

Prenylflavonoid Icariin Induces Estrogen Response Element–Independent Estrogenic Responses in a Tissue-Selective Manner

Liping Zhou,¹ Christina Chui-Wa Poon,¹ Ka-Ying Wong,¹ Sisi Cao,¹ Wenxuan Yu,¹ Xiaoli Dong,^{1,2} Wayne Yuk-Wai Lee,³ Yan Zhang,⁴ and Man-Sau Wong^{1,2}

¹Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, PR China; ²State Key Laboratory of Chinese Medicine and Molecular Pharmacology (Incubation), The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen 518057, PR China; ³Department of Orthopaedics & Traumatology, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, PR China; and ⁴Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200000, PR China

ORCID numbers: 0000-0002-9941-7812 (L. Zhou); 0000-0002-7413-295X (S. Cao); 0000-0002-0729-8618 (M.-S. Wong).

Icariin, a flavonoid phytoestrogen derived from *Herba epimedii*, has been reported to exert estrogenic effects in bone and activate phosphorylation of estrogen receptor (ER) α in osteoblastic cells. However, it is unclear whether icariin selectively exerts estrogenic activities in bone without inducing undesirable effects in other estrogen-sensitive tissues. The present study aimed to investigate the tissue-selective estrogenic activities of icariin in estrogen-sensitive tissues in vivo and in vitro. Long-term treatment with icariin effectively prevented bone of ovariectomized (OVX) rats from estrogen deficiency–induced osteoporotic changes in bone structure, bone mineral density, and trabecular properties. Moreover, icariin regulated the transcriptional events of estrogen-responsive genes related to bone remodeling and prevented dopaminergic neurons against OVX-induced changes by rescuing expression of estrogen-regulated tyrosine hydroxylase and dopamine transporter in the striatum. Unlike estrogen, icariin did not induce estrogenic effects in the uterus and breast in mature OVX rats or immature CD-1 mice. In vitro studies demonstrated that icariin exerted estrogen-like activities and regulated the expression of estrogen-responsive genes but did not induce estrogen response element–dependent luciferase activities in ER-positive cells. Our results support the hypothesis that icariin, through its distinct mechanism of actions in activating ER, selectively exerts estrogenic activities in different tissues and cell types.

© Endocrine Society 2019.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Key Words: phytoestrogens, icariin, estrogenic activity, estrogen receptor, estrogen deficiency, tissue-selectivity

The dramatic decrease in circulating estradiol during menopause results in various negative physiological consequences in target tissues that lead to uterine and breast atrophy, and increase the risk of developing osteoporosis and neurodegenerative disorders [1]. Replenishing

Abbreviations: ALP, alkaline phosphatase; BMD, bone mineral density; BS/BV, bone surface over bone volume; BV/TV, bone volume over total volume; C3, complement component 3; DAT, dopamine transporter; ERE, estrogen response element; ERs, estrogen receptors; HH3, histone H3; HRT, hormone replacement therapy; IL-1 β , interleukin-1 β ; μ CT, microcomputed tomography; OCN, osteocalcin; OPG, osteoprotegerin; OVX, ovariectomy; PD, Parkinson disease; RANKL, receptor activator of nuclear factor kappa-B ligand; SERMs, selective estrogen receptor modulators; Tb.N, trabecular number; Tb.Th, trabecular bone thickness; Tb.Sp, trabecular bone separation; TH, tyrosine hydroxylase.

Received 28 April 2019

Accepted 23 November 2019

First Published Online 25 November 2019

Corrected and Typeset 8 February 2020.

February 2020 | Vol. 4, Iss. 2

doi: 10.1210/jendso/bvz025 | Journal of the Endocrine Society | 1–17

this drop with exogenous estrogen (or in combination with progestin) carries considerable benefits and at the same time inevitable risks to postmenopausal women. Although the potential benefits of hormone replacement therapy (HRT) for treatment of vasomotor symptoms and osteoporosis has been highlighted by the North America Menopause Society, the increased risk of reproductive cancers, stroke, and cardiovascular diseases remains a major concern both for menopausal women and the medical community [2].

Selective estrogen receptor modulators (SERMs) are estrogen receptor ligands that serve as either full or partial agonists in some tissue, such as bone, but antagonists in other tissue, such as uterus and breast. Tamoxifen and raloxifene, two of the most widely used SERMs, have been shown to effectively reduce vertebral fracture in postmenopausal women [3]. However, the long-term clinical use of SERMs for prevention and management of postmenopausal symptoms is limited by their reported undesirable side effects, such as uterine pathology in women prescribed tamoxifen, increased thromboembolic events for those prescribed raloxifene and, in some cases, carcinogenesis in postmenopausal women using SERMs [4]. Thus, it is not surprising to find that menopausal women who are unwilling to continue HRT or SERMs would turn to the use of complementary and alternative medicine [5].

Phytoestrogens derived from natural plants are one of the most popular alternative approaches for management of postmenopausal syndrome [6]. The most common examples of phytoestrogens are isoflavones, flavonoids, and lignans [7]. Recent reports indicated that phytoestrogens can attenuate bone loss associated with estrogen deficiency both in animal and human studies [8-10]. The majority of actions of estrogen in the human body are mediated by estrogen receptors (ERs), ER α , ER β , and more recently, G-protein coupled estrogen receptor [11]. Estrogen-dependent signaling pathways that trigger cellular events can be classified as either genomic or nongenomic [11]. In genomic signaling, ERs on binding with their ligands are activated and induce alteration of gene transcription via interaction with estrogen response elements (EREs) or interaction with other transcription factors like activator protein or Sp-1 and then binding to their cognate DNA element. In contrast, nongenomic signaling [11] is mediated by extranuclear ER and requires unique posttranslational modifications and protein-protein interactions of the receptor with adaptor molecules, G proteins, and kinase [12]. These nongenomic pathways will account for rapid changes in cellular events in estrogen-sensitive tissues induced by estrogen. Because the in vivo effects of phytoestrogens are similar to estrogens and their actions are mediated through similar ERs, there are increasing concerns for the safety of using phytoestrogens [7]. Thus, the ideal phytoestrogens for management of menopause-associated symptoms should exhibit selectivity in estrogen-sensitive tissues without undesirable stimulation of the growth of reproductive tissues.

Icariin is a flavonoid glucoside isolated from *Epimedium brevicornum* Maxim. (*Herba epimedii*, HEP), the most frequently prescribed herb used for clinical management of bone diseases in China. Our studies [13-15] as well as others [16] have shown that icariin is the bioactive compound that accounts for the osteoprotective effects of HEP. A daily dose of a preparation containing 60 mg icariin, 15 mg daidzein, and 3 mg genistein was shown to be effective in preventing bone loss in late postmenopausal women in a 24-month randomized, double-blind, and placebo-controlled trial [10]. Most important, no incidence of breast cancer or cardiovascular events was reported in the postmenopausal women after 2 years of icariin treatment [10], suggesting that icariin could exert tissue-specific effects in bone. Furthermore, our in vitro studies demonstrated that the stimulatory effects of icariin on osteoblastic functions were ER dependent. However, unlike genistein, icariin did not stimulate ER α - or ER β -mediated, ERE-dependent luciferase activities but were able to rapidly induce ER α phosphorylation at serine 118 and serine 167 in osteoblastic cells [13]. These results suggest that icariin is a unique class of phytoestrogens that can activate ER through rapid nongenomic signaling pathways in bone cells. However, it is unclear whether icariin exerts tissue-selectively bone-protective effects without inducing adverse effects in other estrogen-sensitive tissues.

The present study was designed to characterize the estrogenic effects of icariin *in vivo* using mature ovariectomized (OVX) rats and immature CD-1 mice model and *in vitro* using 4 estrogen-sensitive cell lines.

1. Methods and Materials

A. Animal Experiment 1: Long-term Estrogenic Activities of Icariin in Mature Ovariectomized Rats

A-1. Preparation of control diets containing icariin

Icariin was purchased from Shanghai Winherb Medical Technology Co, Ltd (catalog No. 25810, Shanghai, China) and formulated into diets at 3 doses by Research Diet, Inc (New Brunswick, NJ, USA). The purity of icariin is 99.3%, as shown in the high-performance liquid chromatography profile of the icariin preparation (Fig. 1) [17].

