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Antiosteoporotic activity of echinacoside in ovariectomized rats

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ABSTRACT

Purpose: Echinacoside (ECH), isolated from *Cistanche tubulosa* (*Schrenk*) R. Wight stems, has been reported to enhance bone regeneration in MC3T3-E1 cells *in vitro*. The objectives of this study were to investigate the antiosteoporotic effect of ECH on bone metabolism in the ovariectomized (OVX) rat model of osteoporosis *in vivo*.
Methods: Fifty-six aged 6 months female Sprague-Dawley rats were randomly assigned into sham-operated group (SHAM) and six OVX subgroups ($n = 8$ each). The OVX rats were then subdivided into six groups treated with vehicle (OVX), Xian-ling-gu-bao (XLGB, 0.5 g/kg body weight/day, orally), 17β-estradiol (E2, 50 μg/kg body weight/day, orally) or ECH (30, 90, and 270 mg/kg body weight, daily, orally) for 12 weeks respectively. We evaluated the pharmacological effects of E2, XLGB and ECH against osteoporosis by evaluating the body weight, uterus wet weight, serum and urine biochemical parameters, bone mineral density (BMD), bone biomechanical properties, bone microarchitecture, bone histomorphology and uterus immunohistochemistry.
Results: In OVX rats, the increases of body weight, serum hydroxyproline (HOP) levels, and the decreases of uterus wet weight and BMD were significantly reversed by ECH treatment. Moreover, three dosages of ECH completely corrected the increased urine concentration of calcium (Ca), inorganic phosphorus (P), and HOP observed in OVX rats. Furthermore, Micro-CT analysis results of distal femur showed that all ECH-treated groups notably enhanced bone quality compared to OVX group ($p < 0.05$). Consistent with this finding, total femur BMD and biomechanical strength of tibia were significantly improved ($p < 0.05$) after 12 weeks ECH administration. Histological results also showed the protective activity of ECH through promotion of bone formation and suppression of bone resorption. In addition, the ECH administration also significantly enhanced the expression of ER in the uteri according to immunohistochemical evaluation ($p < 0.05$). Those findings, based on the serum and urine biochemical, BMD, Micro-CT, biomechanical test, histopathological and immunohistochemical parameters, showed that ECH has a notable antiosteoporotic effect, similar to estrogen, especially effective for prevention osteoporosis induced by estrogen deficiency.
Conclusion: These results suggest that ECH, as a new class of phytoestrogen, has a remarkable antiosteoporotic activity, and may be a promising candidate for treatment of postmenopausal osteoporosis induced by estrogen deficiency in a natural way through herbal resources.

Introduction

Osteoporosis, the most frequent bone remodeling disease especially for post-menopausal women in any racial or ethnic group, is defined by low bone mass and a high risk of fractures, and has become a well-known major pubic threat accompanying with increasing social-economic burden in our aging society (Wang et al. 2012). The prevalence of osteoporosis was estimated to be approximately 200 million people worldwide with attendant costs exceeding 10 billion dollars per annum (Reginster and Burlet 2006;

Abbreviations: BMD, bone mineral density; BV/TV, bone volume over total volume; Ca, calcium; Ct.Th., cortical bone thickness; E2, estradiol; ECH, echinacoside; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; H & E, hematoxylin and eosin; HOP, hydroxyproline; HRT, hormone replacement therapy; i.p., intraperitoneal; Micro-CT, microcomputed tomography; N.OB., number of osteoblast; N.OC., number of osteoclast; N.Tb., number of trabecular; OVX, ovariectomized; P, inorganic phosphorus; PBS, phosphate-buffered saline; SMI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th., trabecular bone thickness; Tb.Th, trabecular thickness; VOI, volume of interest.

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Katrina and McDonald 2009). Most of osteoporotic patients are postmenopausal women, and the estrogen deficiency is the predominant cause of rapid hormone-related bone loss during the first decade after menopause.

There are various treatment options available that can reduce osteoporosis induced fracture risk but each has limitations. For example, the benefit of hormone replacement therapy (HRT) has been confirmed in terms of osteoporosis treatment, however, adverse outcomes of long-term HRT such as higher incidence of endometrial cancer, mammary cancer, and increased risk of coronary heart disease or other cardiovascular diseases have been identified (Persson et al. 1999; Davison and Davis 2003). Bisphosphonates reduce risk of fracture by about a factor of 2 with the potential adverse effects of leading to atraumatic fracture of bone as a consequence of an adynamic state similar to that described in patients on chronic maintenance hemodialysis (Cranney et al. 2002; Odvina et al. 2005) There are other therapies for osteoporosis and reduction risk of fracture such as selective estrogen receptor moderators like raloxifen and droloxifen, strontium ranelate, calcitonin and synthetic parathyroid hormone. Each treats osteoporosis and exhibits its own advantages and disadvantages. Hence, it would be most helpful to explore natural alternatives for prevention bone loss and fracture risk induced by osteoporosis with less undesirable side effects.

Many plant-derived compounds have the potential to counteract the deleterious effects of estrogen deficiency on bone. The *Cistanche tubulosa* (*Schrenk*) R. Wight (Orobanchaceae parasitic plant), an important traditional Chinese herbal medicine used widely as a promoting agent of blood circulation, treatment for kidney deficiency and neurodegenerative diseases in China, is widely distributed in North Africa and Asian countries (Kobayashi et al. 1987). Recently, it has been used in many Chinese anti-osteoporosis formulae. Echinacoside (ECH, $C_{35}H_{46}O_{20}$, molecular weight: 786.72, Fig. 1a), as a natural polyphenolic compound, is considered to be the major bioactive component of *Cistanches tubulosa* (*Schrenk*) R. Wight and has a broad range of therapeutic applications in antioxidative, anti-inflammatory, neuroprotective, nitric oxide radical-scavenging, and cardioactive (Tu et al. 1997; Pennacchio et al. 1996). It was reported that echinacoside stimulated bone regeneration in MC3T3-E1 cells *in vitro* (Li et al. 2012). Such *in vitro* evidence implies that echinacoside may have a role in the treatment and/or prevention of osteoporosis and suggests that an *in vivo* assessment of the potential bone protective effect of echinacoside should be carried out.

