REVIEW

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A systematic review of the efficacy and pharmacological profile of *Herba Epimedii* in osteoporosis therapy

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Received December 4, 2012, accepted February 8, 2013

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Pharmazie 68: 713–722 (2013) *doi: 10.1691/ph.2013.2900*

The purpose of this systematic review is to assess the efficacy and pharmacological profiles of *Herba Epimedii* in osteoporosis therapy. Four databases were extensively retrieved that include two Chinese electronic databases (VIP Information and CNKI) and two English electronic databases (CA and MEDLINE). *Herba Epimedii* has been an important traditional herbal medicine for centuries in China and other Asian countries. Recently, quite a few pharmacological effects of *Herba Epimedii*, its extracts and active components have been identified that include improving bone health and cardiovascular function, regulating hormone level, modulating immunological function, and inhibiting tumor growth. The anti-osteoporosis activity of *Herba Epimedii* and its extracts have attracted world-wide attention. The literature search has revealed that a lot of studies have recently been carried out related to the bone-strengthening activity of *Herba Epimedii* and some of its active compounds, such as total flavonoids and icariin. Pharmacokinetic and toxicity studies have confirmed the efficacy and safety of *Herba Epimedii* and its most abundant active component icariin, while only a few authors have reviewed the anti-osteoporosis properties of the plants. So we summarize the work of various investigators on the effects of *Herba Epimedii*, its extracts and active components against osteoporosis. The underlying mechanism of osteoprotective action, derivatives of icariin, animal models and cell lines used in the research were also reviewed in this paper.

1. Introduction

Osteoporosis is a worldwide bone disease defined as deterioration in bone mass and micro-architecture, with increasing risk of fragility fractures (Raisz and Rodan 2003). As osteoporosis is results from the imbalance of bone formation and bone resorption, researchers have developed drugs to maintain this balance. Among them, there are bisphosphonates, calcitonin, and selective estrogen receptor modulators, which can only prevent the further loss of bone but not stimulate new bone formation. The only FDA approved compound capable of stimulating new bone formation is parathyroid hormone (PTH), however high doses of PTH were shown to develop osteosarcomas (Khosla et al. 2008). Another method to prevent osteoporosis is hormone replacement therapy (HRT) which can prevent postmenopausal osteoporosis due caused by low hormone levels. However, the side effects of HRT is a big concern, which could increase the risk of invasive breast cancer, heart disease, stroke and pulmonary embolism (Vahle et al. 2002). So more people turned to the search for alternative or natural therapy for osteoporosis (Rossouw et al. 2002).

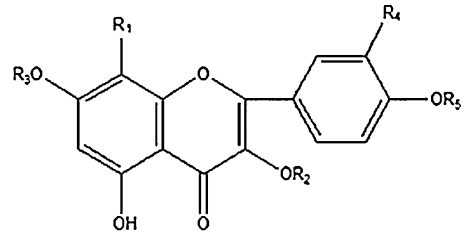
Traditional Chinese Medicine (TCM) may be an option, which includes a series of traditional medical practices originating in China, and now remain as a form of primary care throughout the most Asian countries while it is a complementary or alternative medical system in most western countries. In TCM, the understanding of the human body is based on the holistic understanding of the universe and the treatment of illness is based primarily on the diagnosis and differentiation of syndromes. In TCM theories, osteoporosis also named as bone atrophy is related to the function of kidney and liver. In short, the kidney controls the bone and stores the essence of life, which nourishes the bone marrow to support the bone. So we believe that osteoporosis is due to the deficiency of "Kidney-Yang" which cannot warm up the body, the deficiency of essence and Qi cannot nourish the bone marrow and support the bone (Sun and Ma 2010).

Abbreviations: PTH, parathyroid hormone; HRT, hormone replacement therapy; TCM, Traditional Chinese Medicine; CHM, Chinese Herbal Medicine; OVX, ovariectomized; CORT, Corticosterone; BMSCs, bone marrow mesenchymal stem cells; XLGBC, Xianling Gubao capsule; EXD, Er-Xian Decoction; XZD, Xian-Zhen Decoction; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IL-1β, interleukin-1β; IL-6, interleukin-6; ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein-2; OPG, osteoprotegrin; RANKL, receptor activator of NF-kB ligand; HEF, total flavonoids of *Herba Epimedii*; Runx2, Runt-related transcription factor-2; BSP, bone sailoprotein; PPARγ2, peroxisome proliferator-activated receptor gamma-2; DKK1, Dickkopf-1; OPN, oesteopontin; BMD, bone mineral density; Cbfα1, Core binding factor alpha1; CFU-F, colony unit forming-fibroblasts; IGF-1, insulin-like growth factor-1; TRAP, tartrate-resistant acid phosphatase; KO, knockout; WT, wild type; LPS, lipopolysaccharide; IκBα, I-kappa-B-alpha.

