		0.222 ± 0.008	15.6 ^{***}
	1.0×10^{-6}		0.227 ± 0.007	13.7 ^{***}
	1.0×10^{-7}		0.230 ± 0.010	12.5 ^{***}
	1.0×10^{-8}		0.236 ± 0.013	10.3 ^{**}
	1.0×10^{-9}		0.253 ± 0.019	3.8

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, significant as compared to blank; # Proliferation ratio (%).

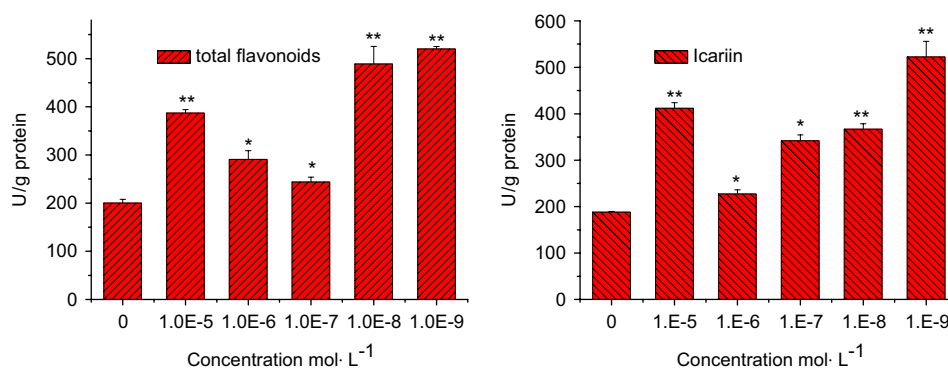


Fig. 2. Effects of icariin and total flavonoids on the alkaline phosphatase activity. $n = 6$, * $p < 0.05$; ** $p < 0.01$; significant as compared to blank.

a transplantable rat osteosarcoma often used as a developed osteoblast model (Meng et al., 2005a, b; Xie et al., 2005). This could be the cause of different cell type (cell line vs. primary osteoblasts) and culture medium (effects of steroids on growth). Meanwhile, the crude extract and flavonoids have been found effective in preventing osteoporosis in animal studies (Chen et al., 2004; Huang and You, 1997; Xie et al., 2005); but no appreciable effect was observed when osteoblasts were exposed to flavonoids in vitro. However, serum isolated from rats administered flavonoid extract from *E. sagittatum* orally was able to significantly stimulate the proliferation as well as the differentiation of ROB cells, which indicated that flavonoids may enhance the development of osteoblasts through their active metabolites (Chen et al., 2004). It is very interesting that the total flavonoids and icariin, a typical and main constituent of *E. koreanum* Nakai, inhibited the proliferation, and meanwhile, promoted the differentiation of osteoblasts. This result indicates that a full-scale evaluation of the effects of agents to osteoblasts should be undertaken, consisting of proliferation, differentiation and mineralization assays. Osteoblast differentiation is a crucial aspect of bone formation and remodeling, a process that is severely compromised in osteoporosis. As the relationship between osteoblastic proliferation and differentiation was obscure, the mechanism involved in the whole process must be studied further.

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