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European Journal of Pharmacology 504 (2004) 147–153

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Proliferation-stimulating effects of icaritin and desmethylicaritin in MCF-7 cells

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Received 18 March 2004; accepted 4 October 2004

Abstract

Icariin, icaritin and desmethylicaritin are constituents of *Epimedium* with a similar structure to genistein and daidzein. Using the modified MCF-7 cell proliferation assay (E-SCREEN assessment system), these compounds were tested for their estrogen-like activities. Icaritin and desmethylicaritin, but not icariin, strongly stimulated the proliferation of MCF-7/BUS cells. Cell cycle analysis revealed that the proliferation stimulatory effect was associated with a marked increase in the number of MCF-7/BUS cells in S phase and a significant increase in the G2/M population, with effects similar to those of estradiol. These actions were dose dependent (range from 1 nM to 10 μM) and could be significantly inhibited by the specific estrogen receptor antagonist ICI 182,780 [7 α-[9(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl)-estra-1,3,5(10)-triene-3,17β-diol)]. The estrogen receptor-regulated progesterone receptor and PS2 mRNA levels were increased by treatment with icaritin or desmethylicaritin within 24 h and the effects were also reversed by ICI 182,780. It was concluded that icaritin and desmethylicaritin are novel phytoestrogens and that the estrogenic effects of icaritin and desmethylicaritin are mediated by the estrogen receptor.

Keywords: Icariin; Icaritin; Desmethylicaritin; Phytoestrogen; Estrogenic activity; Cell cycle analysis; S phase; Reverse transcriptase-polymerase chain reaction (RT-PCR)

1. Introduction

Icariin, icaritin and desmethylicaritin are constituents of *Epimedium*, a Traditional Chinese Herbal Medicine which has a broad range of therapeutic applications in genital, cardiovascular, endocrine, bronchial, urinary and immune systems (Wu et al., 2003). Both the herb and the total flavonoid extracts have been shown to possess many kinds of biological functions, especially in hormone regulation, immunological function modulation, and anti-tumor, anti-aging and anti-atherosclerosis activities (He et al., 1995). Some studies have investigated the pharmacological activities of the monomers in *Epimedium* (Lee et al., 1995; Li et al., 1997; Mao et al., 2000; Zhang et al., 2000). However, the mechanisms of the pharmacological effects are not yet known.

Many flavone glycosides, including isoflavones such as genistein and daidzein, are structurally similar to mammalian estrogens and are believed to possess the estrogenic activities associated with the estrogen receptor-dependent pathway and estrogen receptor-independent pathway (Collins-Burow et al., 2000). The estrogenic activities of phytoestrogens are closely associated with their structures, and the activity of some flavone glycosides is strikingly increased by removal of their glycoside moieties (Miksicek, 1995). The number and position of hydroxyl groups in the molecular structure are other important factors which contribute to strong binding to estrogen receptors and the resulting estrogenic activity (Calliste et al., 2001). Interestingly, the structures of icariin, icaritin and desmethylicaritin seem to satisfy the requirements of estrogen mimetics (Fig. 1).

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doi:10.1016/j.ejphar.2004.10.002

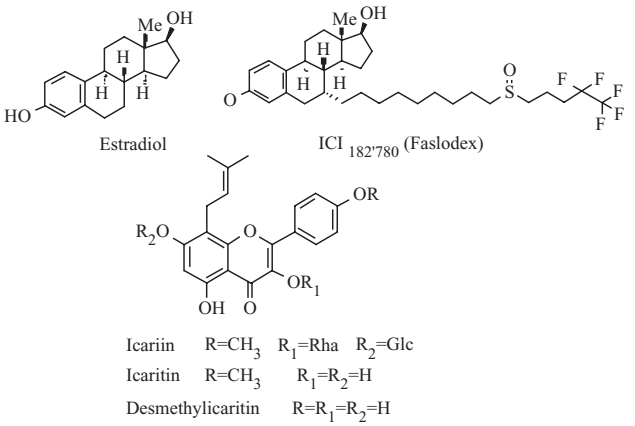


Fig. 1. Chemical structures of related compounds.

Icariin is the quality control standard in many Traditional Chinese Medicine at present. According to the major metabolic route of orally administered icariin, there are at least eight series of icariin compounds which can be converted into desmethylicaritin by the elimination of the sugar moiety and/or demethylation in vivo (Wu et al., 2003). The structure of desmethylicaritin can be superimposed on that of luteolin, a well-known anti-estrogenic flavoid (Hiremath et al., 2000). Therefore, it is suggested that the icariin series may have estrogenic/anti-estrogenic activities.

