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Icariin from *Epimedium brevicornum* Maxim promotes the biosynthesis of estrogen by aromatase (CYP19)

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ABSTRACT

Ethnopharmacological relevance: *Epimedium brevicornum* Maxim has long been used for the treatment of osteoporosis in China and other Asian countries. However, the mechanism behind the antiosteoporotic activity of this medicinal plant is not fully understood.
Aim of the study: The present study was designed to investigate the effects of five widely used antiosteoporotic medicinal plants (*Epimedium brevicornum*, *Cuscuta chinensis*, *Rhizoma drynariae*, *Polygonum multiflorum*, and *Ligustrum lucidum*) on the production of estrogen, and identify the bioactive compounds responsible for the estrogen biosynthesis-promoting effect.
Materials and methods: Human ovarian granulosa-like KGN cells were used to evaluate estrogen biosynthesis, and the production of 17β-estradiol was quantified by a magnetic particle-based enzyme-linked immunosorbent assay (ELISA) kit. Further, the mRNA expression of aromatase was determined by a quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR), and the protein expression of aromatase was detected by western blotting. The activity of alkaline phosphatase (ALP) in rat osteoblastic UMR-106 cells was measured using *p*-nitrophenyl sodium phosphate assay.
Results: Among the 5 antiosteoporotic medicinal plants, the extract of *Epimedium brevicornum* was found to significantly promote estrogen biosynthesis in KGN cells. Icariin, the major compound in *Epimedium brevicornum*, was identified to be the active compound for the estrogen biosynthesis-promoting effect. Icariin promoted estrogen biosynthesis in KGN cells in a concentration- and time-dependant manner and enhanced the mRNA and protein expressions of aromatase, which is the only enzyme for the conversion of androgens to estrogens in vertebrates. Further study showed that icariin also promoted estrogen biosynthesis and ALP activity in osteoblastic UMR-106 cells.
Conclusions: These results show that the promotion of estrogen biosynthesis is a novel effect of *Epimedium brevicornum*, and icariin could be utilized for the prevention and treatment of osteoporosis.

1. Introduction

Estrogens play a crucial role in the normal physiology of a variety of tissues, including the mammary glands, reproductive tract, central nervous system, and skeleton. The actions of estrogens are mediated by the estrogen receptors (ER α/β), which function as ligand-regulated transcriptional factors (Heldring et al., 2007). Selective estrogen receptor modulators (SERMs), pharmaceutical agents that selectively activate or inhibit ERs, are used in the treatment of estrogen-related diseases, such as breast cancer or osteoporosis, depending on the target tissues (Bryant, 2002). The phytoestrogens in medicinal plants, mainly flavonoids, have long been considered to exert beneficial effects on estrogen-related diseases by acting as SERMs (Oseni et al., 2008).

The biosynthesis of estrogen in humans is controlled by aromatase (encoded by *CYP19A1*), which is the only enzyme that catalyzes the formation of estrogens by using androgens, such as testosterone and androstenedione as substrates (Simpson et al., 2002). Although the coding region of an aromatase is always the same, the transcriptional control of aromatase is tissue-specific because of the presence of different promoters (Simpson, 2004). For example, in the ovary aromatase expression is mediated by promoter II, which is regulated by gonadotropins, through the stimulation of cyclic adenosine monophosphate (cAMP) generation (Michael et al., 1995). In osteoblasts, aromatase expression is driven by promoter I.4, which is mainly regulated by class I cytokines (Ribot et al., 2006).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; ER, estrogen receptor; SERM, selective estrogen receptor modulator; cAMP, cyclic adenosine monophosphate; ALP, alkaline phosphatase; BCA, bicinchoninic acid; BuOH, *n*-butanol; EtOAc, ethyl acetate; PE, petroleum ether; PDE, phosphodiesterase; FSH, follicle-stimulating hormone; PKG, cGMP-dependent protein kinase

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Pharmaceutical agents that inhibit aromatase activity have been widely used for the treatment of hormone-dependent breast cancer in postmenopausal women, where they show superior efficacy to conventional anti-ER drugs such as tamoxifen (Johnston and Dowsett, 2003). However, pharmaceutical agents that can promote estrogen biosynthesis by activating aromatase expression or activity are rarely reported. Although some herbicides, fungicides, and insecticides have been found to promote aromatase expression in a cAMP-dependent or -independent manner (Sanderson et al., 2000; You et al., 2001; Morinaga et al., 2004), further studies are required to identify potent aromatase agonists that produce less side-effects. Estrogen deficiency is the major cause of osteoporosis, a disease affecting over 200 million people worldwide (Riggs et al., 1998). Although estrogen supplementation is an established regimen for the prevention of these diseases, the side effects associated with its long-term use, such as increased risks of breast, ovarian, and endometrial cancers, limit its clinical use (Davison and Davis, 2003). Thus, alternative methods that can improve the therapeutic efficacy and safety by locally promoting estrogen biosynthesis should be developed for the prevention and treatment of diseases caused by estrogen deficiency.

