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Determination of rat urinary metabolites of icariin *in vivo* and estrogenic activities of its metabolites on MCF-7 cells

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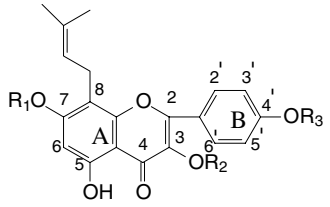
To confirm that the estrogenic activity of icariin is based on the close relationship between the structures of its metabolites and the effects of their binding to target hormone receptors, the metabolism of icariin in rat urine was analyzed *in vivo*, and the estrogenic activity of its metabolites was measured in cultured MCF-7 human breast cancer cells, respectively. By CZE analysis, peaks corresponding to the relative positions of desmethylicaritin and icaritin were observed in the urine sample. Structural analysis following LC-ESI-MS revealed molecular ions $[M\text{-}H]^-$ of 512.8, 353.3, and 367.0 for metabolites consistent with those of icariside II, desmethylicaritin, and icaritin. Icariin, icaritin, and desmethylicaritin were analyzed for their estrogenicity using MCF7-cell proliferation (E-screen test). MCF-7 cells were cultured in an estradiol free medium and then exposed to 10^{-8} to 10^{-5} mol/L icariin and its metabolites, icaritin and desmethylicaritin, for 6 days. Icaritin and desmethylicaritin significantly increased cell proliferation, and the cell number increased from 1.61 to 4.14 fold compared with the untreated control, but the parent compound icariin failed to exhibit this effect. These results indicate that icariin is converted to icariside II, desmethylicaritin, and icaritin *in vivo*, and that the latter two act as a weak xenoestrogen on MCF-7 cells.

1. Introduction

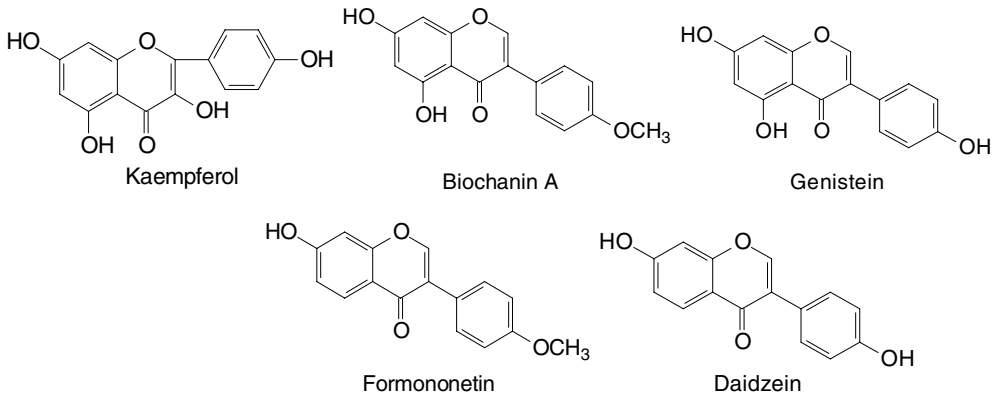
Several herbs belonging to the genus *Epimedium* (Yin Yang Huo) have been widely used in the traditional Chinese medicine (TCM) as a tonic, aphrodisiac, and antirheumatic (China Pharmacopeoia 2000). The flavonoid glycosides of *Epimedium* species have been studied as major active ingredients in clinical treatments (Lenoble et al. 2002; Xin 2000; Ji et al. 2000; Guo et al. 1996). Among them icariin, a flavonoid glycoside, is known as an indicative constituent of the *Epimedium* genus, and has been used as a marker compound in various composite formulas owing to its appearing in every Epimedium species (Wu et al. 2003).

Epimedium has been investigated *in vivo* for several decades to assess its estrogen-like activity. It has been reported that the total flavonoids of *Epimedium* increase femur weight as well as femur matrix apparent density in a rat model of osteoporosis induced by retionoic acid (Ji et al. 2000), and improve bone density, and enhance E2 level as well as decrease IL-6 concentration in serum of ovariectomized rats (Jiang et al. 2002). In addition *Epimedium* significantly increased lumbar bone mineral density and lowered IL-6 mRNA expression of ovariectomized rats (Wang et al. 2000). However, the estrogen-like activity of icariin has not been well understood so far.