An excellent animal model to study the development and treatment possibility of postmenopausal osteoporosis is the ovariectomized (OVX) mature rat model (French et al. 2008; Lelovas et al. 2008). The aim of the study was to evaluate the effects of ECH in preventing osteoporosis, ameliorating bone loss, improving bone strength and bone quality in OVX rats.

Materials and methods

Substances

Echinacoside (ECH) was separated and purified from an ethanol extract of *Cistanche tubulosa* (*Schenk*) R. Wight by our laboratory, and its structure was confirmed by UV, IR, MS, and NMR

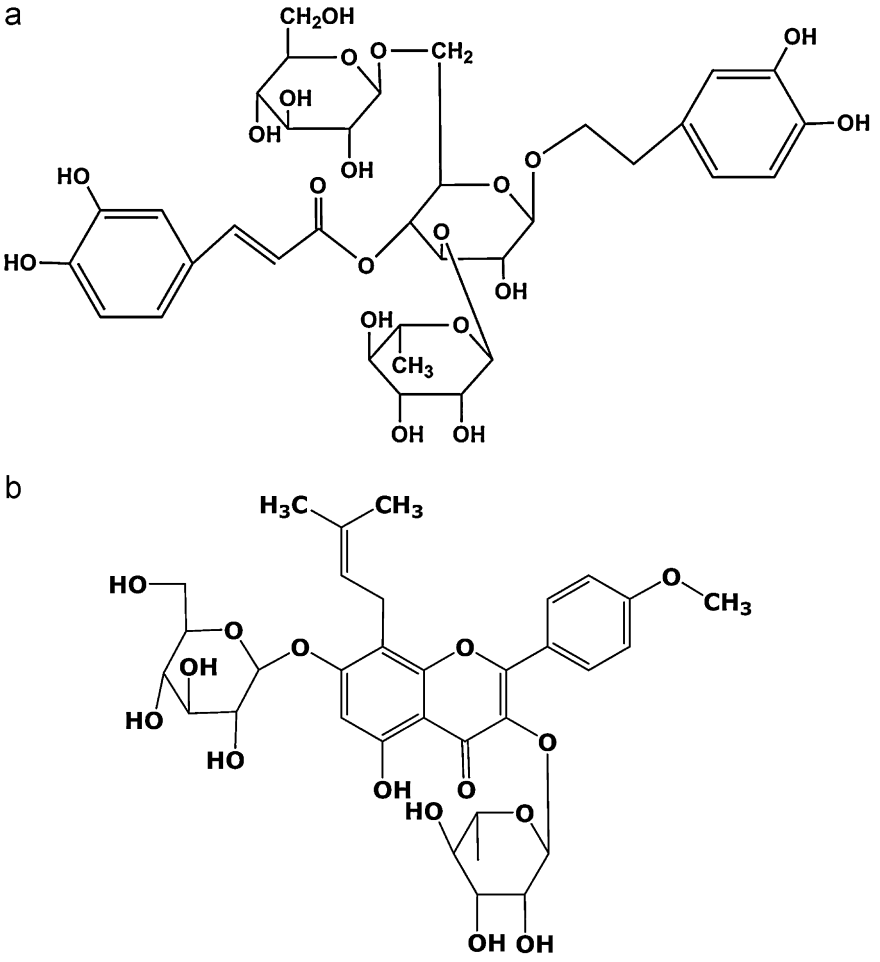

Fig. 1. (a) Chemical structure of echinacoside (ECH) and (b) chemical structure of icariin (ICA).

spectroscopy. Its purity (98.5%) was determined by Agilent 1260 Series HPLC with DAD detector (Agilent Scientific, Co., USA). Xian-ling-gu-bao (XLGB, containing icariin (Fig. 1b) 7–8.75 mg/g, Guizhou, China) and 17β-estradiol (E2, Sigma; purity ≥ 98%) were used as positive controls.

Animal experiments

All animal experiments were strictly carried out according to the Guide for the Humane Use and Care of Laboratory Animals and were approved by the Animals Ethics Committee of the China Pharmaceutical University. 48 female Sprague-Dawley rats (Nantong University, China), aged 6 months with the body weight of 280 ± 20 g, were allowed to acclimatize for 7 days before the start of the experiment. Every four animals were kept in one cage with a standard laboratory diet and tap water under climate-controlled conditions (25 °C, 55% humidity, and 12 h of light alternating with 12 h of darkness).

After 7 days of acclimatization, the rats were anesthetized with intraperitoneal (i.p.) injection of 300 mg/kg chloral hydrate (Sinopharm®, China) and underwent either bilateral laparotomy (SHAM, $n = 8$) or bilateral OVX (OVX, $n = 48$) at week 0. The surgical procedure was performed under aseptic conditions following the China Pharmaceutical University Animal Care protocol. Rats were left untreated for 4 weeks to allow rats to recover and develop osteopenia. After 4 weeks, the OVX rats were randomly divided into 5 groups: ovariectomized treated with vehicle (OVX, $n = 8$), OVX treated with Xian-ling-gu-bao (XLGB, $n = 8$, 0.5 g/kg body weight/day), 17β-estradiol (E2, $n = 8$, 50 μg/kg body weight/day), and OVX treated with low dosage (ECH-L, $n = 8$, 30 mg/kg body weight/day), with medium dosage (ECH-M, $n = 8$, 90 mg/kg body weight/day), or with high dosage ECH (ECH-H, $n = 8$, 270 mg/kg body weight/day). The experimental dose for XLGB, E2 and ECH in the present study was equivalent to the corresponding clinical prescription dose for a 70 kg human subject. Vehicle, XLGB, E2 and ECH were administrated orally, which started on 5th week after OVX and lasted for 12 weeks. Body weight was measured weekly, and the ECH, XLGB or E2 dose adjusted accordingly.

After 12 weeks of intervention and on the last day of treatment, urine was collected from overnight fasted animals by metabolic cages and preserved at −20 °C until further analysis. All animals were anesthetized with i.p. injection of 300 mg/kg chloral hydrate, and blood was collected from the carotid artery in the early morning. The blood was allowed to clot, and centrifuged at 3000 × *g* for 10 min. Serum was harvested and stored at −20 °C until use for biochemical assays. Then, femora, tibiae and uteri were collected and the adherent connective tissues were completely removed before using for measurement of BMD, trabecular microarchitecture by microcomputed tomography (Micro-CT), bone biomechanical quality by three-point bending test, bone histomorphometry and uterus immunohistochemistry respectively.