Many medicinal plants have been shown to have antiosteoporotic effects *in vitro* and *in vivo* and are used clinically for the prevention and treatment of osteoporosis (Zhang et al., 2006). *Epimedium brevicornum*, a traditional Chinese medicine, has been widely used in China for the treatment of cardiovascular diseases, infertility, impotence, amnesia, lumbago, arthritis, and numbness and weakness of the limbs for thousands of years (Ma et al., 2011). Icariin is the main flavonoid present in *Epimedium brevicornum*, used for herbal quality control (Chinese Pharmacopoeia Commission, 2010), and is considered the major bioactive component of this plant (Meng et al., 2005; Ma et al., 2011). Several studies have shown that both *Epimedium brevicornum* and icariin have antiosteoporotic effects *in vitro* and *in vivo* by stimulating osteoblast proliferation and function; these findings support the wide use of *Epimedium brevicornum* in many Chinese formulas for treating osteoporosis (Zhang et al., 2007, 2008; Qin et al., 2008; Nian et al., 2009; Hsieh et al., 2010). Additionally, *Cuscuta chinensis*, *Rhizoma drynariae*, *Ligustrum lucidum*, and *Polygonum multiflorum* exhibit antiosteoporotic effects both *in vitro* and *in vivo*, and are also widely used in many Chinese formulas to treat osteoporosis (Li et al., 2001; Zhang et al., 2006). Flavonoids are generally considered as potent inhibitors of aromatase activity (Balunas and Kinghorn, 2010). However, one dietary flavonoid, hesperetin, was shown to increase aromatase expression in breast MCF-7 cancer cells (Li et al., 2011). Thus, it is possible that some medicinal plants, which have seldom been studied previously, could prevent or treat osteoporosis by promoting estrogen biosynthesis.

To elucidate the effect of antiosteoporotic medicinal plants on the regulation of estrogen biosynthesis, we performed estrogen biosynthesis-promoting guided isolation of active compounds from the extracts of *Epimedium brevicornum*, *Cuscuta chinensis*, *Rhizoma drynariae*, *Ligustrum lucidum*, and *Polygonum multiflorum*. Then, we assessed the effects and mechanisms of action of the isolated active compounds on human ovarian granulosa cells and osteoblastic cells, which are the major sources of estrogen in premenopausal and postmenopausal women respectively.

2. Materials and methods

2.1. Materials

Testosterone, letrozole, sildenafil, forskolin, and icariin were purchased from Sigma (Shanghai, China). The magnetic particle-based 17β-estradiol enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bio-Ekon Biotechnology (Beijing, China). The assay kit for alkaline phosphatase (ALP) was purchased from Nanjing Jiancheng Bioengineering Institute, China. RPMI 1640 and DMEM/F12 media, and fetal calf serum were products of Gibco-Invitrogen (Carlsbad, CA, USA).

2.2. Plant extraction

An ethanolic extract of *Cuscuta chinensis* and water extract of *Ligustrum lucidum* were prepared as described in previous studies (Yang et al., 2011; Chen et al., 2012). Dried leaves of *Epimedium brevicornum*, dried rhizomes of *Rhizoma drynariae*, and dried roots of *Polygonum multiflorum* were purchased from Tongrentang Chinese Medicine (Beijing, China) and voucher specimens were deposited in the herbarium of Herpetology and Herbarium, Chengdu Institute of Biology, Chinese Academy of Sciences (Voucher nos., SC1705, SC1706, and SC1707). The dried leaves of *Epimedium brevicornum* (5 kg) were extracted using distilled deionized water (3 × 30 L) under reflux to obtain the residue (735 g), which was suspended in water (1.5 L) and was successively fractionated using petroleum ether (3 × 1.5 L), ethyl acetate (3 × 1.5 L), and *n*-butanol (3 × 1.5 L) to give 38.9 g, 61.0 g, and 357.2 g extracts respectively. The residue was concentrated under reduced pressure to give a 251.6 g extract. The dried rhizomes of *Rhizoma drynariae* (5 kg) were extracted using 50% ethanol (3 × 30 L) at room temperature to obtain the residue (560 g). The dried roots of *Polygonum multiflorum* were extracted using 70% ethanol (3 × 30 L) at room temperature to obtain the residue (730 g).

2.3. Cell culture

Human ovarian granulosa-like KGN cells (supplied by Professor Yiming Mu, Chinese PLA General Hospital, Beijing, China) were maintained in DMEM/F-12 medium supplemented with 5% (v/v) fetal bovine serum (Gibco-Invitrogen), penicillin (100 U/mL), and streptomycin (0.1 mg/mL). Rat osteoblastic UMR-106 cells were maintained in RPMI 1640 medium supplemented with 1% penicillin/streptomycin and 10% fetal calf serum.

2.4. Cell-based estrogen biosynthesis assay

The assay was performed as described in a previous study (Lu et al., 2012). KGN cells or UMR-106 cells were seeded in 24-well plates and cultured overnight. The next day, the medium was replaced with serum-free medium and the cells were pretreated for 24 h with the test chemicals. Testosterone (10 nM) was then added to each well and the cells were incubated for another 24 h. At the end of this incubation, the culture supernatants were collected and stored at −20 °C. Levels of 17β-estradiol in the supernatants were quantified by magnetic particle-based ELISA according to the manufacturer's instructions (Bio-Ekon Biotechnology). Optical densities were measured at 550 nm using a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). Results were normalized to the total cellular protein content and expressed as percentage 17β-estradiol production in comparison with the control samples. The protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Bestbio, Shanghai, China).

2.5. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

The qRT-PCR analysis of aromatase expression was conducted as described previously (Lu et al., 2012). Total cellular RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Total RNA (2 μg) was reverse-transcribed using Super-Script III Reverse Transcriptase (Invitrogen) with oligo$(dT)_{18}$ primers. Equal amounts of cDNA were subjected to qRT-PCR with the