In terms of chemical structure, icaritin (the aglycone of icariin) and desmethylicaritin (the demethylation product of icaritin) are similar to kaempferol, a well-known estrogen-like flavonoid, which enhances MCF-7 (estrogen receptor-positive breast cancer cell line) proliferation. Its EC_{50} value (concentration needed to enhance the MCF-7 proliferation by 50%) is 1.1 $\mu mol \cdot L^{-1}$, which is close to EC_{50} values of the classical phytoestrogens, daidzain (0.7 $\mu mol \cdot L^{-1}$) and genistein (0.3 $\mu mol \cdot L^{-1}$) (Breinholt and Larsen 1998). Upon ingestion, the aglycones are efficiently liberated from their glycoside forms and subjected, in part, to demethylation metabolism by colonic bacterial flora of the large bowel (Bingham et al. 1998). Moreover, it is known that biochanin A and formononetin can be



	R_1	R_2	R_3	M_r
icariin	glucose	rhamnose	CH_3	676
icaritin	H	H	CH_3	368
desmethylicaritin	H	H	H	354
icariside	H	rhamnose	CH_3	514



metabolized to genistein and daidzein, respectively, by gut micro-organisms (Bingham et al. 1998), and genistein and daidzein have stronger estrogenic activities than biochanin A and formononetin (Kuiper et al. 1998). It is suggested that the estrogenic activity of icariin may be due to the close relationship of the structures of its metabolites and effects of their binding to target hormone receptors.

The understanding of the absorption mechanism and metabolic pathways of icariin is limited. Whether the active ingredient *in vivo* is the parent compound or its metabolites has not been established. Determining the biological activities of its metabolites is of great importance for understanding the biological functions and mechanism of both icariin and *Epimedium*.

Accurate methods to detect icariin and its active metabolites are necessary to conduct thorough pharmacological studies on them. Capillary electrophoresis (CE) has been reviewed many times (Zhang et al. 2000; Huang et al. 1997; Nemutlu and Kir 2003; Zhou et al. 2002; Mauri et al. 2003) and clearly continues to be a very active separation method in research since this technique often provides higher resolving power, shorter analysis time and lower operating cost than HPLC. An interesting aspect of CE is that the separation mechanism is based on the migration of charged analytes, which is different from traditional chromatographic techniques. Indeed, it has been shown that CE techniques are complementary to chromatographic techniques (Tagliaro et al. 1996; Boone et al. 1999). This is especially important for toxicological analysis, since the identification of unknown compounds in biological matrices should be made and/or confirmed by at least two analytical techniques that are based on different principles. HPLC-MS has been used widely to detect and identify metabolites of phytoestrogens (Grace et al. 2003; O'Leary et al. 2003; Mohsen et al. 2002). HPLC with on-line MS offers several advantages for such biotransformation studies, since conjugated metabolites may be analyzed without prior hydrolysis or derivatization and peaks from chromatograms may be matched to permit selection of the appropriate parent ions for structural analysis by MS.

One of the most rapid and sensitive *in vitro* tests of estrogenicity is based on the use of MCF-7 human breast cancer cells (E-screen assay) (Kavlock et al. 1996). The E-screen assay reflects the affinity of xenoestrogens for binding with and stimulating an estrogen receptor (ER) by measuring the estrogen-dependent proliferation of MCF-7 cells (Payne et al. 2000).

In order to confirm whether the parent compound or the metabolites show estrogenic activity, we investigate the