In the present paper, a modified MCF-7 cell proliferation assay (E-SCREEN method) (Soto et al., 1995) was used to identify the estrogenic-like activity of these compounds. The possible mechanism of action was investigated by cell cycle analysis and mRNA measurements. The estrogen receptor antagonist ICI 182, 780 was used as a tool.

2. Materials and methods

2.1. Materials

Icariin (>99% purity) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). ICI 182, 780 [7 α-[9(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl)-estra-1,3,5(10)-triene-3,17β-diol)] was purchased from Tocris, Cookson, United Kingdom. 17β-Estradiol, genistein (>99% purity), daidzein (>99% purity), dimethylsulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromile (MTT), phenol red-free Dulbecco's modified Eagle' medium (phenol red-free DMEM), Triton-x 100, ribonuclease A and propidium iodide were purchased from Sigma-Aldrich (St. Louis. MO, USA). Dextron T-70 was provided by Pharmacia (NJ, USA). DMEM medium and fetal bovine serum were obtained from Gibco BRL Life Technologies, Scotland. Human serum was provided by Blood Central of Zhejiang Province (Hangzhou, China).

2.2. Preparation and identification of icaritin and desmethylicaritin

Icaritin was prepared by the cellulose hydrolysis method (Liggins et al., 2000) from icariin, and icaritin was demethylated with boron tribromide (Mcomie et al., 1968) to form desmethylicaritin. Purification was performed by preparative high-performance liquid chromatography (HPLC) and identification was conducted by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS). The purity of both compounds was greater than 98%.

2.3. Cell culture

Estrogen receptor-positive human breast adenocarcinoma MCF-7/BUS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin), supplemented with 10% fetal bovine serum. Cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Medium was renewed 2–3 times per week.

2.4. Charcoal–dextran stripped human serum preparation

In order to minimize the estrogenic activity of serum, steroid hormones were stripped from pooled human serum by treatment with charcoal and dextran, using an existing modified protocol (Payne et al., 2000). The charcoal–dextran stripped human serum was filtered and stored at −20 °C until used.

2.5. Proliferation assay of MCF-7 cells

Confluent MCF-7/BUS cells were washed twice with D-Hanks solution before the addition of 0.25% trypsin–EDTA. The flask was left for 2–3 min at room temperature (close to 20 °C), after which the cells were detached, resuspended in full medium, counted and seeded into 24-well plates at a density of 1×10^4 cells/well in normal growth medium. After 48 h, the cells were completely attached to the well bottom. Then the cells were washed with D-Hanks and the estrogen-free medium (phenol red-free DMEM with 5% charcoal–dextran stripped human serum) was added. Following another 48-h pretreatment, the cells were exposed to increasing concentrations of estradiol and other test compounds. Cell proliferation was assessed after 7 days, during which the medium was changed every 3 days. In the assessment method, cells were incubated with 50 μl of 5 mg ml^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromile (MTT) solution for 4 h. Following this, the medium was discarded, and 500 μl DMSO was added. Absorbance at 570 nm was determined with an ELx800 universal microplate reader (Bio-Rad, USA). By MTT method, cell numbers were obtained as absorbance values. The results

are expressed as proliferation compared with that induced by treatment with 1 nM estradiol.

2.6. Interaction with ICI 182,780

Some phytoestrogens, such as genistein and daidzein, act both as agonists and antagonists at estrogen receptors. In the present study, we examined the effects of icariin, icaritin and desmethylicaritin as well as their interaction with ICI 182,780, a pure estrogen receptor antagonist. The concentration of icariin, icaritin and demethylicaritin was set as 1 μM, and the effect of cotreatment with 0.1 μM ICI 182,780 on cell proliferation was assessed with the MTT method after 7 days, as described above.