Urine and serum parameters

Urine was subjected for the estimation of calcium (Ca), inorganic phosphorus (P) and hydroxyproline (HOP) levels using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) on a microplate reader (Thermo Fisher Scientific, Co., USA). Serum samples were also subjected for the measurement of Ca, P, and HOP by commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for the *in vitro* determination. The levels of serum estradiol (E2) were also determined with a sandwich enzyme-linked immunosorbent assay (ELISA) assay kit (R&D Systems Inc., USA). ELISA assays were performed for serum E2 according to manufacturer's instruction.

Measurement of bone mineral density (BMD)

The BMD of the right total femur was measured by using dual energy X-ray Absorption Spectrometry (DEXA, Hologic Inc., Boston, MA, USA) equipped with appropriate software (edition 13.1.2) using the small animal scan mode. The investigator performing the measurement was unaware of the treatments the rats had received. The measurements obtained were expressed as grams of mineral contents per cm^2 of surface area as described elsewhere (Tu et al. 1999).

Micro-CT analysis

The right distal femora from each group were scanned by Micro-CT system (μCT-Sharp, ZKKS-MCT, China) with ZKKS Micro-CT 3-D analysis software of version 3.0 for scanned images reconstruction. The scanning system was set to 60 kV, 40 W, with an isotropic voxel size of 22 μm. Bone morphometric parameters including bone volume over total volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and structure model index (SMI) were obtained by analyzing the volume of interest (VOI).

Biomechanical evaluation

The freshly isolated tibiae were assessed for the three-point bending test (Peng et al. 1994) using CSS-4420 material testing machine (Changchun Research Institute for Testing Machines Co. Ltd., China). The force and displacement data were automatically recorded into a computer which was interfaced to the material testing machine and the load-deformation curve was plotted simultaneously. The results of measurements of ultimate load (newtons, N), extrinsic stiffness (newtons per millimeter, N/mm) and energy to yield point (mJ) were calculated with the load-deformation curve.

Histopathology of femur bone

For histological analysis, right femora ($n = 4$/group) were fixed in 10% neutral formalin for 2 days at room temperature and then decalcified in 10% ethylenediaminetetraacetic acid (pH 7.2–7.4, changed every 3 days) for 4 weeks. Decalcified tissues were then washed, dehydrated in gradient alcohol, embedded in paraffin wax, and cut into longitudinal sections of 4 μm thickness through the proximal femur with a microtome (Leica RM2235, Germany). Sections were stained with hematoxylin and eosin (H & E). Then, the micro-architectural changes were observed and the number of positively stained cells in sections was enumerated under microscope (Leica DM 100, Germany) with Mini See 1.0.9.37 image analyzing system. The following parameters were measured: cortical bone thickness (Ct.Th., mm), trabecular bone thickness (Tb.Th., mm), number of trabecular (N.Tb., n/mm^2), number of osteoblast (N.OB., n/HPF) and number of osteoclast (N.OC., n/HPF).

Immunohistochemical staining

Selected uteri ($n = 4$/group) were fixed in 10% formalin for 2 days at room temperature for immunohistochemical evaluation of estrogen receptor (ER) expression. The tissues were then washed, dehydrated in gradient alcohol, embedded in paraffin wax, and cut into serial sagittal sections (4 μm thick) with a microtome (Leica RM2235, Germany). Immunohistochemical localization of ER was performed with ER polyclonal antibody (Bioss, Beijing, China) according to the manufacturer's instructions. In the negative control, phosphate-buffered saline (PBS) was substituted for

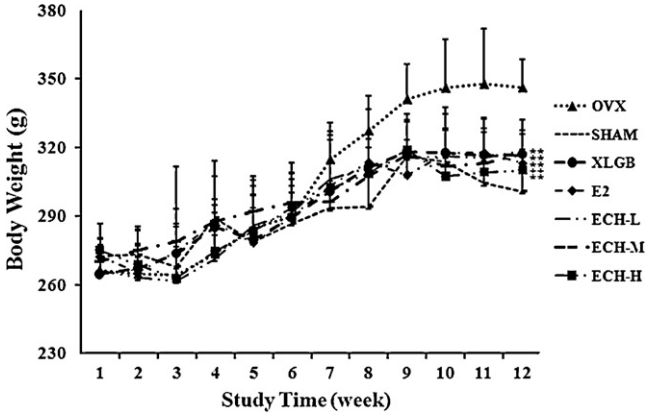

Fig. 2. Body weight changes in the OVX model of osteoporosis. OVX rats received no treatment (OVX), E2 (50 μg/kg/day), and ECH (30, 90 and 270 mg/kg/day) for 12 weeks. Values are mean ± SD, error bars in the figure are presented as SD, $n = 8$. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* sham group, $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* OVX group at the same time point as evaluated by ANOVA.

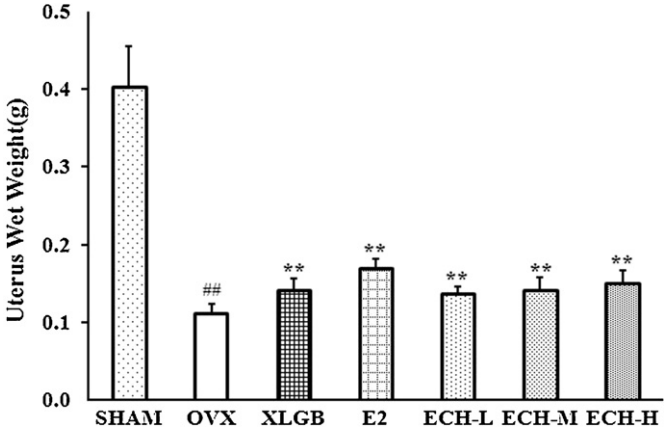

Fig. 3. Uteri were isolated and weighed after euthanized and uteri wet weight changes were recorded. OVX rats received no treatment (OVX), E2 (50 μg/kg/day), and ECH (30, 90 and 270 mg/kg/day) for 12 weeks. Values are mean ± SD, error bars in the figure are presented as SD, $n = 8$. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* sham group, $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* OVX group at the same time point as evaluated by ANOVA.

the primary antibodies. Stained sections were examined qualitatively under light microscopy (Leica DM 100, Germany) with Mini See 1.0.9.37 image analyzing system.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by a *post hoc* multiple comparison using Fisher's least significant difference (LSD) *t*-test. All calculations were performed using SPSS Version 15.0 for Windows (SPSS, Chicago, IL, USA). The statistical significance was defined as $p < 0.05$.