Table 1: Chinese traditional formulas contains *Herba Epimedii* for treating bone disorders

Formula	*Ingredients*	*Target*	*Main outcome*	*Authors*
Xianling Gubao capsule	*Epimedium brevicornum* Maxim., *Radix Dipsaci*, *Fructus Psoraleae*, *Radix Rehmanniae*, *Radix Salviae* Miltiorrhizae., *Anemarrhena asphodeloides,* et al.	OVX rats; Menopausal women	enhance the bone mass and BMD, improve the biomechanical property and the level of estrogen in the blood serum of OVX rats; increased the serum levels of IGF-1 and osteocalcin and BMD after taked by the menopausal women.	Xing et al., 2012; Wang et al., 2010; Zhang et al., 2004.
Er-Xian Decoction	*Epimedium brevicornum* Maxim., *Curculigo orchioides* Gaertn., *Anemarrhena asphodeloides* Bge., *Phellodendron chinense* Schneid., *Morinda officinalis* How., *Angelica sinensis* (Oliv.) Diels et al.	osteoblastic UMR-106 cells; osteoclastic RAW 264.7 cells; OVX rats.	enhanced osteoblastic UMR-106 cell proliferation, ALP activity and bone nodules formation; decreased TRAP activity and the bone resorption action of osteoclasts induced from RAW 264.7 cells; increase the calcium, phosphorus, and estradiol in serum and biomechanical strength in OVX rats.	Zhu et al., 2010; Nian et al., 2006
Xian-Zhen Decoction	*Epimedium brevicornum* Maxim., *Radix Astragali* Mongolici., *Fructus Ligustri* Lucidui., *Radix Rehmanniae* preparata., et al.	OVX rats; Postmenopausal women.	promote the bone mechanics; slow down the bone loss; inhibit the osteoblast apoptosis.	Zhang et al., 2008; Zhou et al., 2006; Zhou et al., 2004.
Migu Tablet	*Epimedium brevicornum* Maxim., *Cortex Eucommiae*, *Fructus Psoraleae*, Walnut and *Pyritum* et al.	primary osteoblasts; Postmenopausal women; aged males.	stimulate the secretion and synthesis of TGF-β1 in primary osteoblasts; increase the ALP, OC in the serum and BMD in patients with postmenopausal osteoporosis; prevent bone loss in aged males.	Xia et al., 2006; Dai et al., 2007; Xie et al., 2003.
Bushen Jiangu Decoction	*Cortex Eucommiae*, *Radix Polygoni* Multiflori., *Epimedium brevicornum* Maxim., *Radix Salviae* Miltiorrhizae., *Radix Rehmanniae* preparata., et al.	OVX rats; primary osteoblasts.	decreased the content of TNF-α; stimulate the proliferation of osteoblasts, enhance the synthesis of ALP and osteocalcin.	Li et al., 2005; Xing et al., 2001.
Bushen Tongluo Decoction	*Epimedium brevicornum* Maxim., *Radix Dipsaci, Fructus Psoraleae*, *Radix Rehmanniae* preparata., *Rhizoma Chanxiong, Poria, Scolopendra, et al.*	Human with primary osteoporosis; OVX rats.	Improve the functional indicators, bone metabolic indicators and bone density; increase the serum calcium and bone density, while decrease the serum phosphorus and alkaline phosphatase of OVX rats.	Shi et al., 2006; Min et al., 2010.
Gushu Dan	*Epimedium brevicornum* Maxim., *Rhizoma Drynariae, Radix Salviae* Miltiorrhizae., *Fructus Cnidii,* et al.	Osteoporosis rats after administrated prednisolone.	increase bone density, bone biomechanics, blood BGP content, bone calcium, and bone phosphorus.	Zhang et al., 2008.
Bushen Ningxin Decoction	*Radix Rehmanniae*, *Fructus Psoraleae*, *Epimedium brevicornum* Maxim., *Radix Morindae* Officinalis., *Semen Ziziphi* Spinosae., *Fructus Lycii*, et al.	Postmenopausal osteoporosis Mice.	Stimulate the expression of IFN-γ and IL-4, increase the BMD, area of bone trabecula and OPG mRNA expression in postmenopausal osteoporosis mice.	Wang et al., 2005.

Another important organ related to osteoporosis is the liver, which governs the tendons in TCM theories. The tendons, relying on the liver for blood supply, attached to the bones, are nourished by the kidney-essence and are the tissue basis of exercise.The liver, storing blood, is the root of physical stamina; while kidney, storing essence, is the official manager of hard work. The essence and blood sharing with the same origin, provide energy for exercise (Lu et al. 2009). Chinese Herbal Medicine (CHM) is an important component of the typical TCM therapies (Zhou and Qu, 2009) and CHM for osteoporosis treatment is mainly related to nourishing kidney and livers, strengthening the tendons and bones and improving the exercise capacity. Among these CHM, there are *Herba Epimedii, Rhizoma Drynariae,or Psoralea corylifolia*. *Herba Epimedii* is most frequently used in classic formulas for treatment of osteopenia and bone fractures, so we here review the research on progress of *Herba Epimedii* into the treatment of osteoporosis. An online search of published articles related to *Herba Epimedii* and its active components in the treatment of osteoporosis was conducted and abstracts or full articles in English or Chinese