2.7. Cell cycle analysis

Exponentially growing cells were plated in 150-mm Petri dishes in phenol red-free DMEM containing 10% charcoal–dextran stripped human serum for 48 h and treated with test compounds. After 24 h, the cells were harvested and 1×10^6 cells were placed into a polypropylene tube and centrifuged. The supernatant was removed and 1 ml 4 °C 70% EtOH was added dropwise to the cell pellet during vortexing. The cells were kept at 4 °C until DNA staining. Fixed cells were treated with 100 μg/ml RNase A in phosphate-buffered saline solution (PBS) for 1 h, followed by staining with 50 μg/ml propidium iodide in PBS. Flow cytometric analysis of cell cycle distribution and apoptosis was performed with a BD FACSCalibur with a 488-nm (blue) argon laser (Becton Dickinson, San Jose, CA). Data acquisition was performed with CellQuest 3.1 software and data were analyzed with ModFit LT 3.0 software (Variety Software House, Topsham, ME).

2.8. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

MCF-7 cells were grown in 150-mm Petri dishes in phenol red-free DMEM containing 10% charcoal–dextran stripped human serum for 48 h and treated with solvent or different concentrations of icaritin and desmethylicaritin. After 24 h, the cells were harvested and total RNA was isolated with Trizol reagent (Gibco BRL) according to the manufacturer's instructions. After extraction, RNA was precipitated by recommended procedures and dissolved in 0.1% diethylpyrocarbonate solution. To synthesize first strand cDNA, 7 μl total RNA was incubated in 0.5 μg of oligo(dT)6 primer (Sangon, China) and 5 μl deionized water at 65 °C for 15 min. Reverse transcription reactions were performed using 200 units of M-MuLV reverse transcriptase (Gibco BRL) in 5× reaction buffer (250 mM Tris–HCl; pH 8.3 at 25 °C, 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol) and 1 mM deoxynucleoside triphosphate (dNTP) mixture containing 10× PCR buffer (100 mM Tris–HCl; pH 8.3 at 25 °C, 500 mM KCl, 15 mM $MgCl_2$), 25 units Taq polymerase (Sangon, China), 1 μl of 10 mM dNTP mixture, and 30 pmol of each primer. The final volume was adjusted to 50 μl. The specific primer pairs were designed as follows: progesterone receptor sense primer 5′-AGTTGTGAGAGCACTGGATGC-3′, progesterone receptor antisense primer 5′-GATCTGCCACATGGTAAGGC-3′, PS2 sense primer 5′-TGGAGAACAAGGTGATCTGC-3′ PS2 antisense primer 5′-ATCTGTGTTGTGAGCCGAGG-3′, ERα sense primer 5′-AGACATGAGAGCTGCCAACC-3′, ERα antisense primer 5′-GCCAGGCACATTCTAGAAGG-3′, β-actin sense primer 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, β-actin antisense primer 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′ (Otsuki et al., 2000). The PCR reactions were initiated by 3 min of denaturation at 94 °C, followed by amplification at 94 °C for 45 s, and 55 °C for 45 s, 72 °C for 45 °C; 30 cycles for progesterone receptor, 25 cycles for PS2, and 20 cycles for β-actin using Mastercycler gradient (Eppendorf, Germany). The PCR products were analyzed by 1.5% agarose gel electrophoresis, visualized with ethidium bromide staining, and quantified using a bio-imaging analyzer (Bio-Rad). The density of the products was quantitated using Quantity One version 4.2.2 software (Bio-Rad).

2.9. Data analysis

All data are expressed as means±standard deviation (S.D.), and the level of significance between two groups was assessed with Student's *t*-test. *P* values of less than 0.01 were considered to be statistically significant.

3. Results

3.1. Stimulation of MCF-7 cell proliferation

The estrogenic effect of the selected compounds was examined by E-SREEN assay in MCF-7 cells. In the E-SCREEN assay, the maximum cell proliferative effect was observed with 1 nM estradiol. The proliferative effect of the compounds relative to that of estradiol (1 nM, 100%) is expressed as Relative Proliferative Effect (RPE) (Fig. 2). Both icaritin and desmethylicaritin were able to significantly stimulate MCF-7/BUS cell proliferation at concentrations of 10^{-10} M to 10^{-5} M in a dose-dependent manner ($P<0.01$) (Fig. 2), although icariin demonstrated no significant effect on MCF-7/BUS cell proliferation compared with the vehicle control. The maximal proliferative effect of both icaritin and desmethylicaritin was achieved at 10^{-6} M and was almost