Results

Body weight and uterus wet weight

The weekly average body weights of rats in each group throughout the experimental period are depicted in Fig. 2. There was no significant difference in the mean body weight initially in each group, however, at sacrifice (after 12 weeks), the rats in XLGB, E2 and ECH groups, weighed significantly less than the OVX rats ($p < 0.01$). The body weight of the ECH group at all doses increased less than the OVX group, which were increased by 65.88%, 59.00% or 43.66%, respectively ($p < 0.01$), compared to the OVX group. The body weights of the rats in the XLGB and E2 group were also lower than the OVX group by 51.02% and 65.42%, respectively ($p < 0.01$).

As shown in Fig. 3, the uterine weight of the SHAM and OVX rats differed significantly ($p < 0.01$). The weight of the ECH-H group was higher than the OVX group by 34.72% and the difference was statistically significant ($p < 0.01$). However, the difference among ECH groups had no statistical significance. Similarly, XLGB and E2 treated groups demonstrated a significantly enhanced uterus wet weights by 27.41% and 52.37% ($p < 0.001$) as compared to OVX group.

Serum and urine biochemistry

The effects of the ECH on serum and urine biochemical parameters of osteoporosis in rats after 12 weeks are shown in Table 1. Ovariectomized (OVX) rats showed statistically significant ($p < 0.01$) increased levels of serum hydroxyproline (HOP) by 36.41%, urinary excretion levels of calcium (Ca) by 92.23%, inorganic phosphorus (P) by 66.67% and HOP by 34.43% compared to SHAM group. However, there were no significant changes in the levels of serum Ca and P levels. Treatment with ECH, XLGB and E2 significantly prevented the elevation of serum HOP levels compared to OVX group ($p < 0.05$). Moreover, elevations of urinary excretion of Ca, P and HOP levels were prevented significantly in all ECH-treated and E2-treated groups ($p < 0.05$).

Bone mineral density

As shown in Fig. 4, the right total femora bone mineral density (BMD) of OVX rats significantly declined to 0.222 ± 0.008 mg/cm^2 compared to 0.281 ± 0.009 mg/cm^2 of SHAM rats ($p < 0.01$). This indicated that the ovariectomy decreased the BMD by 7.83% after 12 weeks. In a same period of time, treatments of ECH at a dosage of 90 mg/kg (ECH-M) or 270 mg/kg (ECH-H) notably enhanced the BMD by 16.82% ($p < 0.05$) or 24.02% ($p < 0:01$), respectively, in comparison to OVX group. Similarly, XLGB treated and E2-treated rats also increased total femora BMD by 15.92% and 16.67% compared

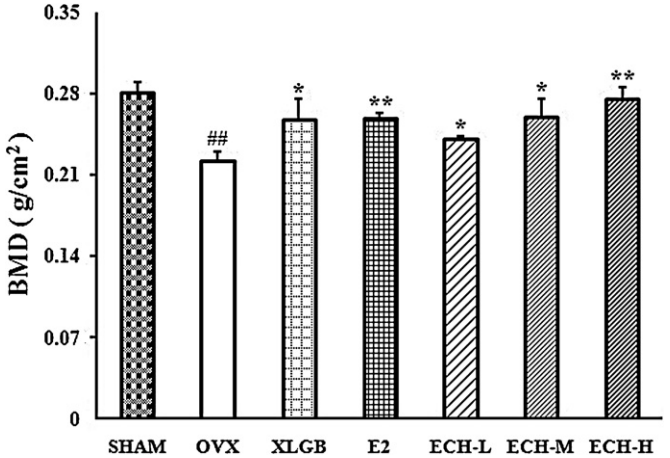

Fig. 4. Effects of 12-week treatment with ECH or E2 on the total right femur bone mineral density (BMD) in the OVX rats. Data were expressed as mean ± SD, error bars in the figure are presented as SD, $n = 4$ specimens/group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* sham group, $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* OVX group at the same time point as evaluated by ANOVA.

Table 1
Serum and urine parameters, Micro-CT analysis and histomorphometric evaluation.