Compounds	R_1	R_2	R_3	R_4	R_5	M.W
Hexandraside E	$-CH_2CH=C(CH_3)_2$	-glc	-glc	-H	-H	678
Hexandraside F	$-CH_2CH=C(CH_3)_2$	-rha(3-1)glc	-glc	-H	$-CH_3$	838
Epimedin A	$-CH_2CH=C(CH_3)_2$	-rha(2-1)glc	-glc	-H	$-CH_3$	838
Epimedin B	$-CH_2CH=C(CH_3)_2$	-rha(2-1)xyl	-glc	-H	$-CH_3$	808
Epimedin C	$-CH_2CH=C(CH_3)_2$	-rha(2-1)rha	-glc	-H	$-CH_3$	822
Epimedoside C	$-CH_2CH=C(CH_3)_2$	-H	-glc	-H	-H	516
Baohuoside I	$-CH_2CH=C(CH_3)_2$	-rha	-H	-H	$-CH_3$	514
Baohuoside II	$-CH_2CH=C(CH_3)_2$	-rha	-H	-H	-H	500
Baohuoside VII	$-CH_2CH=C(CH_3)_2$	-rha(4-1)glc	-H	-H	$-CH_3$	676
Sagittatoside A	$-CH_2CH=C(CH_3)_2$	-rha(2-1)glc	-H	-H	$-CH_3$	676
Sagittatoside B	$-CH_2CH=C(CH_3)_2$	-rha(2-1)xyl	-H	-H	$-CH_3$	646
Icariin	$-CH_2CH=C(CH_3)_2$	-rha	-glc	-H	$-CH_3$	676
Caohuoside C	$-CH_2CH=C(CH_3)_2$	-rha	-H	-OH	$-CH_3$	530
2"-*O*-Rhamnosyl icariside II	$-CH_2CH=C(CH_3)_2$	-rha(2-1)rha	-H	-H	$-CH_3$	660
Kaempferol-3-*O*-rhamnoside	-H	-rha	-H	-H	-H	432

Glc=β-D-glucose Rha=α-L-rhamnose Xyl=β-D-xylose M.W.=Molecular Weight

Fig. 1: Chemical structures of 15 flavnoids in different *Epimedium* species.

were included for the preparation of this review. The following electronic resources were used: MEDLINE (1980 to 2012); CA (1980 to 2012); Chinese National Knowledge Infrastructure (CNKI) (until Nov 2012) and VIP Database (until Nov 2012).

2. Botany, history and ethno-pharmacology of *Herba Epimedii*

Epimedium is the largest genus of herbaceous Berberidaceae. As a temperate, old world genus, *Epimedium L.* (Berberidaceae) disperses from Japan to Algeria and mainly occurs in eastern Asia and the Mediterranean lands (Zhang et al. 2008). The corolla characteristics such as petal type, the form and relative size of the inner sepals and petals, and flower dimension are important characters used in the classification of *Epimedium* species. So based on the diameter of flowers, *Epimedium* can be divided into two taxas, small-flowered taxa (less than 1 cm) and large-flowered taxa (more than 1 cm). It includes more than 57 species, but about 47 species specifically exist in China only except *E. koreanurn Nakai* which also exists in Japan and North Korea (Zhang et al. 2008; Xu and He, 2005; He et al. 2005; Sun et al. 2005). Most of these species are distributed mainly in central, southwest and northeast of China, include Shaanxi, Hubei, Hunan, Gansu, Sichuan, Guizhou, and the three provinces in northeast China (Li et al. 2005; Zhang et al. 2009; Ying, 2002). Although the genius *Epimedium* has so many species, only eight species have been used for centuries in traditional Chinese herbal formulations to treat a wide range of diseases (Wu et al. 2003). There are five *Epimedium* species classified under the same yinyanghuo herb name, namely *E. brevicornu* Maxim, *E. koreanum* Nakai, *E. sagittatim* (Sieb & Zucc.) Maxim, *E. pubescens* Maxim and *E. wushanense* T.S.Ying according to the Chinese Pharmacopoeia (Chinese-Pharmacopoeia-Commission 2005; Sze et al. 2010; Guo and Xiao 2003).

Herba Epimedii has a long history as a medicinal plant to treat a wide range of complaints. As far as we know, the earliest record was in *Shen Nong Materia Medica* which has been written in the Eastern Han Dynasty. In this famous medical classic, it was stated that the root and leaves can all be used for treatment, but the details are not very clear. *Annotation of Materia Medica* is one of the pharmacopoeias published by the government of the Tang Dyansty, but the record is also very simple. The characteristics of *Herba Epimedii* were exactly stated in the *Compendium of Materia Medica* that has been completed by Li Shi-Zhen in the Ming Dyansty. It was stated that *Herba Epimedii* can tonify pneuma, strengthen bones and muscles, reinforce liver and kidney, enhance psychic energy. PRC codex also records that *Herba Epimedii* has all these effects and can be used for therapy of some related diseases. Modern pharmacological research displayed that *Herba Epimedii* and its extracts have many kinds of bioactivities that include stimulation of the osteoblastogenesis and suppression of the activity of osteoclasts (Zhang et al. 2012; Hsieh et al. 2010). It inhibits the invasion and migration of cancer cells (Wang et al. 2010), improves the sexual function (Chen and Chiu 2006), enhances the memory (Urano and Tohda 2010), increases the activity of phytoestrogens (Kang et al. 2012), promotes the immunological ability and has anti-inflammatory propesties (Liu et al. 2010; Wu et al. 2012). So *Herba Epimedii* is always used for the treatment of osteoporosis, tumors, erectile dysfunction, Alzheimer disease and menopausal syndrome in clinical practice.