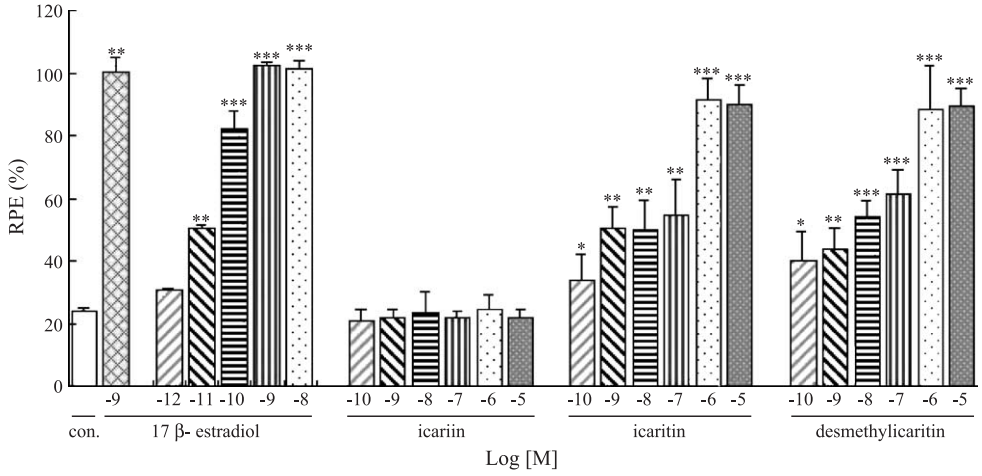


Fig. 2. Estrogenic activity of icariin, icaritin and desmethylicaritin in MCF-7/BUS cells. The cells were incubated in phenol red-free DMEM supplemented with hormone-free human serum with 17-β estradiol or other test compounds for 7 days. Incubation with DMSO alone was performed as control (con) and the final concentration of vehicle, DMSO, in the medium never exceeded 0.1%. After incubation for 7 days, the MTT assay was performed to measure cell proliferation. The proliferative effect relative to estradiol (1 nM, 100%) is expressed as Relative Proliferative Effect (RPE). Results are expressed as means±S.D. of five separate experiments for each data point. Significant difference was set at *P<0.05, **P<0.01, ***P<0.001 vs. DMSO.

equivalent to the effect of 1 nM estradiol (The RPE was 91.7±6.1 and 88.0±14.5, respectively).

3.2. Suppression by ICI 182,780

Then, an experiment was designed to determine whether the proliferation–stimulatory effect induced by icaritin and desmethylicaritin could be inhibited by ICI 182,780, the pure estrogen receptor antagonist. The proliferative effects of 1 nM estradiol, 1 μM icaritin or 1 μM desmethylicaritin were blocked by addition of 100 nM ICI 182,780 (Fig. 3). The RPE of estradiol, icariin and desmethylicaritin after treatment with ICI 182,78 decreased to 17.0±7.0, 13.6±5.7 and 13.7±4.6, respectively.

3.3. Effects of icaritin and desmethylicaritin on cell cycle distribution

Cell cycle analysis showed that in hormone-free medium, almost all cells were in G1 phase and the proportion was even higher after coadministration of 100 nM ICI 182,780. As shown in Fig. 4, we observed a dramatic increase in the number of cells in S phase after treatment with 1 nM estradiol, 1 μM icaritin or 1 μM desmethylicaritin for 24 h