	SHAM	OVX	XLGB	E2	ECL-L	ECH-M	ECH-H
Serum parameters				*n* = 8/group			
Serum Ca	1.249 ± 0.097	1.274 ± 0.099	1.256 ± 0.086	1.258 ± 0.094	1.266 ± 0.092	1.258 ± 0.046	1.270 ± 0.053
Serum P	1.972 ± 0.212	2.064 ± 0.173	1.922 ± 0.325	1.969 ± 0.233	1.910 ± 0.456	1.923 ± 0.242	1.966 ± 0.185
Serum HOP	26.343 ± 6.446	35.934 ± 7.999$^{\#}$	27.110 ± 4.796*	25.320 ± 9.518*	27.749 ± 6.873*	24.936 ± 7.331*	22.251 ± 9.673**
Urine parameters				*n* = 8/group			
Urine Ca	1.117 ± 0.237	2.148 ± 0.374$^{\#\#}$	1.724 ± 0.233*	0.717 ± 0.136**	1.088 ± 0.189**	1.047 ± 0.199**	0.895 ± 0.244**
Urine P	7.888 ± 2.379	13.146 ± 2.626$^{\#\#}$	6.132 ± 2.500**	4.573 ± 1.588**	7.186 ± 1.578**	6.267 ± 1.283**	3.934 ± 0.557**
Urine HOP	31.771 ± 8.993	42.708 ± 8.364$^{\#}$	31.597 ± 12.354	31.424 ± 8.200*	32.465 ± 9.413*	28.646 ± 8.784**	29.167 ± 11.221*
Micro-CT analysis				*n* = 4/group			
BV/TV (%)	58.60 ± 7.80	17.56 ± 3.15$^{\#\#}$	27.99 ± 4.47**	33.64 ± 3.53**	24.94 ± 2.67*	25.20 ± 1.04*	29.72 ± 2.13**
Tb.N (1/mm)	3.99 ± 0.15	2.04 ± 0.48$^{\#\#}$	2.98 ± 0.32*	3.01 ± 0.10*	3.03 ± 0.19*	3.15 ± 0.34*	3.21 ± 0.45*
Tb.Sp (μm)	101.23 ± 12.64	468.41 ± 69.42$^{\#\#}$	271.89 ± 81.98*	222.22 ± 10.74**	341.83 ± 39.24*	317.09 ± 32.91*	277.91 ± 28.06*
Tb.Th (μm)	161.55 ± 15.17	80.66 ± 2.90$^{\#\#}$	106.84 ± 14.78*	116.06 ± 8.91**	98.24 ± 8.73*	117.74 ± 20.42*	119.56 ± 8.16**
SMI	0.74 ± 0.09	2.93 ± 0.46$^{\#\#}$	2.05 ± 0.23*	1.37 ± 0.29**	2.18 ± 0.06*	1.79 ± 0.45*	1.88 ± 0.22*
Histomorphometric evaluation				*n* = 4/group			
Ct.Th. (mm)	0.064 ± 0.005	0.042 ± 0.005$^{\#\#}$	0.047 ± 0.009	0.050 ± 0.003*	0.063 ± 0.008**	0.060 ± 0.006**	0.065 ± 0.010**
Tb.Th. (mm)	0.46 ± 0.007	0.35 ± 0.002$^{\#}$	0.45 ± 0.03**	0.43 ± 0.05*	0.41 ± 0.02**	0.42 ± 0.05*	0.42 ± 0.05*
N.Tb. (n/200×)	9.75 ± 1.71	6.50 ± 1.73$^{\#}$	7.75 ± 1.26	7.50 ± 0.58	10.00 ± 1.75*	10.75 ± 2.50*	12.50 ± 4.36*
N.OB. (*n*/HPF)	46.00 ± 10.20	26.15 ± 4.56$^{\#}$	32.00 ± 10.67	41.25 ± 2.99**	34.58 ± 2.11	37.40 ± 1.80**	39.00 ± 2.39**
N.OC. (*n*/HPF)	10.50 ± 3.00	16.00 ± 3.27$^{\#}$	12.50 ± 5.00	10.00 ± 2.83*	11.00 ± 3.83	9.00 ± 3.83*	8.50 ± 0.52*

The data are expressed as mean ± SD.

* $p < 0.05$ *versus* OVX group at the same time point as evaluated by ANOVA.

** $p < 0.01$ *versus* OVX group at the same time point as evaluated by ANOVA.

$^{\#}$ $p < 0.05$ *versus* SHAM group.

$^{\#\#}$ $p < 0.01$ *versus* SHAM group.

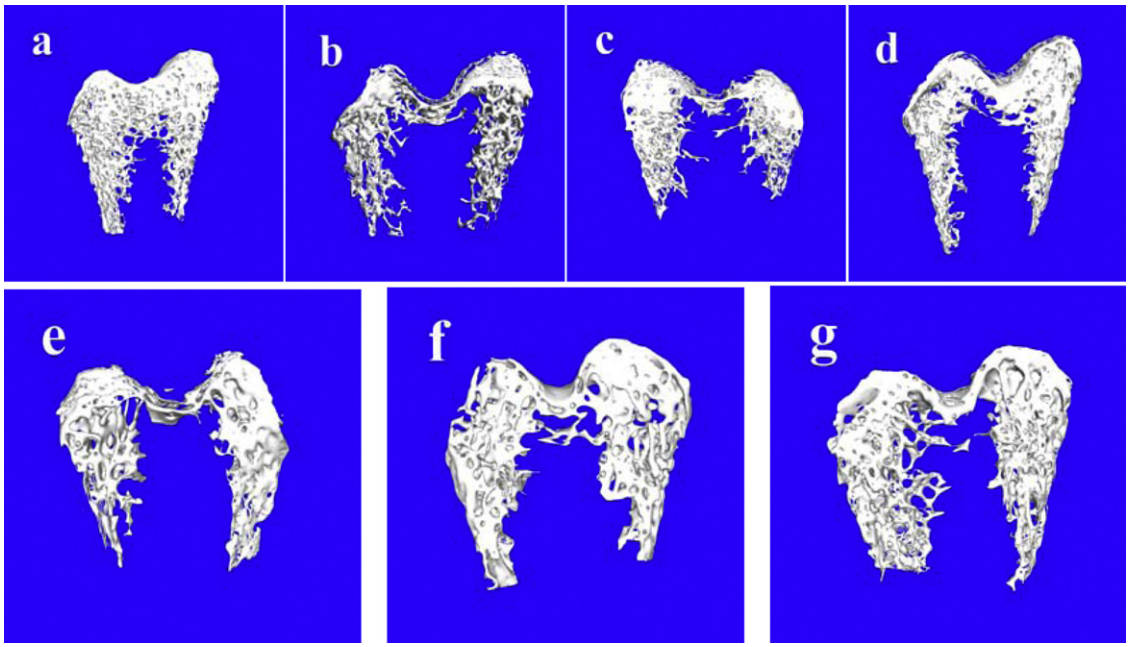

Fig. 5. Representative Micro-CT images of trabecular bone microarchitecture in the distal femurs. (a) SHAM group; (b) OVX group; (c) E2 group; (d) ECH-L group; (e) ECH-M group; (f) ECH-H group. The OVX rats presented notable reduction in the trabecular number, trabecular area compared with the SHAM rats. ECH and E2 partially prevented OVX-induced trabecular bone loss and significantly improved trabecular bone mass and microarchitecture.

to OVX rats. Additionally, the significant difference was observed between ECH groups.

Micro-CT analysis

To investigate the role of ECH in bone metabolism, we determined structural changes in distal femur trabecular bone with Micro-CT. The quantitative results were expressed as BV/TV, Tb.N, Tb.Sp, Tb.Th and SMI in Table 1. Analysis of the distal femur morphometric parameters indicated that deterioration of the microarchitecture in the OVX group compared to SHAM group, evidence by significant decreasing trabecular BV/TV, Tb.N, and Tb.Th ($p < 0.01$) and increasing Tb.Sp and SMI ($p < 0.01$). Treatment with ECH, XLGB or E2 reversed the above mentioned indices and all were statistically significant as compared to OVX group ($p < 0.05$). However, the significant difference was not observed between ECH groups. The preventive effects of ECH on trabecular bone mass and microarchitecture deterioration are further proved by the 3D Micro-CT images (Fig. 5). OVX group presented notable reduction in the trabecular number and trabecular area when compared with SHAM group. ECH partially prevented OVX-induced bone loss and significantly improved the trabecular bone mass and microarchitecture after 12 weeks treatment, similar as XLGB and E2.