3. Animal models and cell lines used in anti-osteoporosis studies of *Herba Epimedii*

There are many animal models and cell lines in studies to demonstrate the osteoprotective effects of *Herba Epimedii*. The main cause of postmenopausal osteoporosis is estrogen deficiency,

so aged ovariectomized (OVX) rats are a successful animal model to mimic postmenopausal osteoporosis. Corticosterone (CORT) and other glucocorticoids, such as dexamethasone have also been used in animal models to imitate the secondary osteoporosis.

BMSCs (bone marrow mesenchymal stem cells) are pluripotent progenitor cells that give rise to osteoblasts, adipocytes, chondrocytes and myocytes (Prockop 1997; Pittenger et al. 1999). There is a reciprocal relation between the differentiation of adipocytes and osteoblasts. Clinical studies found an increase of differentiation of BMSCs into adipocytes instead of osteoblasts in a variety of osteoporosis models (Stenderup et al. 2001). It has been reported that the osteogenic potential of BMSCs of postmenopausal women is substantially lower than that of healthy control women, and that gene expression during apidogenic differentiation of BMSCs is increased, thus leading to the increase of adipose tissue and decrease of the trabecular bone (Verma et al. 2002; Sekiya et al. 2004). Therefore, the enhancement of osteogenesis with a concomitant decrease in adipogenesis may provide a therapeutic target in the treatment of osteoporosis by increasing bone formation through diverting the adipogenesis in BMSCs to osteogenesis (Fu et al. 2008). So whether or not the drug can modulate the differentiation of BMSCs from an adipogenic lineage to an osteogenic lineage becomes an important question in molecular pharmacology research.

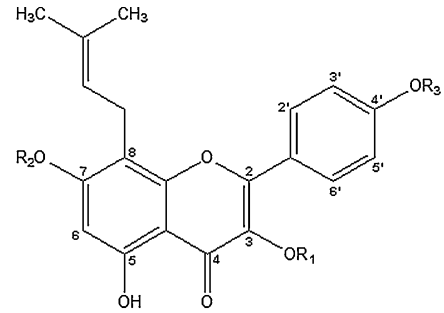
As we know that the main reason of osteoporosis is the imbalance of osteoblast and osteoclast activity, so osteoblast-like cells (such as MC3T3-E1, UMR-106) and osteoclast-like cells (such as RAW264.7) were usually used in the basic research of osteoporosis. Studies on the bone formation activity of osteoblasts and bone resorption activity of osteoclasts are also the main methods to evaluate the efficacy of *Herba Epimedii*.

4. *Herba Epimedii* used in the osteoporosis treatment formula in TCM

Although many studies have proved that *Herba Epimedii* can tonify the kidney and strengthen the bones, it is always accompanied with other CHM for bone diseases treatment and have left many classic formulas in history. Now these formulas are still used in China and some Asian countries. Xianling Gubao capsule (XLGBC) is one of the successful formulas that can prevent osteoporosis and has been available for many years. The main component of this product is *Herba Epimedii* and its quality control is based on detecting the contents of total flavonoids and icariin by HPLC (Wang and Guo 2000). Er-Xian Decoction (EXD), a popular Chinese medicinal formula include *Herba Epimedii*, has been used for the treatment of osteoporosis, menopausal syndrome and age-associated diseases in the past 50 years (Chen et al. 2008). Xian-Zhen Decoction (XZD) is among other, another popular Chinese traditional medicinal formula that contains *Herba Epimedii* for bone disorders (Table 1).

5. Osteoprotective effects of *Herba Epimedii* aqueous extracts

Decocting method is often used in TCM therapy, the main aim is to dissolve the active substances. So many experts studied the osteoprotective effects of *Herba Epimedii* aqueous extracts. Although the decocting method was adopted, the final concentrations were not quite consistent. Different final doses, such as 0.1, 0.2, 0.75, 1 g/ml (1 g/ml means 1 milliliter is equal to 1 gram medicinal material), were all applied research and got similar results. Ye et al. studied the changes on the spongy bone by OVX in rats after administration of *Herba Epimedii* aqueous extracts (dose: 1 g/ml, 1 ml per 100 g body weight), and



	R_1	R_2	R_3
Icariin	Rhamnose	Glucose	CH_3
Icaritin	H	H	CH_3
Icariside I	H	Glucose	CH_3
Icariside II	Rhamnose	H	CH_3

Fig. 2: Chemical structures of icariin and its metabolites.

found that the bone trabecular thickness decreased obviously at the 4th week and the percentage of bone trabecular area also decreased apparently at the 12th week after the operation, however the numbers of multi-nuclei/osteoclast like cells increased obviously in the tendency group, while these markers in the experimental group (took the extracts by oral administration) were similar to control group (the ovaries were not removed), suggesting that *Herba Epimedii* aqueous extracts can prevent the occurrence of OVX-induced osteoporosis by inhibiting the generation of the matrix in spongy bone of OVX rats (Ye et al. 2004; Ye et al. 2008). At the same time, they also found that *Herba Epimedii* aqueous extracts decreased the expression of tumor necrosis factor-α (TNF-α) in monocytes and stimulated the expression of TGF-β (transforming growth factor-β) in bone microenvironment, leading to a prevention of bone loss due to the lack of estrogen (Ye et al. 2006). Another groups found that *Herba Epimedii* aqueous extracts at the same concentration can improve the content of calcium and phosphorus, enhance the activity of alkaline phosphatase (ALP) and inhibit the contents of IL-1β (interleukin-1β), IL-6(interleukin-6), TNF-α in degenerated cervical vertebra, thus inhibiting the formation of osteophyte and delay cervical bone degeneration (Zhao and Xu, 2008; Zhao et al. 2008).