A-2. Animal study and sample collection

The present experiment was conducted under the animal license issued by the Department of Health, Hong Kong Special Administrative Region Government, and the Hong Kong Polytechnic University Animal Subjects Ethics Sub-committee (ASESC Case: 13/18). Sixty 6-month-old female Sprague-Dawley rats weighing 230 ± 20 g were purchased from The Chinese University of Hong Kong and housed in centralized animal facilities of The Hong Kong Polytechnic University on a 12-hour light and dark cycle. Water and food were available *ad libitum*. After 1 week of acclimatization, all the rats were given bilateral OVX or sham operation. During the preliminary experiment, rats were allowed to consume diets *ad libitum* and the daily amount of intake was recorded for 5 days. Based on the preliminary study, the mean daily intake of diet for each rat was established as 15 g (the minimal amount of daily intake) in the present study. On recovery for 2 weeks, the OVX rats were randomly subjected to oral administration with vehicle (OVX), 17 β -estradiol (E2, 1.0 mg/kg/d), and icariin at 3 doses of 50 ppm (ICA-50 ppm, D15061901, 0.05 g of icariin in 1 kg of diet), 500 (ICA-500 ppm, D15061902, 0.5 g/kg diet) and 3000 ppm (ICA-3000 ppm,

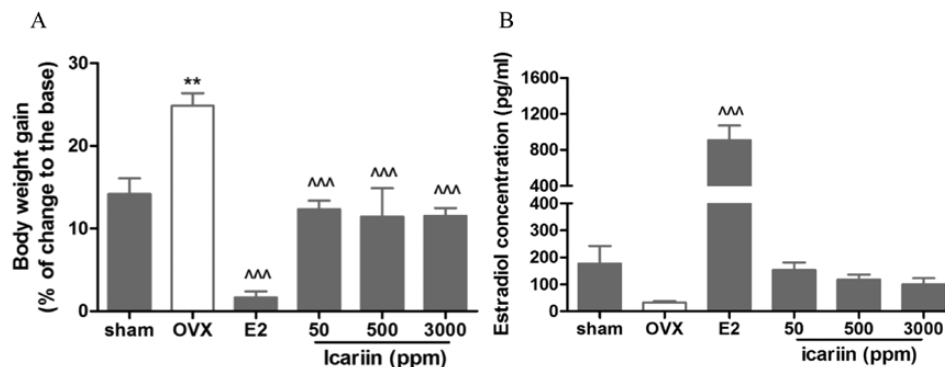


Figure 1. Body weight gain and serum estradiol in mature ovariectomized rats in response to treatment with icariin or 17 β -estradiol. Mature Sprague Dawley rats were given ovariectomy (OVX) or sham operation. After 2 weeks' recovery, OVX rats were treated with either vehicle, 17 β -estradiol (E2, 1.0 mg/kg/d), or icariin at 50 ppm, 500 ppm, and 3000 ppm for 12 weeks. Body weight was measured every 2 weeks. On treatment, serum level of estradiol was measured by using an EIA kit (CayMan). A, Body weight gain and percentage of changes to baseline were compared between groups. B, On treatment, circulating estradiol level and serum estradiol level. Data are expressed as mean \pm SEM. n = 6 to 12. Differences between groups were determined by 1-way analysis of variance followed by Tukey test for post hoc comparison. ** $P < .01$ vs sham; ^^ $P < .001$ vs OVX.

D15061903, 3.0 mg/kg diet) in the form of icariin-containing diets for consecutive 12 weeks. The sham-operated rats were administered vehicle (sham). During the whole recovery period and treatment, the rats in the sham, OVX, and E2 treatment groups were paired-fed the control phytoestrogen-free diet (AIN93-M, D00031602) to remove the influences of phytoestrogens. The flowchart for the animal experiment and ingredients of the diets have been provided in Supplemental data file (Fig. 2 and Table 1) [17]. During the entire treatment, body weight were measured every 2 weeks to investigate the effects of icariin on body weight gain. One day before the rats were humanely killed, each rat was housed individually in a metabolic cage. Twenty-four-hour urine was collected in metabolic cages, aliquoted, and stored at -80°C . The animals were humanely killed under anesthesia with ketamine/xylazine, blood was collected from the rats' abdominal aortas at death, and aliquots of serum were stored at -80°C . Serum level of estradiol was measured by an Estradiol EIA Kit according to the manufacturer's instruction (CayMan, catalog No. 582251). The uterus was freshly collected, weighed, and stored at -80°C for further measurement. Bilateral striata were freshly isolated and stored at -80°C . The whole left leg and spine were collected and stored at -20°C for microcomputed tomography (μCT). The right leg was freshly collected and stored at -80°C after removal of all soft tissues. Breast tissue together with its surrounding skin was collected in 4% paraformaldehyde and fixed for 6 hours for hematoxylin-eosin (H&E) staining.

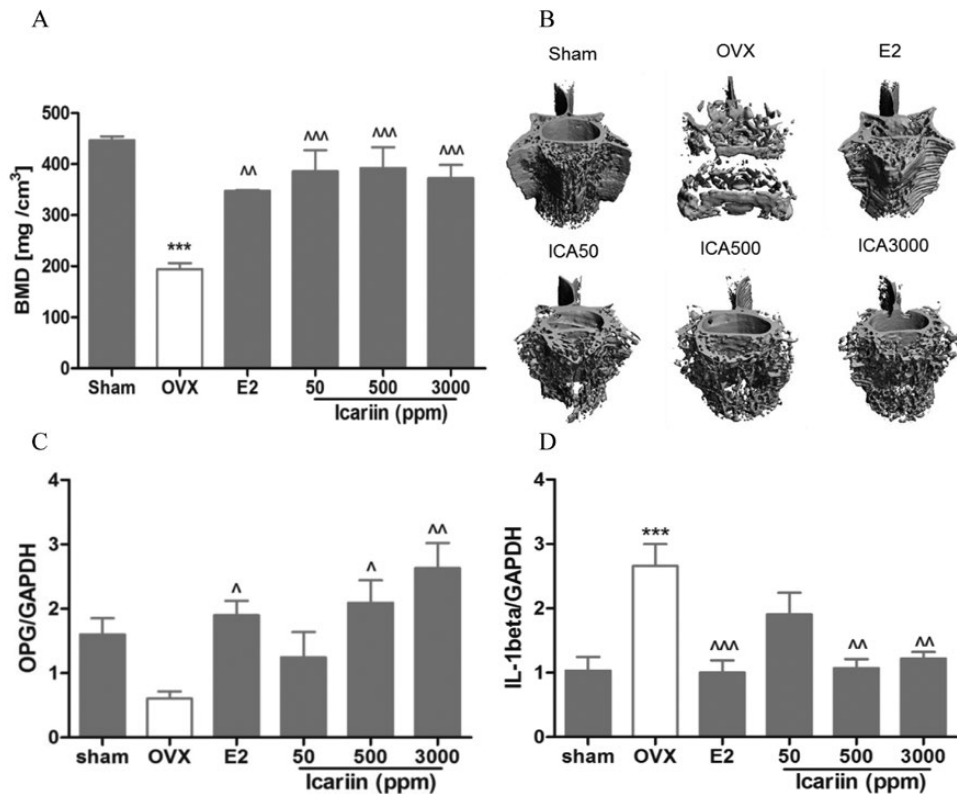


Figure 2. Bone properties and messenger RNA expression of estrogen-sensitive genes in mature ovariectomized rats in response to treatment with icariin or 17 β -estradiol. Mature Sprague Dawley rats were given ovariectomy (OVX) or sham operation. After 2 weeks' recovery, OVX rats were treated with either vehicle, 17 β -estradiol (E2, 1.0 mg/kg/d), or icariin at 50, 500, and 3000 ppm for 12 weeks. A, Bone mineral density (BMD) and B, microarchitecture of lumbar vertebrae were determined by microcomputed tomography. C, Osteoprotegerin (*OPG*) and D, interleukin 1 β (*IL-1 β*) messenger RNA expression in the right tibial head were measured by real-time reverse transcriptase polymerase chain reaction. Data are expressed as mean \pm SEM. n = 5 to 12. Differences between groups were determined by 1-way analysis of variance followed by Tukey test for post hoc comparison. *** $P < .001$ vs sham; ^ $P < .05$, ^^ $P < .01$, ^^ $P < .001$ vs OVX.

Table 1. Effects of icariin on trabecular bone properties at lumbar spine of ovariectomized rats

Groups	BS/BV (1/mm)	BV/TV	Tb.N (1/mm)	Tb.Th (mm)	Tb.Sp (mm)
Sham	18.10 ± 0.26	0.442 ± 0.012	4.12 ± 0.09	0.160 ± 0.003	0.136 ± 0.006
OVX	23.96 ± 0.76 ^a	0.093 ± 0.016 ^a	0.62 ± 0.17 ^a	0.114 ± 0.009	1.313 ± 0.026 ^a
E2	17.89 ± 1.19 ^d	0.351 ± 0.010 ^d	3.71 ± 0.06 ^d	0.235 ± 0.039	0.176 ± 0.006 ^d
Icariin-50	17.40 ± 0.66 ^d	0.138 ± 0.011	0.95 ± 0.04 ^b	0.160 ± 0.015 ^c	0.993 ± 0.036 ^d
Icariin-500	16.44 ± 1.11 ^d	0.200 ± 0.039 ^c	1.07 ± 0.09 ^c	0.170 ± 0.011 ^c	0.888 ± 0.063 ^d
Icariin-3000	17.49 ± 1.50 ^c	0.151 ± 0	1.15 ± 0.12 ^d	0.162 ± 0.012 ^c	0.929 ± 0.065 ^d

Trabecular bone properties of lumbar vertebra were determined by microcomputed tomography. Data are expressed as mean ± SEM. n = 8 to 12. Differences between groups were determined by 1-way analysis of variance followed by Tukey test for post hoc comparison.