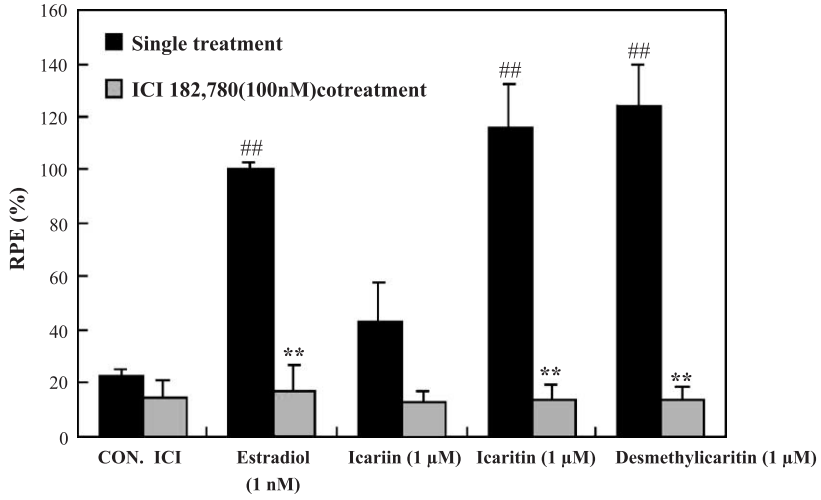
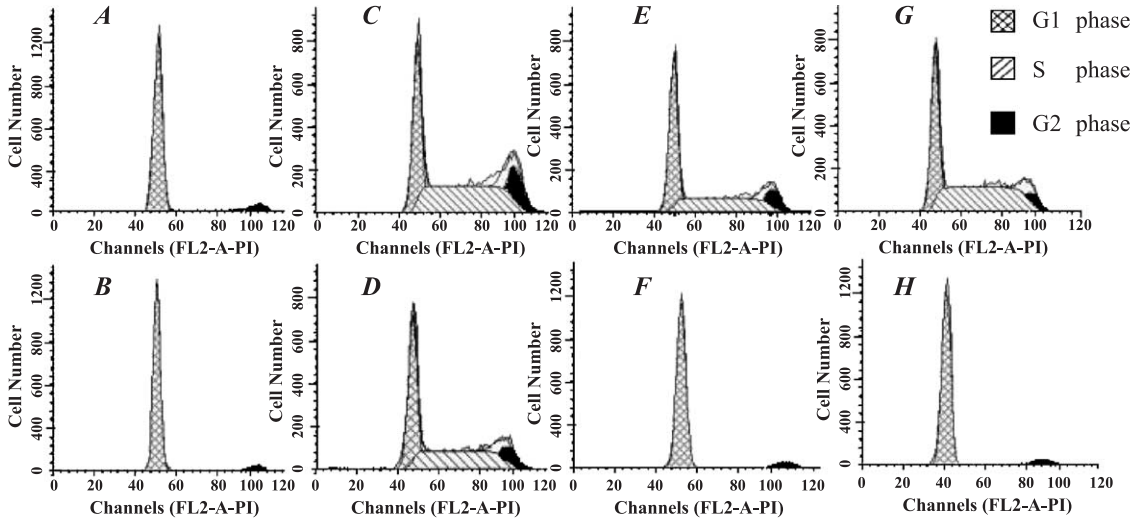


Fig. 3. Effect of cotreatment with pure antiestrogen ICI 182,780 on cell proliferation induced by icaritin and desmethylicaritin in MCF-7/BUS cells. The cells were incubated in phenol red-free DMEM supplemented with hormone-free human serum without or with 100 nM ICI 182,780 for 7 days. Incubation with DMSO alone was performed as control (con) and the final concentration of vehicle, DMSO, in the medium never exceeded 0.1%. After incubation for 7 days, the MTT assay was performed to measure cell proliferation. The proliferative effect relative to estradiol (1 nM, 100%) is expressed as Relative Proliferative Effect (RPE). Results are expressed as means±S.D. of five separate experiments for each data point. Significance was set at **P<0.01 vs. solvent control and $^{\#\#}P$<0.01 vs. the same dose of compound without treatment of ICI 182,780.



Groups \ Cell Cycle Phase	G1 Phase (%)	S Phase (%)	G2 Phase (%)
a. Solvent Control	81.72	11.39	6.82
b. 10 nM ICI 182,780	93.29	2.89	3.83
c. 1 nM 17-β estradiol	33.24	47.81	18.96
d. 1 nM 17-β estradiol+ 100 nM ICI 182,780	47.67	38.53	13.81
e. 1 μM icaritin	43.88	43.51	12.60
f. 1 μM icaritin + 100 nM ICI 182,780	92.16	2.54	5.30
g. 1 μM desmethylicaritin	37.76	53.15	9.09
h. 1 μM desmethylicaritin + 100 nM ICI 182,780	92.12	2.13	6.51

Fig. 4. Effects of icaritin and desmethylicaritin on cell cycle distribution of MCF-7 cells. DMSO was used as solvent control. MCF-7 cells were cultured in DMEM with 10% fetal bovine serum for 24 h. The medium was then changed to medium without hormone and incubation was continued for another 24 h with DMSO and test compounds. Cell-cycle analysis was performed by Flow Cytometry. Results are expressed as percentage of cells in G1 phase, S phase and G2 phase. (A) Solvent Control; (B) 100 nM ICI 182,780; (C) 1 nM 17-β estradiol; (D) 1 nM 17-β estrodial+100 nM ICI 182,780; (E) 1 μM icaritin; (F) 1 μM icaritin+100 nM ICI 182,780; (G) 1 μM desmethylicaritin; (H) 1 μM desmethylicaritin+100 nM ICI 182,780. These data are representative of at least two separate experiments.