Herba Epimedii aqueous extracts not only can prevent osteoporosis, but can also help to cure bone fractures. Animal models of rabbits with 3 mm fracture in half radius were established and found that *Herba Epimedii* aqueous extracts could stimulate callus formation, remodel and the generation of collagen fiber, cartilage tissue, bone trabecula and bone matrix. Even the bone densities and biomechanical properties were improved compared with blank group, the effects were similar to the use of bone morphogenetic protein-2 (BMP-2) (Wang et al. 2011).

Glucocorticoid treatment may lead to loosened femoral heads, elevated blood lipids, thinning and sparse trabecular bone, and increased medulla cavity fatty cells, which may induce osteoporosis, or even femoral head necrosis. Some researchers found that *Herba Epimedii* extracts (dose: 0.2 g/ml or 0.4 g/ml, 3 ml per day) not only can improve blood flow changes during femoral head necrosis, improve metabolic disorder, promote the accumulation of fat metabolism, but also can promote the osteoblast proliferation and differentiation into mature bone cells, inhibit osteoclast activity, promote femoral head bone regeneration and prevent steroid-induced necrosis of the femoral head (Li and Meng 2010). The underling mechanism may be related to the effect of *Epimedium* on antagonizing glucocorticid-induced abnormal expression of osteoprotegrin (OPG) and receptor acti-

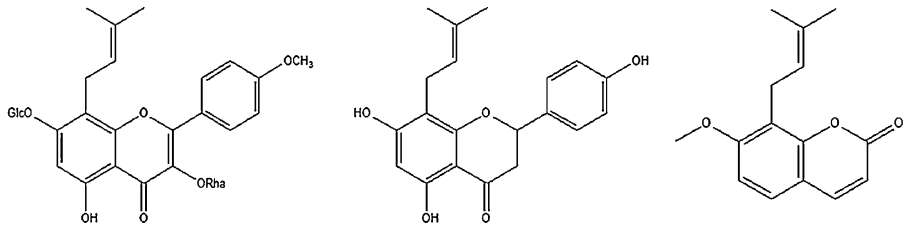


Fig. 3: Structures of icariin, 8-prenylnaringenin and osthole.

vator of NF-kB ligand (RANKL) mRNA expression *in vivo* (Wang et al. 2011). Similar results were also obtained in rat osteoblast-like UMR 106 cells. The extracts can significantly stimulate cell proliferation in a dose-dependent manner and increase ALP activity at dose of 200 mg/ml. It also modulated osteoclastogenesis by increasing OPG mRNA and decreasing RANKL mRNA expression, resulting in a dose-dependent increase in OPG/RANKL mRNA ratio (Xie et al. 2005). So the *Herba Epimedii* aqueous extracts can effectively suppress the OVX-induced increase in bone turnover possibly through increasing osteoblastic activities and decreasing osteoclastogenesis. The serum pharmacology is one of the most important methods of study on bone metabolism. The serum of the aged rats treated with *Herba Epimedii* aqueous extracts can stimulate the proliferation and differentiation of neonatal rat osteoblasts in vitro (Ma et al. 2002). The extracts also played a role on suppressing the adipogenic differentiation of BMSCs (Liu et al. 2011).

6. Osteoprotective effects of the total flavonoids of *Herba Epimedii*

Although *Herba Epimedii* aqueous extracts can prevent bone loss, the active ingredients are still not clear. Flavonoids are one of the most important type of compounds in Chinese herbs, and it is reported that the total flavonoids of *Herba Epimedii* (HEF) have a wide range of pharmacological and biological activities, such as improving bone health and cardiovascular function, regulating hormone level, modulating immunological function, and inhibiting tumor growth (Zhang et al. 2009; Shen et al. 2007; Huang et al. 2006; She et al. 2003). In fact, most of the 130 compounds identified in different species of *Epimedium*, most of them are the flavonoids (Wu et al. 2003; Xie and Sun 2006). Chen et al. analyzed and compared the contents of 15 flavonoids in 37 samples from 17 species of *Epimedium*, which included hexandraside E, hexandraside F, epimedin A, epimedin B, epimedin C, epimedoside C, baohuoside I, baohuoside II, baohuoside VII, sagittatoside A, sagittatoside B, icariin, caohuoside C, 2''-*O*-rhamnosyl icariside II and kaempferol-3-*O*-rhamnoside (Fig. 1). Determination of these flavonoids is most commonly used in quality control of *Epimedium* preparations (Chen et al. 2008). Because the *Herba Epimedii* flavonoids (HEF) are so complex, HEF are adopted in many experiments, such as anti-osteoporosis, the estrogen like activity and anti-tumor actions, no matter what models (cell culture or animal models) are used. After HEF treatment, the differentiation of rat BMSCs was significantly modulated, the mRNA level of Runt-related transcription factor-2 (Runx2) and bone sailoprotein (BSP) were significantly higher, while peroxisome proliferator-activated receptor gamma-2 (PPARγ2) was significantly lower in BMSCs from HEF-treated rats versus soluble vehicle-treated OVX rats. HEF also could significantly increase the ALP activity and Runx2 mRNA expression while decreased adipocyte number and PPARγ2 mRNA expression in human BMSCs culture (Peng et al. 2009). Xu et al. (2011) confirmed that HEF down-regulated the expression of Dickkopf-1 (DKK1) protein in an osteogenic induction medium and inhibited up-regulation of DKK1 protein in an adipogenic induction medium, balanced the osteogenic and adipogenic differentiation by regulating the expression of DKK1 protein. Besides, HEF could also shorten the total time needed for osteogenic differentiation of BMSCs, up-regulate the mRNA expression of several marker genes and osteogenic regulators, such as BMP-2, osteocalcin, oesteopontin (OPN) and Runx2, and inhibit osteoclastogenesis of BMSCs by enhancing the ratio of OPG/RANKL (Zhang et al. 2009).