Three-point bending test

The effects of ECH on biomechanical parameters of osteoporosis in rats after 12 weeks using three-point bending experiment are shown in Fig. 6a–c. Twelve weeks of estrogen deficiency, the biomechanical parameters of tibia, such as ultimate load, stiffness, and energy absorption, decreased significantly in the OVX group as compared to the SHAM group ($p < 0.01$). A statistically significant increase in biomechanical strength was observed in ECH, XLGB and E2 treatment groups as compared to OVX group ($p < 0.01$), evidenced by increased levels of ultimate load, stiffness, and energy absorption, but not XLGB for stiffness. However, no significant difference was observed between ECH-treated groups.

Histomorphology

Histologically the sections of distal femur in the region proximal to the epiphyseal growth plate were examined for the changes. The alterations of bone histology and morphology were observed including changers in cortical bone thickness, trabcular bone thickness, trabcular number, osteoblasts and osteoclasts number. As shown in Table 1, in the absence of ECH administrations, significant reductions on cortical bone thickness, osteoblasts number, trabcular thickness and number were observed in the OVX rats ($p < 0.05$), as well as significant enhancement of osteoclasts number ($p < 0.05$) in comparison to SHAM rats. XLGB, E2 and all ECH dosages were able to recover the changes on bone histology and morphology in the OVX rats. In general, analyzing the indices above, there were no significant changes between the different treatment groups.

Immunohistochemistry

The ER expression appeared as a yellow-brown staining in the cytoplasm. The major locations of positive ER expression were all the endometrial epithelial cells (Fig. 7). Fig. 7 displays the optical microscopy images for ER staining. A significant decrease in ER labeling was already observed in OVX group compared with SHAM group, whereas the ER expressions of the ECH-treated groups were significantly higher than that of the OVX group ($p < 0.05$) after 12 weeks administration, similar to the E2-treated group ($p < 0.01$). ECH-M group and ECH-H group significantly increased the ER expression by 96.97% and 112.12% respectively, compared to OVX group, similar as XLGB and E2 groups which increased by 81.82% and 228.79% respectively.

Discussion

In this study, we explored the effects of ECH on OVX-induced osteoporosis in rats. Our study clearly demonstrates the beneficial effects of ECH in the prevention of bone loss induced by ovariectomy. It is well known that estrogen deficiencies are important risk factors in the pathogenesis of osteoporosis. Ovariectomy results

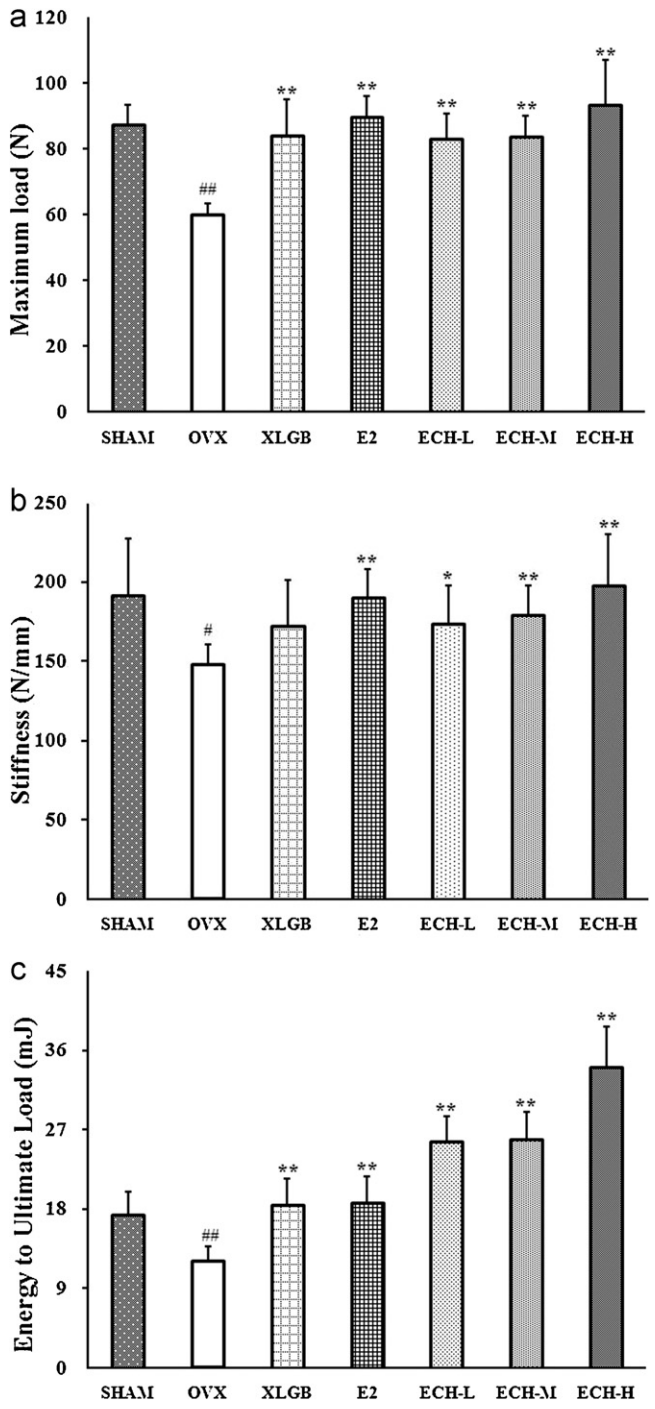

Fig. 6. Ultimate load, stiffness, and energy absorption determined from three-point bending test on right tibiae after sacrifice at 12 weeks. (a) Ultimate load. (b) Stiffness. (c) Energy absorption. Data were expressed as mean ± SD, error bars in the figure are presented as SD, $n = 6$ specimens/group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* sham group, $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* OVX group at the same time point as evaluated by ANOVA.

in a dramatic decrease in body weight, uterine wet weight, BMD, biomechanical strength, and bone quality, and these changes are in part due to estrogen deficiency (Nian et al. 2009). Our data showed that OVX decreased the weight of the uterus and increased the body weight when compared with the SHAM group, clearly showing the effectiveness of ovariectomy. ECH treatments significantly increased the uterine weight and serum E2 levels, as well as decreased body weight and the serum HOP levels in OVX rats. In line with this finding, the ECH administration also significantly enhanced the expression of ER in the uteri according to immunohistochemical evaluation. Moreover, the ECH treatment significantly enhanced total femora BMD, biomechanical strength, and bone quality for 12 weeks. These results suggested that ECH may act as an estrogen analog, as it had a similar effect as estrogen on uterine weight, serum E2 levels and even on the prevention of bone loss. It implies that ECH possess a marked antiosteoporotic activity on OVX induced osteoporosis in rats may by phytoestrogen effect, at least partly, thereby indicating a potential therapeutic usefulness as an anti-osteoporotic agent.