compared with the control. The proportion of cells in S phase increased from 11.39% (solvent control) to 47.81% (1 nM estradiol), 43.51% (1 μM icaritin) and 53.15% (1 μM desmethylicaritin) respectively. These effects were inhibited by 100 nM ICI 182,780. Icaritin and desmethylicaritin, added for 24 h, also caused a significant increase in the G2/M population (Fig. 4). These results were in accordance with the data obtained from the cell proliferation analysis and possibly demonstrated that icaritin and desmethylicaritin exert their effect on cell proliferation at S phase entry.

3.4. Progesterone receptor and PS2 mRNA expression

The expression of the progesterone receptor and PS2 genes in MCF-7 cells is regulated in a characteristic manner by estrogenic compounds. These genes can be stimulated by agonistic estrogens such as the natural ligand estradiol (Hall et al., 2001; Frasor et al., 2003), whereas ICI 182,780 is known to antagonize the stimulatory effect of estradiol in MCF-7 cells (Howell et al., 2000; Oliveira et al., 2003). To further characterize the molecular mechanisms, we studied the effects of icaritin and desmethylicaritin on ERα, progesterone receptor and PS2 mRNA expression in MCF-7 breast cancer cells in comparison to estradiol and ICI (Fig. 5). As shown, estradiol (1 nM), icaritin (0.1, 1, 10 μM) and desmethylicaritin (1 μM) significantly increased the expression of mRNA for the progesterone receptor and PS2 compared with control. These effects were inhibited by 100 nM ICI 182,780 (Fig. 5).

4. Discussion

Phytoestrogens are polyphenolic non-steroidal plant-derived compounds with estrogen-like biological activity which have been associated with a variety of changes in the reproductive system and certain hormone-dependent diseases, such as prostate cancer, colon cancer and breast

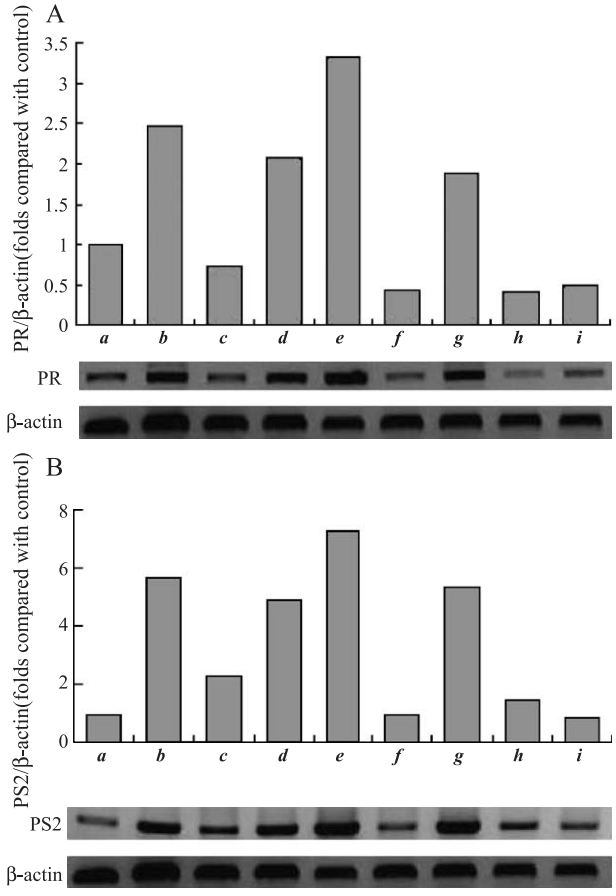


Fig. 5. Effect of icaritin and desmethylicaritin on mRNA expression of estrogen-responsive genes in MCF-7 cells. MCF-7 cells were treated with (a) DMSO; (b) 1 nM 17-β estradiol; (c) 0.1 μM icaritin; (d) 1 μM icaritin; (e) 10 μM icaritin; (f) 1 μM icaritin+100 nM ICI 182,780; (g) 1 μM desmethylicaritin; (h) 1 μM desmethylicaritin+100 nM ICI 182,780; (i) 1 nM 17-β estradiol+100 nM ICI 182,780. After incubation with test compounds for 24 h, total RNA was extracted using TRIzol reagent (Gibco BRL). The mRNA levels of (A) progesterone receptor (PR), (B) PS2 were measured by reverse transcriptase-polymerase chain reaction (RT-PCR) and normalized using β-actin as an internal control. RT-PCR products were run on an ethidium bromide stained 1.5% agarose gel, which was scanned using a bio-imaging analyzer (Bio-Rad) and the density of the products were quantited using Quantity One version 4.2.2 software (Bio-Rad). Results expressed as folds relative to DMSO (solvent control).