Abbreviations: BS/BV, bone surface over bone volume; BV/TV, bone volume over total volume; E2, 17β-estradiol; OVX, ovariectomy; Tb.N, trabecular bone number; Tb.Th, trabecular bone thickness; Tb.Sp, trabecular bone separation.

^a*P* < .001 vs sham. ^b*P* < .05. ^c*P* < .01. ^d*P* < .001 vs OVX.

A-3. Microcomputed tomography analysis

Bone properties of trabecular bone at the lumbar vertebra were determined by μCT (μCT40, Scanco Medical, Switzerland) as previously described [18]. A total of 150 μCT slices were acquired in the middle part of the lumbar vertebra (L4) (middle point ± 75 slices) and the volume of interest was contoured from 100 slices of the middle L4 vertebra. Bone mineral density (BMD, mg/cm³) and bone morphometric properties, including bone surface over bone volume (BS/BV, 1/mm), bone volume over total volume (BV/TV), trabecular bone number (Tb.N, mm⁻¹), trabecular bone thickness (Tb.Th, mm), and trabecular bone separation (Tb.Sp, mm), were evaluated by contoured volume of interest images.

A-4. Hematoxylin-eosin staining

Breast samples were collected and fixed in 4% paraformaldehyde for 6 hours. After dehydration (Leica TP1020), tissues were embedded in paraffin. Then, 8-μm-thick sections were produced for each sample. To observe the structural changes within the breast in response to treatment with icariin, H&E staining was performed. A minimum of 5 sections from each sample were observed using 400× magnification and photographed using a photomicroscope (Olympus BX51).

B. Animal Experiment 2: Short-Term Estrogenic Activities of Icariin in Immature CD-1 Mice

The present experiment was conducted under the animal license issued by the Department of Health, Hong Kong Special Administrative Region Government, and The Hong Kong Polytechnic University Animal Subjects Ethics Sub-committee (ASESC Case: 13/18). Fifty immature female CD-1 mice purchased from The Chinese University of Hong Kong at age 21 days were orally administered vehicle, 17β-estradiol (0.1 mg/kg), and icariin at 0.2, 2.0, and 20 mg/kg for 7 days. This dose range (0.2-20 mg/kg) is at the low level as compared to those (1-750 mg/kg) used to study the in vivo functions of icariin as reported in animals studies [16, 19] and is also consistent with the dose used in a clinical study of icariin for management of osteoporosis [10]. On treatment, the uterus and mammary gland were collected for weight measurement and histology observation.

C. In Vitro Study

C-1. Cell culture and treatment

MCF-7 cells (ATCC HTB-22), Ishikawa cells (kindly provided by Dr Lihui Wei at Peking University People's Hospital), SH-SY5Y cells (ATCC CRL-2266, kindly provided by Prof Wenfang Chen at Qingdao University), and MG-63 cells (ATCC CRL-1427) were routinely cultured according to ATCC's instructions. Cells were seeded in 96-well, 24-well, or 6-well plates at a density of 0.8×10^3 , 2.0×10^4 or 15×10^4 per well, respectively, for different assays. After 24 hours, the medium was changed to phenol red-free (PRF) medium containing charcoal-stripped fetal bovine serum (cs-FBS) for another 24 hours. Then, cells were treated with icariin at various concentrations for 48 hours.

C-2. MTS assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was used as indirect colorimetric measurement of cell proliferation. Briefly, on treatment with icariin (10^{-12} to 10^{-5} M) for 48 hours, medium was discarded and replaced with 100 μ l of MTS/phenazine methosulfate working solution (Promega, Madison, WI, USA). Absorbance at 490 nm was measured with a microplate reader (CLARIOstar, BMG LABTECH) after incubation at 37°C for 1 to 4 hours. Relative absorbance to control group was analyzed and compared between treatment groups and controls.

C-3. Alkaline phosphatase activity assay

Cells were harvested after treatment and lysed in 100 μ l of passive lysis buffer (Promega) by incubating on ice for 20 minutes. The alkaline phosphatase (ALP) activity of cell lysate was measured by a LabAssay ALP kit (Wako, Japan) following the manufacturer's instruction. Total protein concentrations of the cell lysate were measured via the Bradford method to normalize ALP activity.

C-4. Estrogen response element luciferase reporter activity assay

Cells were seeded in 24-well plate at a density of 2.0×10^4 /well. Twenty-four hours later, cells were transfected with 0.4 μ g of ERETKluc plasmid together with 0.01 μ g of an inactive control plasmid pRL-TK, a Renilla luciferase control vector, by Lipofectamine 2000 reagent (Invitrogen) in PRF medium without antibiotics. Six hours after transfection, the medium for transfection was discarded and cells were subjected to treatment with icariin at optimal concentrations determined by the previously listed assays in PRF medium containing cs-FBS for 24 hours. On treatment, the medium was discarded and cells were lysed with 100 μ l of passive lysis buffer and collected for luciferase activity measurement. Luciferase activity was measured by a Dual Luciferase Reporter Assay System (Promega) and the signal detected by a TD-20/20 Luminometer (Turner Design, USA) following the manufacturer's instructions. Results were expressed as the ratio to control.

D. Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction Assay

Samples from animal experiment 1, including the right tibial head, uterus, and striatum, were homogenized in Trizol reagent by using Precellys 24 homogenizer (Bertin, France). Treated cells from in vitro studies were homogenized in Trizol reagent. Total RNA extraction, reverse transcriptase (RT), and quantitative polymerase chain reaction (PCR) were carried out as previously described [18]. Briefly, 2 μ g of total RNA was RT into complementary DNA

(cDNA) by using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) following the manufacturer's instructions. A total of 20 μ l of PCR reaction system consisting of 1 μ l cDNA, 0.4 μ l forward and reverse primers, 8.2 μ l of DNase and RNase-free water, and 10 μ l of SsoFast EvaGreen Supermix (Bio-Rad) was performed by using an Iq5 Multicolor Real-time PCR Detection System (Bio-Rad, IQ5). Sequences and conditions primers are shown in Reference [17].

E. Immunoblotting

Samples from animal experiment 1 were homogenized in Nonidet P-40 buffer (20 mM, Tris-HCl, 150 mM NaCl, 5 mM EDTA, 67 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, and 1% Triton X-100 [vol/vol]; pH 7.5) supplemented with protease inhibitors (2 μ g/ml aprotinin, 2 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) in liquid nitrogen by using Precellys 24 homogenizer (Bertin, France). Cells from in vitro studies were homogenized in Nonidet P-40 buffer. Total protein was collected after centrifugation, and concentrations of protein were measured by Bradford assay (Bio-Rad Lab, USA). A total of 50 μ g of protein was loaded and separated on 10% sodium dodecyl sulfate–polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane (Millipore, Danvers, MA, USA). On blocking with 10% nonfat milk, the membrane was incubated with rabbit tyrosine hydroxylase (TH; 1:1000, Millipore) [20], dopamine transporter (DAT; 1:4000, Sigma) [21], and mouse anti- β -actin (1:1000, Thermo Fisher Scientific, USA) [22] as primary antibody. This was followed by incubation with goat anti-rabbit immunoglobulin G (1:2000, Abcam, USA) [23] or anti-mouse immunoglobulin G (1:2000, Thermo Fisher Scientific) [24] conjugated with horseradish peroxidase as the secondary antibodies. The antigen-antibody complexes were then detected with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) reagent and visualized by an Azure c600 imaging system (Azure Biosystem, Dublin, CA, USA). The intensity of each band was quantified using the Image J program (National Institutes of Health, Bethesda, MD, USA). The expression level of target protein was normalized by β -actin and the relative expression level was compared between groups.

F. Statistical Analysis

Data were reported as mean \pm SEM. Intergroup differences of the in vivo study were analyzed by 1-way analysis of variance with Tukey post hoc test. Differences between the treatment group and controls in the in vitro study were determined by independent t test. A *P* value less than .05 was considered statistically significant.

2. Results

A. Long-Term Estrogenic Activities of Icariin in Mature Ovariectomized Rats

On treatment for 3 months, OVX rats showed the highest body weight gain (24.83%, Fig. 1A, *P* < .05). Exposure to E2 significantly suppressed body weight gain to 1.65% in OVX rats (*P* < .05). Similarly, icariin at 50, 500, and 3000 ppm significantly suppressed the body weight gain of OVX rats to 12.3%, 11.4%, and 11.5%, respectively (*P* < .05), indicating icariin exerted an estrogen-like effect on body weight gain of OVX rats. Serum level of estradiol decreased in OVX rats (32.7 \pm 6.2 pg/ml) compared with 176.7 \pm 65.1 pg/ml in sham rats, indicating the success of the operation. Serum estrogen level was completely reversed to 909.5 \pm 162.7 pg/ml by administration of exogenous estrogen (Fig. 1B, *P* < .05). Icariin appeared to increase the circulating level of estradiol, and the change induced by icariin at 50 ppm reached statistical significance on excluding sham and E2 groups in the 1-way analysis of variance analysis. The increase in serum estradiol might account for the reduction in body weight of OVX rats on treatment with icariin.