HOP is known to be important biochemical markers associated with bone metabolism and their levels are the most common indicators of osteogenic properties. The levels of those markers are increased in osteoporosis and other bone metabolic disorders due to increased bone turn over (Victor 1993). In our study, serum HOP also support the observations from other investigators that elevated serum HOP levels are due to ovarian hormone deficiency and are prevented by estrogen administration (Ke et al. 1997). The results of this study demonstrate that both ECH and E2 inhibited OVX-induced enhancement of serum HOP levels. It is well known that serum E2 levels and urine Ca, P levels are also widely accepted phenotype markers for bone metabolism. In this study, treatment with ECH and E2 completely reversed the changes of serum E2 levels and urine Ca, P levels observed in OVX rats. However, there were no significant changes in the levels of serum Ca and P levels. The unchanged levels of Ca and P in plasma indicate that homeostatic mechanisms were able to maintain plasma levels of these minerals despite ovariectomy.

BMD has been described as a surrogate measure of bone strength and as primary contributor to bone quality (Bouxsein 2003). BMD is markedly decreased due to an increase in bone turnover in the OVX rats compared with SHAM group. In our study, the oral administration of ECH significantly improved the BMD indicates that ECH could prevent the progress of bone loss induced by ovariectomy (Fig. 4). Moreover, ECH treatment demonstrated a dose dependent increase in the BMD in OVX rats. Furthermore, the three point bending test results supported the BMD findings, evidenced by improving bone mechanical properties such as ultimate load (N), stiffness (N/mm) and energy absorption (mJ) (Fig. 6a–c). Our study is also consistent with the above findings in that ovariectomy resulted in decreased BMD as well as in reduced biomechanical strength.

Although BMD is an important determinant of bone strength, it does not take into account the architectural changes occurring in trabecular bone (Snyde et al. 1993; Kleerekope et al. 1995). The measurement of trabecular bone microarchitecture may improve the estimation of bone strength (Laib et al. 2001). Micro-CT as a new highresolution digital imaging technique has recently been widely used in the experimental studies to provide detailed quantitative nondestructive analysis of 3D microscopic bone architecture (Sran et al. 2007).

We evaluated the metaphyseal region close to the growth plate of the distal femur because it is the most recently formed trabecular bone and presumably the most sensitive to dietary factors affecting mineralization. As noted previously in the Micro-CT analysis, normal trabecular bone structure could be remarkably destroyed post-OVX (Qi et al. 2012). Consistent with these findings, our results also demonstrated notable trabecular bone deterioration, evidenced by decreased Tb.N, Tb.Th and BV/TV, and increased Tb.Sp and SMI. Further observation suggested that all three doses of ECH treatment could increase the BV/TV, Tb.N and Tb.Th, and decrease Tb.Sp and SMI compared to OVX group (Table 1 and Fig. 5).

Histomorphometric analysis of distal femora also showed that protective action of ECH appears to be due to an increase in bone formation and reduction in bone resorption, evidenced by increasing of Ct.Th., Tb.Th., N.Tb. N.OB. and decreasing the N.OC. (Table 1).