cancer (Cos et al., 2003). We found that icaritin and desmethylicaritin to have estrogenic activities in MCF-7 cells: (i) They dramatically stimulated the growth of MCF-7 cells. (ii) The proliferation stimulatory effect could be reversed by coadministration of a pure antiestrogen ICI 182,780. (iii) Icaritin and desmethylicaritin increased the expression of mRNA for the progesterone receptor and PS2, two known estrogen receptor-responsive genes. It can be concluded that icaritin and desmethylicaritin possess potential estrogen-like activity in MCF-7 cells.

It has been demonstrated that the proliferative effect of estradiol and phytoestrogens is exerted via an estrogen receptor-dependent pathway and an estrogen receptor-independent pathway (Tsai and O'Malley, 1994; Kuiper et al., 1996). In the estrogen receptor-dependent pathway, phytoestrogens usually interact with chromatin to alter their cytoarchitectural and phenotypic properties including (1) nuclear transcriptional activity of estrogen receptors and related responsive genes, i.e., progesterone receptor or PS2 and (2) intervening activity of signal pathways associated with cell cycle progression and cell proliferation (Castoria et al., 1999). Estrogens also up-regulate some positive proliferation regulators including survivin, multiple growth factors, genes involved in cell cycle progression, and regulatory factor–receptor loops, and down-regulate transcriptional repressors and antiproliferative and proapoptotic genes (Frasor et al., 2003). Together, these mechanisms likely contribute to the stimulation of proliferation and cytotoxicity in MCF-7 cells. Icaritin and desmethylicaritin (0.1 nM–1 μM) had a stimulatory effect and significantly influenced cell cycle changes in MCF-7 cells. Further research should be conducted to explore the possible molecular mechanisms of the proliferative effect induced by icariin and desmethylicaritin.

In our study, saturation ligand binding analysis of estrogen receptors was not conducted. However, coadministration of ICI 182,780 almost completely blocked the proliferation stimulatory effects induced by icaritin and desmethylicaritin. The dramatically up-regulated mRNA levels of progesterone receptor and PS2 were also reversed by ICI 182,780. The complete reversal induced by the pure estrogen receptor antagonist indicates that icaritin and desmethylicaritin exert their effect through estrogen receptors in MCF-7/BUS cells. ERα mRNA expression was not significantly affected by estradiol, icaritin and desmethylicaritin or coadministration of ICI 182,780 in our experiments. A possible explanation for this finding is that the compounds modulate the estrogen receptor level by affecting the turnover of the protein or other mechanisms (Dauvois et al., 1992; Gibson et al., 1991; Reese and Katzenellenbogen, 1992).

Like daidzein, genistein and their precursors (Bingham et al., 1998; Breinholt and Larsen, 1998), icaritin and desmethylicaritin showed enormous variation in estrogenic potency compared with icariin. This was due to the steric hindrance produced by glycoside moieties which prevented docking to the estrogen receptor binding site (molecular simulation performed with DS-MODELING software, Accelry, USA; data not shown). The isopentenyl group in position 8 in flavonoids plays a crucial role in binding at the estrogen receptor (Kitaoka et al., 1998). However, the binding activity of icaritin and desmethylicaritin differed slightly. This may indicate that the polarity of the 4′ -position in the flavone is not the crucial factor determining estrogenic activity but instead the absence of glycosyls in structure.

The stability of icariin in artificial gastric juice and the transformation of icariin in the intestine have been studied, and results show that icariin can be metabolized to icaritin and desmethylicaritin by human intestinal bacteria in vitro (unpublished). It can thus be concluded that icaritin and

desmethylicaritin derived from *Epimedium*, may exert estrogen-like activity and provide protection against osteoporosis and the menopause syndrome.

Acknowledgments

This work was supported by the National Natural Science Foundation of China, No. 30171121, Technology Foundation for Key Project by Ministry of Education of China, No. 03088.

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