Bone and the central nervous system are 2 sensitive tissues of estrogens in which they serve not only as a reproductive hormone. Thus, the estrogenic activities of icariin were evaluated not only in the uterus and breast, but also in bone and brain tissues in OVX rats. The effects of E2 and icariin on bone properties at the lumbar spine of OVX rats are summarized in Table 1 and Fig. 2A and 2B. Compared to sham rats, OVX led to significant bone loss at the lumbar spine of OVX rats as indicated by the 56.5% reduction in BMD (Fig. 2A, $P < .05$) and the deterioration of bone structure (Fig. 2B) and changes in trabecular properties (Table 1), including decreased BV/TV, Tb.N, and Tb.Th, and increased BS/BV and Tb.Sp. Exposure of OVX rats to E2 for 12 weeks dramatically increased BMD by 78.8% (Fig. 2A) and rescued the OVX-induced changes at the lumbar vertebra of OVX rats ($P < 0.05$). Icariin mimicked the effects of E2 on BMD and trabecular bone properties at the lumbar spine of OVX rats at a dose as low as 50 ppm (Table 1, Fig. 2A and 2B, $P < .05$).

The effects of icariin on the expression of interleukin-1 β (*IL-1 β*) and osteoprotegerin (*OPG*), 2 important estrogen-regulated genes, in bone tissues were also evaluated by RT-PCR. As expected, the expression of *OPG* messenger RNA (mRNA) (Fig. 2C) was decreased and the expression of *IL-1 β* mRNA (Fig. 2D) was increased in the tibial head of OVX rats. E2 stimulated *OPG* mRNA (Fig. 2C) and significantly suppressed *IL-1 β* mRNA (Fig. 2D, $P < .05$). Icariin at 500 ppm and 3000 ppm mimicked the effects of E2 to significantly stimulate *OPG* mRNA expression in the tibial head of OVX rats (Fig. 2C, $P < .05$), whereas icariin at 500 and 3000 ppm mimicked the effects of E2 to markedly inhibit *IL-1 β* mRNA expression in the tibial head of OVX rats (Fig. 2D, $P < .05$).

Striatum, a main part of the substantia nigra-striatum dopaminergic system that regulates reward and movement, was collected to investigate the possible estrogenic activities of icariin on brain tissue. The expression of 2 estrogen-regulated genes, *TH* and *DAT*, in collected striatum were evaluated. The expression of *TH* mRNA was significantly decreased by 64.4%, whereas the expression of *DAT* mRNA was significantly increased by 111.3% in striatum in OVX rats when compared to sham rats (Fig. 3A, $P < .05$). Treatment with E2 completely restored the levels of *TH* and *DAT* mRNA expression in OVX rats to those of sham rats (Fig. 3A, $P < .05$). Similarly, icariin at 500 ppm and 3000 ppm significantly induced *TH* mRNA expression (2.4-fold and 2.3-fold, respectively) and reduced *DAT* mRNA expression (0.51-fold and 0.43-fold, respectively) in striatum of OVX rats (Fig. 3A, $P < .05$). The changes in TH and DAT protein expression in striatum by OVX were also restored by treatment with E2 (Fig. 3B). Most important, icariin at 500 ppm and 3000 ppm mimicked the stimulatory effects of E2 and increased TH protein expression (1.3-fold and 1.5-fold, respectively) (Fig. 3B, $P < .05$), whereas icariin at 50 ppm and 500 ppm mimicked the inhibitory effects of E2 and decreased DAT protein expression in striatum of OVX rats (0.21-fold and 0.57-fold, respectively) (Fig. 3B, $P < .05$).

Fig. 4A shows that uterus weight was dramatically reduced in OVX rats to about 25% that of sham rats, and was markedly restored in OVX rats by E2 to about 80% that of sham rats ($P < .05$). To further investigate the estrogenic activities of icariin in the uterus, mRNA expression of estrogen-responsive genes, complement component 3 (*C3*) and histone H3 (*HH3*), were measured in the uterus by RT-PCR. Treatment with E2 completely restored the OVX-induced decrease in *C3* and *HH3* mRNA expression in the uterus of OVX rats (Fig. 4B and 4C, $P < .05$). Unlike E2, icariin at all 3 doses did not alter either uterus weight or the mRNA expression of *C3* and *HH3*, indicating the action of icariin is distinct from those of E2 in the uterus of OVX rats.

Breast is another major target tissue of E2. To investigate the estrogenic action and potential adverse effects of icariin on breast tissue, the morphology of the mammary gland was visualized by H&E staining. As shown in Fig. 4D, a slight glandular atrophy was observed in OVX rats when compared to sham. Treatment with E2 stimulated mammary glands (shown by the red arrow) of OVX rats as indicated by the increased number and size of mammary ducts (vs OVX rats). In contrast, icariin at all doses did not alter the morphology of breast tissue in OVX rats (Fig. 4D), confirming that the actions of icariin were distinct from E2 in breast tissue.

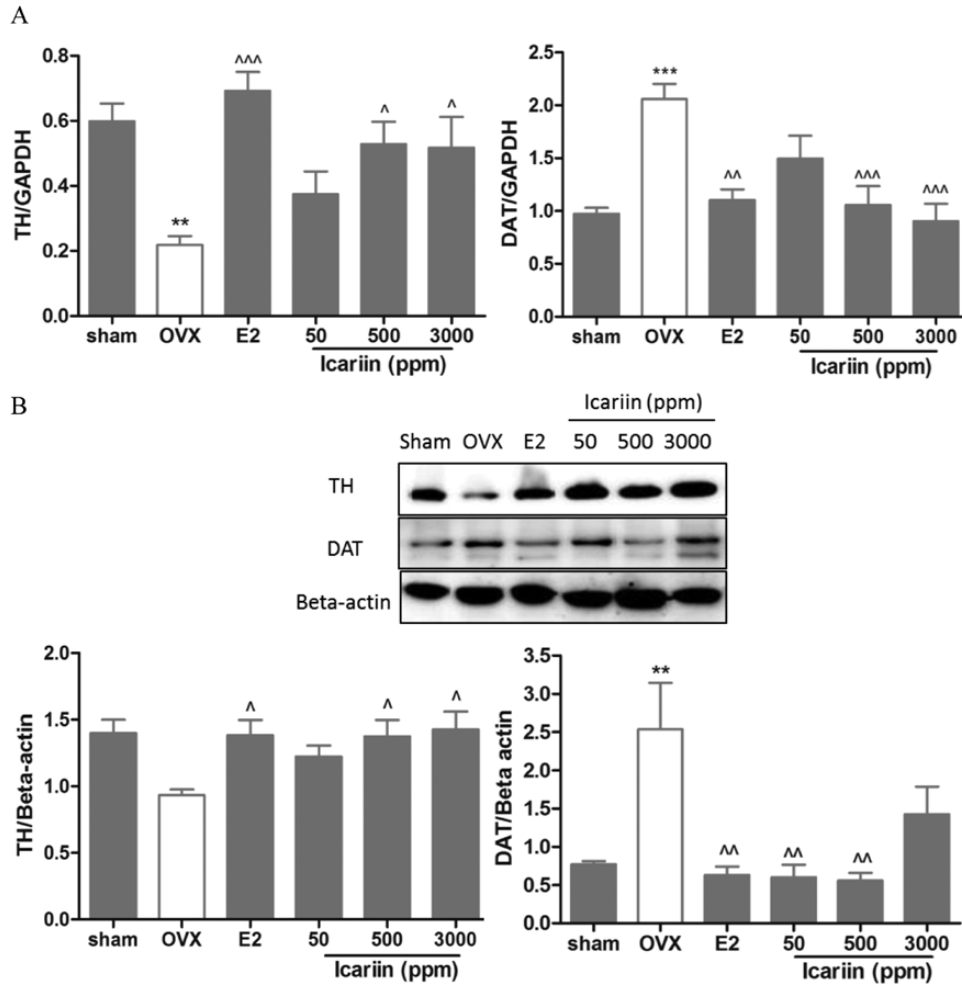


Figure 3. Expression of tyrosine hydroxylase (TH) and dopamine transporter (DAT) in striatum of mature ovariectomized rats in response to treatment with icariin or 17 β -estradiol. Mature Sprague Dawley rats were given ovariectomy (OVX) or sham operation. After 2 weeks' recovery, OVX rats were treated with either vehicle, 17 β -estradiol (E2, 1.0 mg/kg/d), or icariin at 50 ppm, 500 ppm, and 3000 ppm for 12 weeks. A, mRNA expression of *TH* and *DAT* was determined by real-time reverse transcriptase polymerase chain reaction. B, Protein expression of TH and DAT were determined by Western blot. Data are expressed as mean \pm SEM. n = 5 to 12. Differences between groups were determined by 1-way analysis of variance followed by Tukey test for post hoc comparison. ** P < .01, *** P < .001 vs sham; [^] P < .05, ^{^^} P < .01, ^{^^^} P < .001 vs OVX.