HEF have shown to promote the osteogenic differentiation of human BMSCs in above studies. The molecular mechanism may be related to BMP and Wnt/β-catenin signaling pathways, because the mRNA expression of BMP-2, BMP-4, Runx2, β-catenin and cyclinD1 were enhanced apparently, which are BMP or Wnt-signaling pathway-related regulators; while on the other side the expression of noggin (the classical inhibitor of BMP) and DKK-1 (the classic inhibitor of Wnt/β-catenin signaling pathway) were notably inhibited by HEF (Zhang et al. 2010).

OVX-induced osteoporosis was a successful animal model that could mimic bone loss in postmenopausal women, so this animal model was adopted by many researchers to study the anti-osteoporosis effects of HEF. HEF suppressed OVX-induced increase in urinary Ca^{2+} excretion as well as the loss of bone mass and strength at the distal femur in mice in a dose-dependent manner. HEF treatment could stimulate the expression of type I collagen and osteocalcin mRNA and the ratio of OPG/RANKL mRNA, and suppress the increase of IL-6 mRNA induced by OVX in the femur of mice. The deterioration of trabecular bone micro architecture induced by OVX in mice was also prevented by HEF (50 to 100 mg/g body weight) treatment. The mechanism may include that HEF could increase renal Ca^{2+} reabsorption, stimulate the process of osteoblast formation and suppress the process of osteoclastogenesis in OVX mice (Chen et al. 2011). They also found that HEF could increase the femur bone mineral density (BMD) and OPG mRNA expression, inhibiting the differentiation and maturation of osteoclast in OVX rats (Chen et al. 2009). Core binding factor alpha1(Cbfα1, also known as Runx2) is a member of the runt family of transcription factors, which appears to play a pivotal role in regulating the differentiation of osteoblastic precursors and the activity of mature osteoblasts. HEF could increase the expression of Cbfα1 mRNA in the bone of OVX rats in a dose-dependent manner, and enhance osteocalcin expression at a higher dose (160 mg/kg), suggesting that the bone anabolic effects of HEF may be mediated by Cbfα1 (Qian et al. 2006). Other studies suggested that the protective effect of HEF on bone quality in OVX rats may be related to the expression of estrogen receptors. HEF could improve the decrease of serum estradiol level, BMD of vertebra, and the mRNA expressions of ERα and ERβ in hypothalamus and hippocampus in OVX rats, except the wet weight of uterus, suggesting that HEF treatment could prevent bone loss in OVX rats without any side effects on uterus (Wu et al. 2011). Jiang et al. (2002) also found that HEF could improve the bone density and enhance estradiol levels in the serum of OVX rats.

HEF was able to prevent OVX-induced reduction in failure force, as well as pQCT-quantified densitometry, geometry, and micro-CT-quantified 3-D trabecula micro-architecture, but without inducing any increase of uterus wet weight (Zhang et al. 2006). Another experiment from a 24-month randomized, double-blind and placebo-controlled trial found that HEF exerted beneficial effect on preventing bone loss in late postmenopausal women also without any detectable hyperplasia effect on the endometrium (Zhang et al. 2007). Considering the therapeutic efficiency and economical issues, HEF may be a potential candidate for promoting bone regeneration and repair.