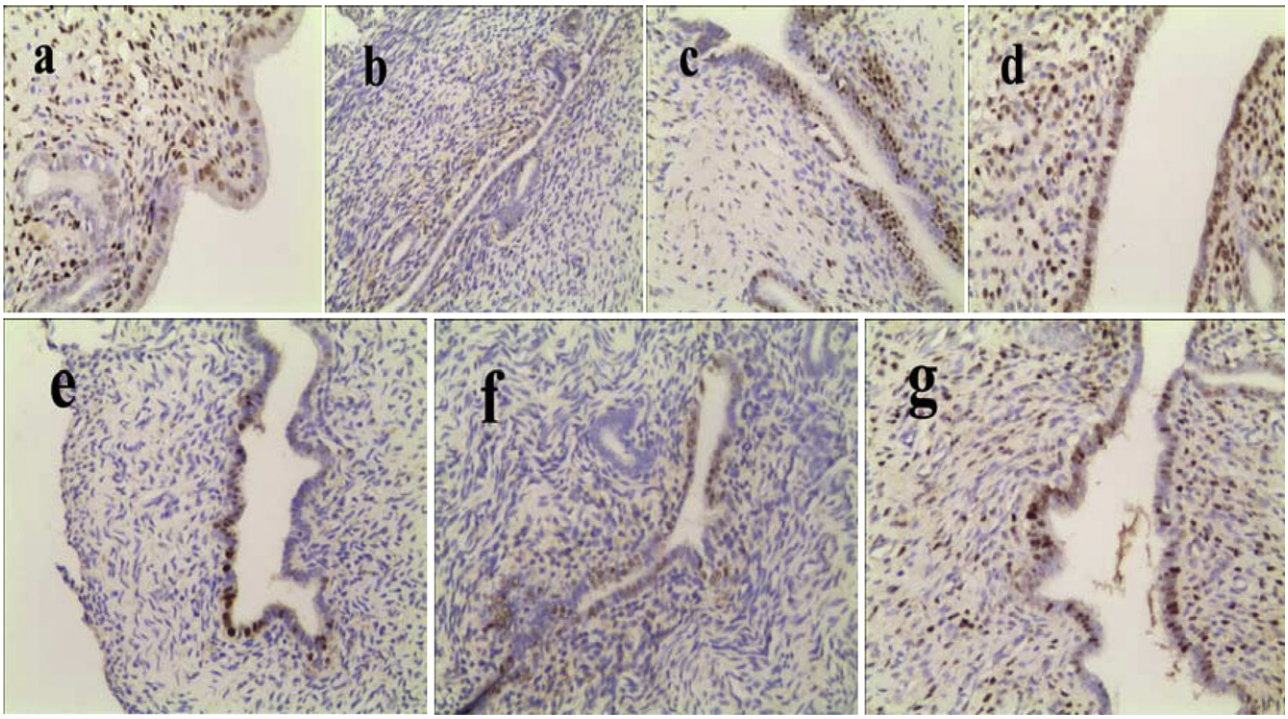

Fig. 7. Representative ER expression of uterus in the endometrium. (a) SHAM group; (b) OVX group; (c) E2 group; (d) ECH-L group; (e) ECH-M group; (f) ECH-H group. The OVX rats presented notable reduction of ER labeling compared with the SHAM rats. ECH and E2 partially prevented OVX-induced ER expression reduction.

Strong evidence indicated that increase in the ratio of osteoblasts to osteoclasts probably accounts for the increased Tb. Th (Ali et al. 2005).

At the molecular level, the effect of estrogen is achieved by acting on the genomic pathway involving estrogen/estrogen receptor interaction. The molecular mechanism of the protective effects of ECH on bone loss may be estrogen response element (ERE)-dependent and mediated through the activation of estrogen receptors (ERs). Thereof, we next examined the levels of ER in the endometrium after 12-week of treatment with a fixed amount of E2 and three doses of ECH. Our results showed that ECH-treated groups for 12 weeks significantly increased the ER expression in the endometrium (Fig. 7), similar as E2 group, which dedicated that the protective effects of ECH from OVX induced bone mass loss by estrogen effect, at least partially.

Our results demonstrated that ECH had a stronger effect than XLGB (containing ICA) on preventing the OVX induced osteoporosis, evidenced by preserving bone mass or preventing bone loss, improving BMD and repairing the femur bone microarchitecture. Recently, the structure activity relationships (SAR) of echinacoside (ECH) and icariin (ICA) have been extensively investigated not only by both theoretical calculations, but also by and experimental studies. SAR, involved in various biological activities, depends on the number and position of different side chains and functional groups (such as prenyl group, hydroxyl groups and methoxy groups). In our study, ECH showed a stronger ability than ICA to preserve bone mass or prevent bone loss, improve BMD and repair the femur bone microarchitecture. The chemical structure of phenylethanoid glycosides (ECH, Fig. 1a) with two pairs of ortho phenolic hydroxyls is more complex than ICA (Fig. 1b) which is a prenylflavonoid glycoside with a glucosyl group on C-3 and a rhamnosyl group on C-7 as well as a methoxyl group on C-4.

Previous reports showed that the antioxidative activity of phenylethanoid glycosides was related to the number and location of phenolic hydroxyl, and the antioxidative activity could be ecnhanced by ortho hydroxyl in the benzoyl ring (Yang et al. 2009). Consistent with that result, some researchers reported that the aglycone part of phenylethanoid glycosides was essential for the potent activity on inhibitory cytotoxicity while 3, 4-dihydroxyl group could also improved the activity (Pan 2011). Meanwhile, other researchers reported that both icaritin (ICT) and desmethylicaritin (DICT), derivatives of icariin (ICA), both markedly enhanced the proliferation of MCF-7 cells, but not ICA (Ye and Lou 2005). Moreover, they The result inferred that while ICA possessing the same features, only the nonconjugated forms (ICT and DICT) exert estrogen-like activities which demonstrated the 7-position hydroxyl on the A ring may be responsible for the estrogen-like activity. However, ICA, biologically inactive *in vitro*, is generally recognized to be the primary effective component in *Epimedii herba* (Feng and Gao 2001). Therefore, the researchers speculated that the pharmacological activity of ICA *in vivo* was related to its active metabolite(s) and suggested that the glucosyl group, rhamnosyl group, and methoxyl group were not necessary for its various activities (Ye and Lou 2005), agreed with that ICA could be metabolized to ICT by human intestinal bacteria (Liu et al. 2000) and the aglycones of flavonoids could readily be released from their sugar components *in vivo* (Kelly and Nelson 1993).

Several studies demonstrate that the phenolic hydroxyl groups in the benzoyl ring, especially in the ortho position, may be highly related to the various activities (Yang et al. 2009; Pan 2011; Ye and Lou 2005), and suggest that the ortho position phenolic hydroxyl groups in the benzoyl ring of ECH are two active groups that take part in preserving bone mass or preventing bone loss, and this may which may be the explainexplanation for why ECH is more potent than ICA in OVX rats in preventing osteoporosis. Furthermore, there was an article reported that additional hydroxyls of the flavone or isoflavone did not decrease activity, while in some instances it may increase binding affinity and the optimum numbers of substituted hydroxyls are 2–4 (Vaya and Tamir 2004).

Therefore, we speculated that the number and location of phenolic hydroxyls, and especially the ortho hydroxyls in the benzoyl ring account for the potent activity of ECH than ICA. As we know,

the traditional category of phytoestrogens contains flavonoid, isoflavonoid, chalconoids and lignans, however, ECH belongs to phenylethanoid glycosides. Therefore, ECH, as a natural derived phenylethanoid glycosides compound and even as a new class of phytoestrogen, could be developed into an effective agent for prevention or treatment of osteoporosis. In conclusion, ECH treatment showed a remarkable antiosteoporotic activity in the adult rat OVX model, may result from the enhancement of bone formation and suppression of bone resorption through estrogen effect, at least partially. Therefore, ECH has promising therapeutic usefulness in prevention or treatment of osteoporosis in humans resulting from estrogen deficiency. Further studies will be focused on predicting the ability of chemicals to bind to the ER with the quantitative structure–activity relationship (QSAR) models, and prediction of agonist/antagonist activities with docking analysis. Moreover, a further step study of ECH QSAR should be undertaken by ECH modification with hydroxylation or methylation in the benzoyl ring. Furthermore, the true mechanisms of ECH on improving bone mass and qualities, and on investigating the role of related protein regulation, as well as target genes and signal pathways are needed to identify.

Conflict of interest statement

Fei Li and Xiaolin Yang contributed equally to this work and should be regarded as co-first authors. Other authors have no conflicts of interest.

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