B. Short-Term Estrogenic Activities of Icariin in Immature Female CD-1 Mice

Estrogenic activities of icariin in reproductive tissues were also evaluated in immature CD-1 mice, which are highly sensitive to estrogenic stimulation because their circulating estrogen levels are prepubertal. In immature CD-1 mice, E2 induced the development of the uterus, as indicated by the increased uterus weight (Fig. 5A) and the thickening of the endometrial granular epithelium (hyperplasia) (Fig. 5B). In contrast, icariin at all doses did not alter uterus weight. However, icariin at 20 mg/kg might have slight effects in altering endometrium epithelial thickness (Fig. 5B). In breast of immature CD-1 mice, E2 induced changes in the morphology (as indicated by arrow) of the mammary glands (Fig. 5C). Icariin at 0.2 and 2.0 mg/kg did not alter the morphology of the mammary glands while appearing to have a moderate effect in stimulating the development of the mammary glands at 20 mg/kg. These results indicate that icariin at a high dose might exert estrogenic effects on the reproductive tissues in immature mice.

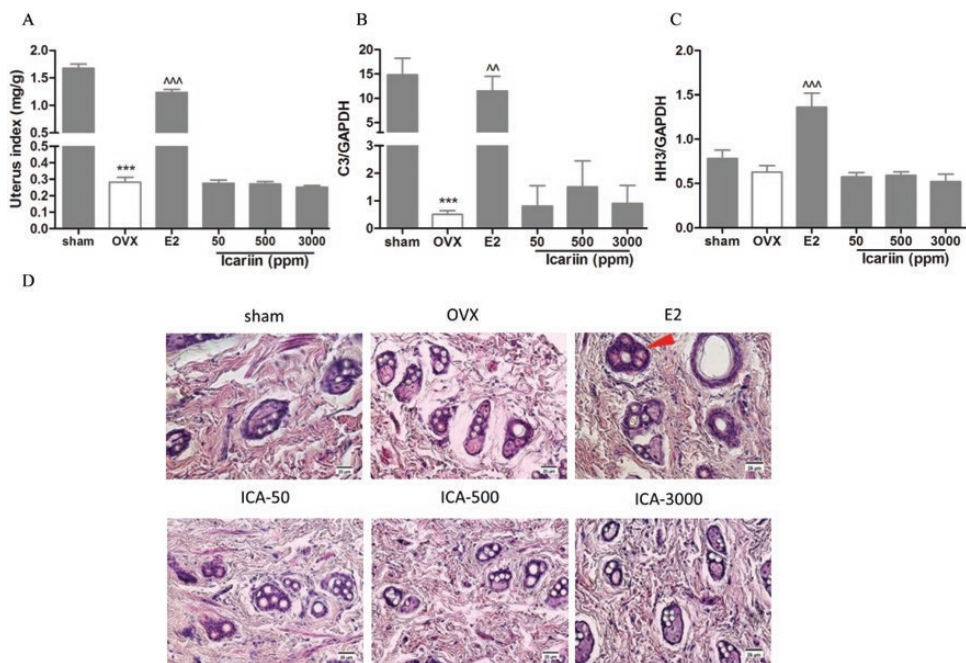


Figure 4. Estrogenic responses to icariin or 17 β -estradiol in the uterus and mammary gland of mature ovariectomized rats. Mature Sprague Dawley rats were given ovariectomy (OVX) or sham operation. After 2 weeks' recovery, OVX rats were treated with either vehicle, 17 β -estradiol (E2, 1.0 mg/kg/d), or icariin at 50 ppm, 500 ppm, and 3000 ppm for 12 weeks. A, Uterus index was calculated as uterus weight/body weight. B, Messenger RNA expression of component complement 3 (C3) and C, histone H3 (HH3) was determined by real-time polymerase chain reaction. D, Histology image of mammary gland (400 \times , indicated by arrow) as revealed by hematoxylin-eosin staining. Data are expressed as mean \pm SEM. n = 8 to 12. Differences between groups were determined by 1-way analysis of variance followed by Tukey test for post hoc comparison. *** P < .001 vs sham; ^^ P < .01, ^^^ P < .001 vs OVX.

C. Direct Estrogenic Activities of Icariin In Vitro

The direct estrogenic effects of icariin were evaluated in human osteosarcoma MG-63 cells, neuroblastoma SH-SY5Y cells, endometrial Ishikawa cells, as well as breast cancer MCF-7 cells, in line with the 4 tissues characterized in the OVX rats. Icariin exerted stimulatory effects on ALP activity in MG-63 cells in a dose-dependent manner, and the optimal effects appeared at 10^{-10} and 10^{-9} M (Fig. 6A, P < .05). In addition, icariin at 10^{-10} and 10^{-9} M mimicked the effects of E2 in inducing the mRNA expression of osteocalcin and *OPG* and suppressing the mRNA expression of *RANKL* (receptor activator of nuclear factor kappa-B ligand) in MG-63 cells (Fig. 6A, P < .05). However, unlike E2, icariin did not alter ERE luciferase activity in MG-63 cells, suggesting that the estrogenic effects induced by icariin are ERE independent. The effects of icariin on cell viability and ERE luciferase activity in SH-SY5Y cells were studied. Icariin mimicked the effects of E2 and dose-dependently promoted cell viability from 10^{-12} to 10^{-5} M in SH-SY5Y cells (Fig. 6B). However, unlike E2, icariin did not induce ERE luciferase activity in transfected SH-SY5Y cells, indicating that the estrogenic effects of icariin are ERE independent.

The estrogenic effects of icariin in Ishikawa cells were evaluated by studying its effects on ALP activity and mRNA expression [25]. Similar to E2, icariin significantly induced ALP activities at 10^{-10} and 10^{-8} M (Fig. 6C, P < .05) and mRNA expression at 10^{-9} and 10^{-8} M in Ishikawa cells (Fig. 6C, P < .05). However, unlike E2, icariin did not activate ERE luciferase activity in Ishikawa cells (Fig. 6C). The estrogenic effects of icariin in MCF-7 cells were evaluated by studying its effects on cell proliferation and *pS2* mRNA expression. Similar to E2, icariin significantly increased cell viability at 10^{-12} to 10^{-5} M and promoted *pS2* mRNA

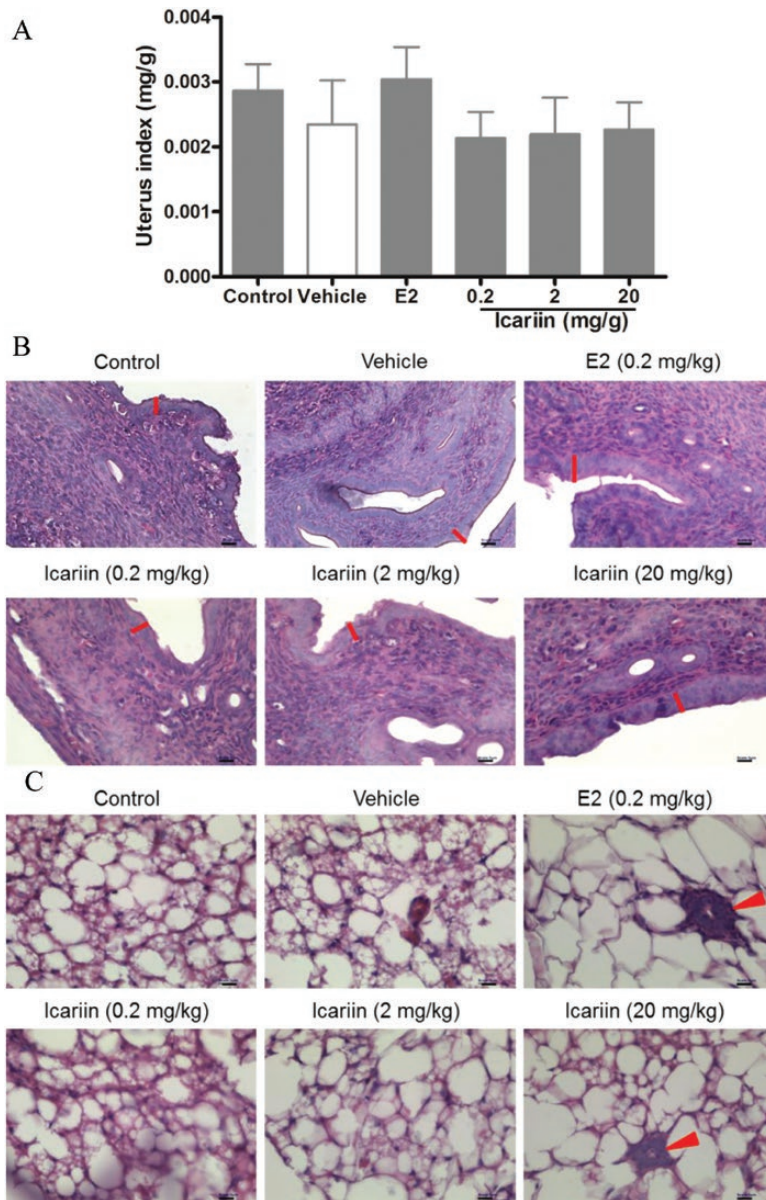


Figure 5. Estrogenic responses to icariin or 17 β -estradiol in the uterus and mammary gland of immature CD-1 mice. Twenty-one day-old female CD-1 mice were orally administered vehicle, 17 β -estradiol (0.1 mg/kg), and icariin at 0.2, 2.0, and 20 mg/kg for 7 days. A, Uterus index. B, Morphology of endometrium (400 \times , thickness of endometrium indicated by length of red line). C, Morphology of mammary gland (400 \times , lactiferous duct indicated by arrow). Data are expressed as mean \pm SEM. n = 7. Differences between groups were determined by 1-way analysis of variance followed by Tukey test for post hoc comparison.

expression at 10^{-8} and 10^{-7} M in MCF-7 cells (Fig. 6D, $P < .05$). However, unlike E2, icariin did not induce ERE luciferase activity in transfected MCF-7 cells (Fig. 6D). These studies indicate that the estrogenic effects of icariin both in Ishikawa and MCF-7 cells were ERE independent.