7. Osteoprotective effects of icariin and its molecular mechanisms

Icariin, a prenylated flavonol glycoside contained in the HEF, is highly related to the therapeutic effects of *Herba Epimedii.* Many Chinese researchers have been focused on icariin for many years, due to its low price, high abundance and multitherapeutic properties. Icariin could improve cognitive deficits and memory impairment, had anti-inflammatory, anti-tumor, and protect DNA damage effects, and even acts as a cGMP-specific prostaglandin E5 inhibitor which might be developed into an agent for the treatment of erection dysfunction (Wu et al. 2012; Urano and Tohda 2010; Wu et al. 2011; Wang et al. 2010; Zhao et al. 2007; Ning et al. 2006). Recently, the bone-strengthening activity of icariin has really attracted world-wide attention. Icariin could stimulate the proliferation of rat BMSCs and increase the number of colony unit forming-fibroblasts (CFU-F) that were stained positively with ALP in a dose-dependent manner. The ALP activity, osteoalcin secretion and calcium deposition level were also enhanced significantly with administration of icariin during the osteogenic induction of rat BMSCs. Moreover, 10 microM of icariin even induced four times more mineralized bone nodules formation than in control group (Chen et al. 2005). We and Sheng et al. confirmed the above results and found that icariin can also significantly elevate the expression levels of marker genes and proteins such as Runx2, Osterix, BMP-2 and insulin-like growth factor-1 (IGF-1) in the osteogenic cultures (Sheng et al. 2008; Zhai et al. 2010). Moreover, Fan et al. (2011) found that icariin had a dose-dependent effect on the proliferation and osteogenic differentiation of isolated human BMSCs when stimulated with different concentrations of icariin (10^{-9} M to 10^{-6} M), while there was a cytotoxic effect if at concentrations above 10^{-5} M. CORT or OVX could induce osteoporosis, while icariin promoted BMSCs differentiation from CORT rats, and increased the secretion of osteocalcin, collagen I and Runx-2 in the OVX model. Gene profile revealed a remarkable shift of gene expression, which potentially targeted cell communication, adhesion, cycle and cytokine secretion, but very few genes were overlapped in these two models, suggesting the effects and molecular mechanisms of icariin on osteoporosis might be pathogen-dependent (Bian et al. 2012).

Icariin could significantly enhance the proliferation and differentiation of human osteoblasts with induction of BMP-2 mRNA and protein expression (Yin et al. 2005). He et al. (2009) found that icariin could promote the differentiation ability of rat osteoblast through up-regulating the cbfα1, BMP-2 and BMP-4 mRNA expression, but showed no effect on the proliferation of osteoblasts. Moreover, there were some other different results. Ma et al. (2011) compared the abilities of genistein and icariin in enhancing differentiation and mineralization of cultured rat calvarial osteoblasts *in vitro*. Although both compounds inhibited the proliferation of osteoblasts in a dose-dependent manner, icariin treatment, compared with genistein at the same dose, always produced higher ALP activity, more and larger areas of CFU-F_{ALP} colonies and mineralized nodules, more osteocalcin secretion and calcium deposition, and a higher level of mRNA expression of osteogenesis-related genes COL1alpha2, BMP-2, Osterix, and Runx-2.

Icariin not only stimulated osteogenic differentiation, but also suppressed osteoclastic differentiation and the activity of bone resportion. It was reported that icariin could significantly reduce the osteoclast formation from RAW 264.7 cells induced by RANKL and the expression of tartrate-resistant acid phosphatase (TRAP), CA II, CTSK and MMP-9, and the inhibitory strength may be higher than HEF (Zhang et al. 2012). It could also reduce the number of multinucleated TRAP-positive cells and the osteoclastic resorption area. Superoxide generation and actin ring formation which are required for osteoclast survival and bone resorption activity were also decreased. It was indicated that icariin inhibited the motility and bone resorption activity of isolated osteoclasts (Huang et al. 2007). Chen et al. (2007) also found icariin could suppress osteoclastogenesis and bone resorption activity in mouse bone marrow culture with significantly lower gene expression level of TRAP, RANK and CTR compared with control group.

The anabolic effect of icariin *in vivo* was confirmed by animal or human studies. The first paper that evaluated the effects of icariin on osteoporotic rats was published in 2005 by Bao et al. They found that icariin at dose of 225 mg/kg per day increased the BMD, maximum load and flexural rigidity in the osteoporotic rats, while the activities of serum TRAP were decreased in the icraiin-fed OVX rats (Bao et al. 2005). Nian et al. (2009) also found that icariin at a dose of 125 mg/kg body weight completely corrected the decreased serum concentration of calcium, phosphorus, and E(2) observed in OVX rats, in addition to significantly increasing biomechanical strength and bone formation when compared with the sham group, indicating an antiosteoporotic effect similar to estrogen. Similarly, Mok et al. (2010) found that icariin suppressed the loss of bone mass and the strength in distal femur of C57BL/6 mice and increased the mRNA expression ratio of OPG/RANKL in tibia following OVX.

Zhao et al. (2010) found icariin-calcium phosphate cement (CPC) tablets could induce new bone and blood vessel formation *in vivo*, which was a strong candidate as an osteogenic compound for use in bone tissue engineering (Zhao et al. 2010). Oral administration of icariin at a dose of 2.5 mg/kg per day for 4 weeks resulted in greater volumes of new bone formation, higher trabecular numbers, and less trabecular separation than in the control group, suggesting that icariin could promote bone formation during mandibular distraction osteogenesis and might be a promising treatment for shortening the course of distraction osteogenesis (Wei et al. 2011). We all know that OPG plays an important role in regulating bone homeostasis by inhibiting osteoclastogenesis and bone resorption. Zheng et al. (2012) used 12-week-old OPG knockout (KO) male mice that were orally administered with icariin (0.3 mg/g) once a day for 8 weeks and found that icariin treatment increased bone formation parameters while decreased bone resorption parameters in wild type (WT) mice; however, the anabolic response of trabecular bone to icariin treatment was diminished in KO mice. The data suggested that the effects of icariin treatment on bone were dependent on up-regulation of OPG, in other words, OPG played an essential role in icariin-mediated beneficial effects on trabecular bone formation.

There is very few clinical evidence for the use of icariin in the treatment for osteoporosis. So further animal and clinical studies are required to investigate the efficacy and safety of icariin in a long-term use to prevent or treat osteoporosis.