3. Discussion

Our previous studies [13-15] demonstrated that icariin is a phytoestrogen that exerts bone-protective effects via the nongenomic rapid estrogen signaling pathway. The present

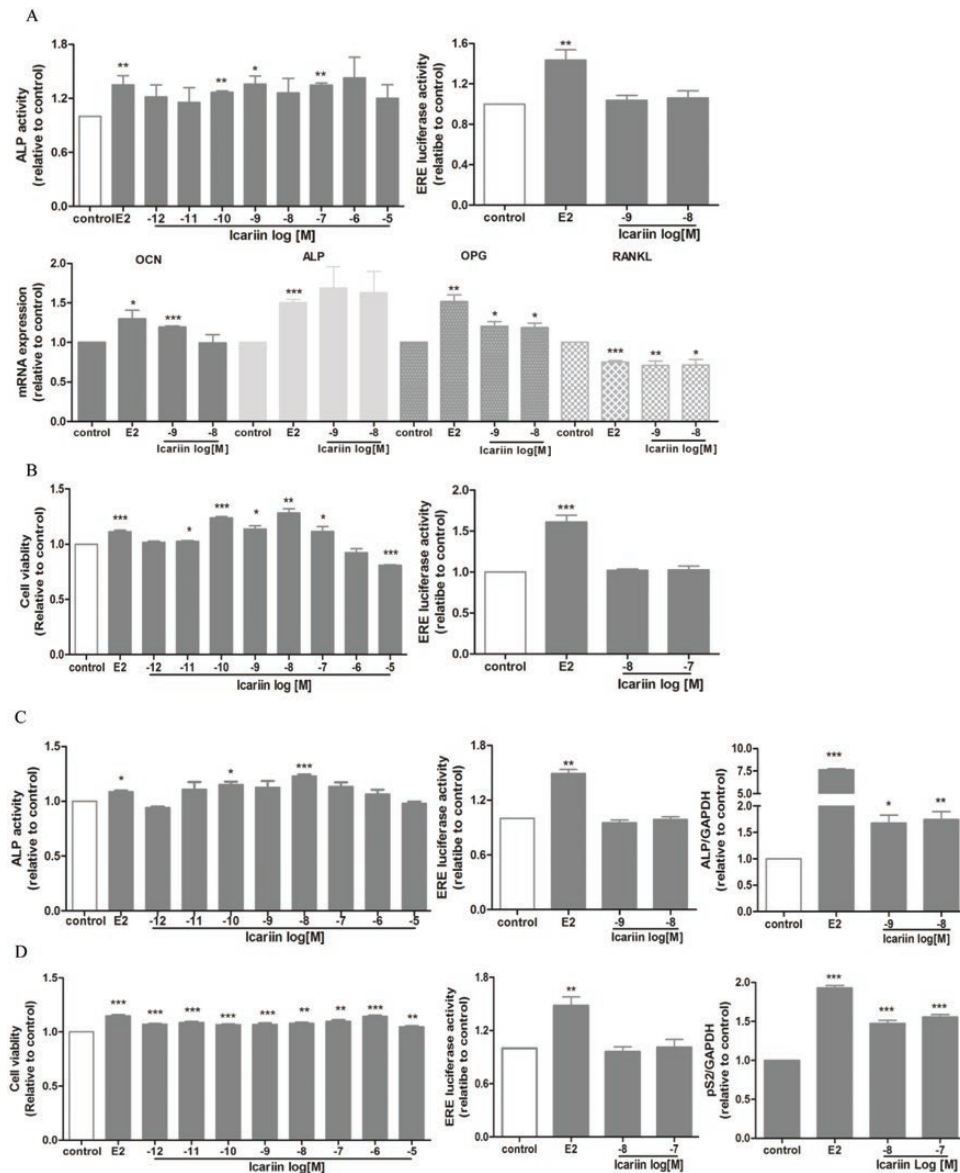


Figure 6. Estrogenic activities in 4 estrogen-sensitive human cell lines. Human osteosarcoma (MG-63), neuroblastoma (SH-SY5Y), endometrial (Ishikawa), and breast cancer (MCF-7) cells were cultured and subjected to treatment with vehicle, estradiol (10^{-8} M) and various concentrations of icariin (10^{-12} to 10^{-5} M) in phenol red-free medium containing 1% charcoal-stripped fetal bovine serum (cs-FBS). A, MG-63 cells: alkaline phosphatase (ALP) activity, estrogen-responsive element (ERE)-dependent luciferase activity, and messenger RNA (mRNA) expression of osteocalcin (*OCN*), *ALP*, osteoprotegerin (*OPG*), and *RANKL* (receptor activator of nuclear factor kappa ligand). B, SH-SY5Y cells: cell viability and ERE-dependent luciferase activity. C, Ishikawa cells: ALP activity, ERE-dependent luciferase activity, and *ALP* mRNA expression. D, MCF-7 cells: cell viability, ERE-dependent luciferase activity, and *pS2* mRNA expression. Data are expressed as mean \pm SEM. $n = 3$ or more. Results were from 2 independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$ vs control.

study further demonstrates that icariin exerts selective estrogenic effects on bone and striatum, but not the uterus and mammary glands, in mature OVX rats as well as ERE-independent estrogen-like effects in ER-positive cells. These results provide insight into understanding the potential mechanism involved in mediating the tissue-selective estrogenic effects of icariin.

A. A Dichotomy of Effects of Estradiol and Icaritin in Nonreproductive vs Reproductive Target Tissues and Cells

Our study attempted to compare and contrast the effects of icaritin with those of estradiol in estrogen-sensitive tissues. Recent studies indicated that both nuclear ER and nonnuclear ER signaling pathways are essential for mediating the effects of estrogen in bone tissues because blocking the ability of localizing ER α to the cell membrane (with point mutation in the palmitoylation site, so-called NOER [nuclear only-ER] mice) greatly reduced the responses of trabecular bone to estrogen in female mice [26]. Our present study suggests that icaritin acts differently from estrogen in bone because it could only partially restore OVX-induced bone loss in OVX rats and did not induce ERE activity in bone cells. Our study also compared the effects of estrogen and icaritin in the striatum, a main part of the substantia nigra-striatum dopaminergic system that regulates reward and movement. Estrogen and icaritin both protected against changes induced by estrogen deficiency in OVX rats and restored *TH* and *DAT* mRNA and protein expression in the striatum, 2 key proteins involved in dopamine metabolism. The present study also showed that icaritin induced cell viability, but not ERE activity, in neuroblastoma SH-SY5Y cells. These results are in line with our previous studies that reported the neuroprotective effects of icaritin in dopaminergic MES23.5 cells mediated by PI3K/Akt and MEK/ERK signaling pathways [27] and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced, Parkinson disease-model OVX mice [28]. Most important, these studies clearly suggest that icaritin mimics estrogen in bone and brain and is a potential candidate for reducing the risk of menopause-related skeletal and neurological disorders.

The present study showed that the effects of icaritin and estrogen on reproductive tissues appear to be different. Our study showed that estrogen deficiency-induced changes in uterine weight and morphology of the mammary glands could be restored only by E2, but not by icaritin at 50 ppm (2.5 mg/kg/d), 500 ppm (25 mg/kg/d), and 3000 ppm (150 mg/kg/d) administered chronically (at a daily diet intake of 15 g) in OVX rats. Our study using immature CD-1 mice showed that icaritin at 20 mg/kg/d (equivalent dose in rats is 10 mg/kg/d) administered orally for 7 days did not affect uterine weight but induced slight endometrial thickening and development of mammary gland. The discrepancies regarding the responses of the 2 models suggest that immature CD-1 mice are more sensitive to estrogenic stimulation than mature OVX rats because the former are at the crucial period for development with a prepubertal level of circulating estrogen [29]. Nevertheless, our chronic (3-month treatment) study showed that icaritin did not induce estrogenic responses in breast or uterus in OVX rats at a dose 15-fold higher than the highest dose used in the CD-1 mice, indicating that the estrogenic actions of icaritin are indeed tissue selective.