The molecular mechanisms of osteoprotective effects by icariin are complicated. Many studies have revealed that the activities of icariin on stimulating bone formation and inhibiting bone resorption may be mediated through several signaling pathways. Hsieh et al. found that icariin at dose of 10^{-8} M increased the proliferation and matrix mineralization of primary osteoblasts and promoted NO synthesis. With icariin treatment, the BMP-2, SMAD4, Cbfα1/Runx2, and OPG gene expressions were up-regulated; while the RANKL gene expression was down-regulated. Concurrent treatment with a BMP antagonist (noggin) or a NOS inhibitor (L-NAME) diminished these effects, suggesting that icariin may act through the induction of BMP-2 and NO pathways (Hsieh et al. 2010). Liang et al. (2012) observed that icariin had beneficial effects on the bone formation in the hFOB 1.19 human osteoblastic cell line and found that icariin up-regulated the expression of BMP-2, Smad4, Cbfα1/Runx2, OPG, RANKL and the OPG/RANKL ratio, indicating that icariin could modulate the process of bone formation via the BMP-2/Smad4 signal transduction pathway in hFOB 1.19 cells. Mok et al. (2010) found that icariin suppressed the loss of bone mass and strength in distal femur and increased the mRNA expression ratio of OPG/RANKL in tibia of OVX mice *in vivo*. Icariin also increased cell proliferation, ALP activity, gene expression of OPG and the OPG/RANKL ratio in UMR 106 cells at ER-dependent manner *in vitro*. Although icariin did not activate ERE-luciferase activity in UMR 106 cells *via* the ERα or the ERβ-mediated pathway, it did increase ERalpha phosphorylation at Ser118.

As to the anti-resorptive effect, Xu et al. (2010) reported that the activation of the PI3K/Akt pathway and the inhibition of NF-κB signaling were involved in the protective effects of icariin on lipopolysaccharide (LPS)-induced acute inflammatory responses and bone loss. Hsieh et al (2011) examined details of the molecular mechanisms of icariin on LPS-induced osteolysis and found that icariin suppressed LPS-mediated activation of the p38 and JNK in osteoclasts but reduced the LPS-induced activation of ERK1/2 and I-kappa-B-alpha (IκBα), and increased the activation of p38 in the osteoblasts. So they believed that icariin inhibits the LPS-induced osteoclastogenesis program by suppressing the activation of the p38 and JNK pathways.

Therefore, icariin may stimulate bone formation and inhibit bone resorption activity through multiple pathways; the real molecular mechanisms are still under discussion.

8. Conclusion

Herba Epimedii has been used in TCM to treat bone fractures and prevent osteoporosis for thousand years, as it is believed that the herb can strengthen bones and tendons. Modern pharmacologic research confirmed that *Herba Epimedii* indeed has osteoprotective effects, no matter the aqueous extracts, or the total flavnoids. Although the efficacy of *Herba Epimedii* has been confirmed, the multi-ingredients restrict its clinical application. Icariin has attracted world-wide attention because it is the main component in *Herba Epimedii* and exerts many kinds of therapeutic effects. The main metabolites of icariin after oral administration are icariside I, icariside II, icaritin (Fig. 2) and other (Xu et al. 2007; Qiu et al. 1999). In our previous study, icariside II (also named baohuoside I) had a stronger bioactivity than icariin in osteogenic differentiation of BMSCs, indicating that icariin administrated orally is transformed into active metabolites (Zhai et al. 2010). In our another study, we proved that icariside II stimulated the osteogenesis of BMSCs via the NO signal pathway and the estrogen signal pathway (Zhai et al. 2011; Zhai et al. 2011). We also found the cross talk between this two pathways that icariside II in combination with ERα activated the downstream factors, such as PI3K and Akt, then stimulated the activity of NOS and increased NO production, and then further activated the NO-cGMP-PKG signal pathway (unpublished data).

Recently, some researchers proposed that the prenyl group is an active group that takes part the in osteogenesis process, such as icariin, 8-prenylnaringenin and osthole (Fig. 3) that can all prevent bone loss and belong to the prenyl compounds (Ming et al. 2012; Humpel et al. 2005). Zhang et al. had compared genistein derivatives (6-prenylgenistein, 8-prenylgenistein or 6, 8-prenylgenistein) in their effects on osteoblastic proliferation, differentiation and mineralization in UMR 106 cells, and found that prenylation at C-8 could be the most effective form to increase the osteogenic activity of genistein (Zhang et al. 2008).

In conclusion, *Herba Epimedii* is one of the most frequently used anti-osteoporosis remedies, the validity of its extracts and total flavonoids have been confirmed in animal and cell line models. Icariin, one of the main prenylflavonoids in *Herba Epimedii,* not only stimulates bone formation, but also inhibits bone resportion. However, whether icariin is indeed more efficient in preserving bone mass and preventing bone loss still needs to be further investigated in animal and clinical studies. In one word, *Herba Epimedii* gives us a better choice and new vision in drug development for osteoporosis therapy, although it is still a long way to go for clinical application.

Acknowledgements: This investigation was supported by the grant (No. 2010CB535002) from National Program on Key Basic Research Project of China (973 Program), and by the National Natural Science Foundation of China (No. 81073037 and No. 81202457).

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