A-1. Selective activation of extranuclear, nongenomic estrogen receptor signaling pathways

Recent studies [30, 31] suggest that the actions of estrogen in uterus and breast tissues are mainly mediated by activation of nuclear ER signaling but not extranuclear rapid ER signaling. Therefore, the lack of effects of icaritin in uterus and breast tissues of OVX rats might be explained by its selective effects in activating extranuclear rapid ER signaling, but not nuclear ER signaling, in these tissues. Indeed, the actions of icaritin appear to be different from those of estrogen as well as other phytoestrogens that exert estrogenic or antiestrogenic activities through its binding to ERs [32]. The most extensively studied phytoestrogen, genistein, is known to exert estrogenic activities by binding to ERs and induce ERE-dependent transcriptional events in estrogen-sensitive tissue [13]. In contrast, icaritin exhibits no affinity for either ER α or ER β and does not induce ERE activity in osteoblastic cells [13, 14]. The present *in vitro* study confirms the ERE-independent estrogenic actions of icaritin in human bone (MG-63) cells and further reports a broad ERE-independent estrogenic action in human neuron-like (SH-SY5Y), breast cancer (MCF-7), and endometrial

cancer (Ishikawa) cells. These results support our *in vivo* observations that the estrogenic action of icariin in ER-positive tissues are not mediated by nuclear ERs. Instead, recent studies by us [13] and others [33–36] show that icariin exerts estrogenic effects in bone cells via nongenomic ER signaling pathways and rapidly activates ER α via MAPK/ERK, PI3K/Akt, and JNK signaling pathways.

A recent study by Gustafsson et al [26] suggested that membrane-initiated ER α signaling plays an important role in mediating tissue-selective estrogen responses in female mice. Pathway preferential estrogen (PaPE) is a newly designed estrogen with lower affinity for both ERs and could preferentially activate the extranuclear ER pathway over the nuclear ER pathway [37]. Our results indicate that icariin mimics the properties of PaPEs to selectively activate the extranuclear-initiated signaling pathway but not the direct nuclear-initiated pathway. PaPE was previously shown to activate MAPK and Akt kinase cascades effectively and regulate the transcription of a subset of ER target genes, despite its very low affinity to ERs and lack of interaction with nuclear ERs [37]. Similarly, our previous published studies [13, 28] indicated that icariin was able to exert estrogenic activities by selectively activating ER α via both MAPK and Akt kinase cascade. Such properties allow PaPEs and icariin to achieve a favorable balance of desired and undesired effects by modulating a pattern of gene regulation, resulting in a series of biological activities both in cells and animals. Unlike E2, PaPEs will not restore the decrease in uterine weight in mature OVX mice nor induce marked ductal growth in OVX mice [37]. Similarly, our study showed that icariin did not induce the growth of uterus and breast tissue in OVX rats and CD-1 mice. Thus, our study suggests that icariin behaves like PaPEs and serves as a naturally occurring extranuclear ER pathway-preferred agent.

A-2. Unique pharmacological properties of icariin

The effects of icariin in the present study were not dose dependent, especially those *in vivo*. Indeed, the bone-protective effects of icariin appeared to be optimal at lower doses than at higher doses, as indicated by the higher BMD in rats treated with 50 ppm and 500 ppm than those with 3000 ppm. Such observation was in agreement with our previously published study regarding the bone-protective effects of total flavonoid (TF) fraction of HEP [38]. In that study, TF fraction of HEP, which also contains icariin, was also more effective at lower doses than at higher doses and its suppressive effects on urinary Ca excretion were associated with changes in renal vitamin D receptor and calbindin 28K mRNA expression. The lack of dose-dependent effects of icariin *in vivo* could be explained by the complex interplay of different physiological systems being affected by the actions of icariin. Our results showed that icariin regulated the circulating estradiol as well as dopamine metabolism, suggesting its effects on the endocrine and neuroendocrine systems. Therefore, apart from its direct estrogenic effects in target tissues, the effects of icariin in other physiological systems might also indirectly modulate its effects *in vivo*.

Icariin appears to act on the endocrine and neuroendocrine systems and significantly increases serum estradiol in OVX rats. Such an induction in serum estradiol might account for the suppressive effects on body weight gain in OVX rats treated with icariin. Indeed, adipose tissues, placenta, and the adrenal gland are a few extragonadal tissues that express aromatase (a key enzyme for the conversion from androgen to estrogen) and become major sources of estrogen in postmenopausal women and OVX rats [39, 40]. Our observation is in agreement with others [41] that demonstrated the increase in circulating estradiol levels by icariin via the increase in biosynthesis of estrogen by inducing aromatase (CYP19) expression. Similar actions on estrogen synthesis through modulation of aromatase expression in extragonadal tissues both in humans and animals have been reported for genistein and other phytoestrogens [42, 43]. Future study will be need to determine whether icariin promotes biosynthesis of estrogen via modulating aromatase activity and expression in extragonadal tissues.

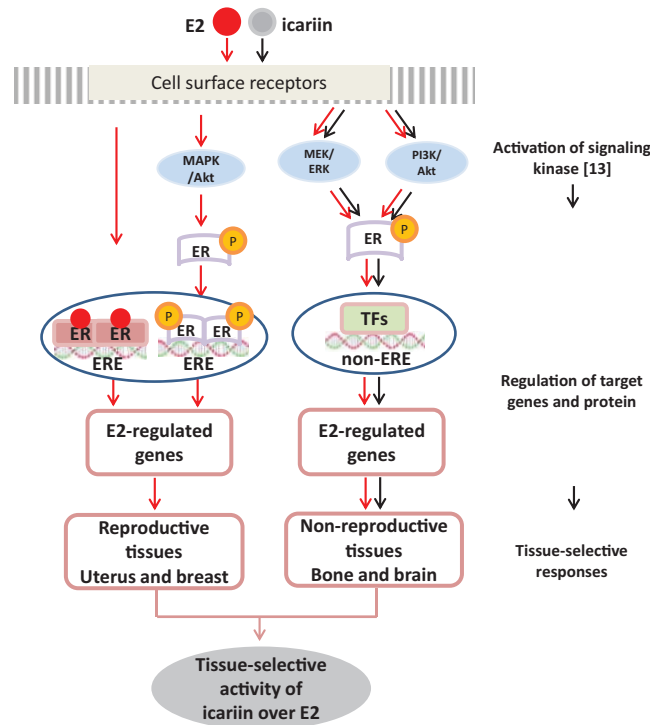


Figure 7. Possible mechanisms of tissue-selective actions of icariin in estrogen-sensitive tissues and estrogen receptor-positive cells. Icariin might 1) rapidly activate the phosphorylation of estrogen receptor (ER α) via phosphorylation of signaling kinase; 2) regulate estrogen-responsive genes via regulating transcription factors through kinase-mediated phosphorylation; and 3) exert estrogenic activities in bone and brain, but not uterus or breast.

4. Conclusions

The estrogenic effects of icariin are distinct from those of estrogen, with presumed different underlying mechanisms that selectively act on nonreproductive target tissues (bone and brain), but not reproductive tissues, by preferential activation of extranuclear ERs. Together with previous findings [13, 36], the mechanisms of tissue-selective actions of icariin in estrogen-sensitive tissues and cells could be summarized as follows (Fig. 7): Icariin might 1) rapidly activate extranuclear ER α via phosphorylation of signaling kinase; 2) alter estrogen responsive genes via regulating transcription factors through kinase-mediated phosphorylation; and 3) exert estrogenic activities in bone and brain, but not the uterus or breast. Our study suggests that icariin is a safe and effective alternative agent for management of menopause-related conditions. However, the mechanism by which icariin exerts membrane-initiated estrogenic actions requires further investigation.

Acknowledgment

We thank the University Research Facility in Life Sciences at The Hong Kong Polytechnic University for the technical support.

Financial Support: This work was supported by the General Research Fund of Research Grant Council (HKSAR; 15103614), Research Studentship support (RTKT) at the Hong Kong Polytechnic University (to L.Z.), Essential Drug Research and Development (2019ZX09201004-003-032) from the Ministry of Science and Technology of China Sanming Project of Medicine in Shenzhen (SZSM201808072), the National Natural Science Foundation of China (81601110), and the Shenzhen Basic Research Program (JCYJ20170818111103886).

Additional Information

Correspondence: Man-Sau Wong, PhD, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Room 806, Lee Shau Kee Building, 11 Yuk Choi Road, Hung Hom, Kowloon, Hong Kong SAR, PR China. E-mail: man-sau.wong@polyu.edu.hk.

Disclosure Summary: The authors have nothing to disclose.

References and Notes

- Obermeyer CM, Reher D, Saliba M. Symptoms, menopause status, and country differences: a comparative analysis from DAMES. *Menopause*. 2007;**14**(4):788–797.
- Shook LL. An update on hormone replacement therapy: health and medicine for women: a multidisciplinary, evidence-based review of mid-life health concerns. *Yale J Biol Med*. 2011;**84**(1):39–42.
- Komm BS, Mirkin S. An overview of current and emerging SERMs. *J Steroid Biochem Mol Biol*. 2014;**143**:207–222.
- Parikh RP, Odom EB, Yu L, Colditz GA, Myckatyn TM. Complications and thromboembolic events associated with tamoxifen therapy in patients with breast cancer undergoing microvascular breast reconstruction: a systematic review and meta-analysis. *Breast Cancer Res Treat*. 2017;**163**(1):1–10.
- Buhling KJ, Daniels BV, Studnitz FS, Eulenburg C, Mueck AO. The use of complementary and alternative medicine by women transitioning through menopause in Germany: results of a survey of women aged 45–60 years. *Complement Ther Med*. 2014;**22**(1):94–98.
- Borrelli F, Ernst E. Alternative and complementary therapies for the menopause. *Maturitas*. 2010;**66**(4):333–343.
- Al-Anazi AF, Qureshi VF, Javaid K, Qureshi S. Preventive effects of phytoestrogens against postmenopausal osteoporosis as compared to the available therapeutic choices: an overview. *J Nat Sci Biol Med*. 2011;**2**(2):154–163.
- Zhang Y, Chen WF, Lai WP, Wong MS. Soy isoflavones and their bone protective effects. *Inflammopharmacology*. 2008;**16**(5):213–215.
- Zhang Y, Li Q, Wan HY, Helferich WG, Wong MS. Genistein and a soy extract differentially affect three-dimensional bone parameters and bone-specific gene expression in ovariectomized mice. *J Nutr*. 2009;**139**(12):2230–2236.
- Zhang G, Qin L, Shi Y. Epimedium-derived phytoestrogen flavonoids exert beneficial effect on preventing bone loss in late postmenopausal women: a 24-month randomized, double-blind and placebo-controlled trial. *J Bone Miner Res*. 2007;**22**(7):1072–1079.
- Vrtačnik P, Ostaneč B, Mencej-Bedrač S, Marc J. The many faces of estrogen signaling. *Biochem Med*. 2014;**24**(3):329–342.
- Banerjee S, Chambliss KL, Mineo C, Shaul PW. Recent insights into non-nuclear actions of estrogen receptor alpha. *Steroids*. 2014;**81**:64–69.
- Ho MX, Poon CC, Wong KC, Qiu ZC, Wong MS. Icaritin, but not genistein, exerts osteogenic and anti-apoptotic effects in osteoblastic cells by selective activation of non-genomic ER α signaling. *Front Pharmacol*. 2018;**9**:474.
- Mok SK, Chen WF, Lai WP, et al. Icaritin protects against bone loss induced by oestrogen deficiency and activates oestrogen receptor-dependent osteoblastic functions in UMR 106 cells. *Br J Pharmacol*. 2010;**159**(4):939–949.
- Xiao HH, Fung CY, Mok SK, et al. Flavonoids from *Herba epimedii* selectively activate estrogen receptor alpha (ER α) and stimulate ER-dependent osteoblastic functions in UMR-106 cells. *J Steroid Biochem Mol Biol*. 2014;**143**:141–151.
- Wang Z, Wang D, Yang D, Zhen W, Zhang J, Peng S. The effect of icaritin on bone metabolism and its potential clinical application. *Osteoporos Int*. 2018;**29**(3):535–544.
- Zhou LP, Wong MS. Data from: Supplementary data-js.2019-00156. *figshare*. 2019. <https://figshare.com/s/fa12441deb0f5519f692>. doi:10.6084/m9.figshare.8051621
- Zhou LP, Wong KY, Yeung HT, et al. Bone protective effects of danggui buxue tang alone and in combination with tamoxifen or raloxifene in vivo and in vitro. *Front Pharmacol*. 2018;**9**:779.
- Feng R, Feng L, Yuan Z, et al. Icaritin protects against glucocorticoid-induced osteoporosis in vitro and prevents glucocorticoid-induced osteocyte apoptosis in vivo. *Cell Biochem Biophys*. 2013;**67**(1):189–197.
- SciCrunch. RRID:AB_390204. https://scicrunch.org/resolver/AB_390204. Accessed July 29, 2019.
- SciCrunch. RRID:AB_1840807. https://scicrunch.org/resolver/AB_1840807. Accessed July 29, 2019.

22. SciCrunch. RRID:AB_2537664. https://scicrunch.org/resolver/AB_2537664. Accessed July 29, 2019.
23. SciCrunch. RRID:AB955447. https://scicrunch.org/resolver/AB_955447. Accessed July 29, 2019.
24. SciCrunch. RRID:AB_2536089. https://scicrunch.org/resolver/AB_2536089. Accessed July 29, 2019.
25. Holinka CF, Hata H, Kuramoto H, Gurbide E. Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). *Cancer Res.* 1986;**46**(6):2771–2774.
26. Gustafsson KL, Farman H, Henning P, et al. The role of membrane ER α signaling in bone and other major estrogen responsive tissues. *Sci Rep.* 2016;**6**:29473.
27. Jiang MC, Chen XH, Zhao X, Zhang XJ, Chen WF. Involvement of IGF-1 receptor signaling pathway in the neuroprotective effects of Icaritin against MPP(+)-induced toxicity in MES23.5 cells. *Eur J Pharmacol.* 2016;**786**:53–59.
28. Chen WF, Wu L, Du ZR, et al. Neuroprotective properties of icariin in MPTP-induced mouse model of Parkinson's disease: involvement of PI3K/Akt and MEK/ERK signaling pathways. *Phytomedicine.* 2017;**25**:93–99.
29. Richter CA, Birnbaum LS, Farabollini F, et al. In vivo effects of bisphenol A in laboratory rodent studies. *Reprod Toxicol.* 2007;**24**(2):199–224.
30. Bartell SM, Han L, Kim HN, et al. Non-nuclear-initiated actions of the estrogen receptor protect cortical bone mass. *Mol Endocrinol.* 2013;**27**(4):649–656.
31. Chambliss KL, Wu Q, Oltmann S, et al. Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. *J Clin Invest.* 2010;**120**(7):2319–2330.
32. Rietjens IMCM, Louisse J, Beekmann K. The potential health effects of dietary phytoestrogens. *Br J Pharmacol.* 2017;**174**(11):1263–1280.
33. Xu Q, Chen G, Liu X, Dai M, Zhang B. Icariin inhibits RANKL-induced osteoclastogenesis via modulation of the NF- κ B and MAPK signaling pathways. *Biochem Biophys Res Commun.* 2019;**508**(3):902–906.
34. Jiao F, Tang W, Huang H, et al. Icariin promotes the migration of BMSCs in vitro and in vivo via the MAPK signaling pathway. *Stem Cells Int.* 2018;**2018**:2562105.
35. Wang Z, Yang L, Xia Y, Guo C, Kong L. Icariin enhances cytotoxicity of doxorubicin in human multidrug-resistant osteosarcoma cells by inhibition of ABCB1 and down-regulation of the PI3K/Akt pathway. *Biol Pharm Bull.* 2015;**38**(2):277–284.
36. Song L, Zhao J, Zhang X, Li H, Zhou Y. Icariin induces osteoblast proliferation, differentiation and mineralization through estrogen receptor-mediated ERK and JNK signal activation. *Eur J Pharmacol.* 2013;**714**(1-3):15–22.
37. Madak-Erdogan Z, Kim SH, Gong P, et al. Design of pathway preferential estrogens that provide beneficial metabolic and vascular effects without stimulating reproductive tissues. *Sci Signal.* 2016;**9**(429):ra53.
38. Chen WF, Mok SK, Wang XL, et al. Total flavonoid fraction of the *Herba epimedii* extract suppresses urinary calcium excretion and improves bone properties in ovariectomised mice. *Br J Nutr.* 2011;**105**(2):180–189.
39. Simpson ER. Aromatization of androgens in women: current concepts and findings. *Fertil Steril.* 2002;**77**(Suppl 4):S6–S10.
40. Zhao H, Tian Z, Hao J, Chen B. Extragonadal aromatization increases with time after ovariectomy in rats. *Reprod Biol Endocrinol.* 2005;**3**:6.
41. Yang L, Lu D, Guo J, Meng X, Zhang G, Wang F. Icariin from *Epimedium brevicornum* Maxim promotes the biosynthesis of estrogen by aromatase (CYP19). *J Ethnopharmacol.* 2013;**145**(3):715–721.
42. Lephart ED. Modulation of aromatase by phytoestrogens. *Enzyme Res.* 2015;**2015**:594656.
43. Ye L, Chan MY, Leung LK. The soy isoflavone genistein induces estrogen synthesis in an extragonadal pathway. *Mol Cell Endocrinol.* 2009;**302**(1):73–80.