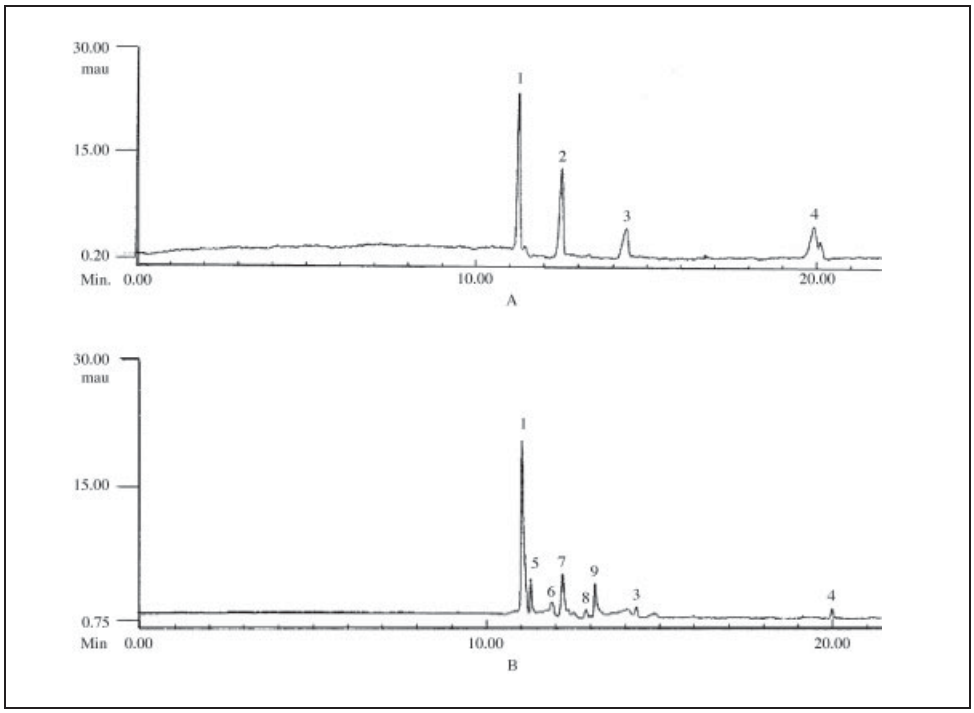


Fig. 1:
Typical electropherograms of a urine standard spiked with carbamazepine, icariin, desmethylicaritin and icaritin (A) and a urine sample (carbamazepine added) from a rat treated with 120 mg icariin/kg by gavage (B), (1) carbamazepine (internal standard); (2) icariin; (3) desmethylicaritin; (4) icaritin; (5), (6), (7), (8), and (9) other unknown metabolites or endogenous compounds

metabolites of icariin in rat urine, examine the potential estrogenic activities of icariin and its metabolites on MCF-7 cells, and discuss the mechanism of the effects of icariin and its metabolites.

2. Investigations and results

2.1. Detection of icariin metabolites in rat urine by HPCE

Ideal separation was achieved under the optimal CE conditions. A typical electropherogram of urine spiked with icariin, icaritin, and desmethylicaritin standard is shown in Fig. 1A. Carbamazepine (internal standard) appeared first, followed by icariin, desmethylicaritin and then finally icaritin.

An electropherogram of a 96 h urine sample is presented in Fig. 1B. Metabolites from rat urine resulted in the formation of a complex mixture separated by CZE. Nine basic analytes (including metabolites and endogenous compounds) were separated from each other within 20 min. No peak corresponding to icariin was observed in the sample. The peaks corresponding to the relative positions of desmethylicaritin and icaritin, respectively, were observed in the 96 h urine sample. However, the presence of the two related metabolites in rat urine could not be ascertained only on the basis of comparison with the relative positions of the standard solution. This prompted us to carry out further studies to identify their structures. For this purpose, an LC/MS method was used to elucidate the metabolites of icariin.

2.2. Characterization of icariin metabolites by LC-ESI-MS

The urine samples were analysed by HPLC and LC-ESI-MS. The UV 270 nm chromatogram is depicted in Fig. 2.

HPLC product B (retention time 22.61 min) coeluted with an authentic sample of desmethylicaritin. LC/ESI/MS analysis of this product produced a molecular ion $[M\text{-}H]^-$ at m/z 353.3, corresponding to desmethylicaritin (Fig. 3A). HPLC product C (retention time 40.78 min) coeluted with icaritin and exhibited molecular mass of m/z 367.0 $[M\text{-}H]^-$ in the negative electrospray ionisation mode, corresponding to icaritin (Fig. 3B). The unknown peak labelled as product A (retention time 14.22 min) was confirmed by ESI mass spectrometry as icariside II; a deprotonated molecular ion of mass 512.8 was observed (Fig. 3C).

Analysis of the 96 h urine sample revealed that the major metabolites of icariin were icariside II, icaritin, and desmethylicaritin.

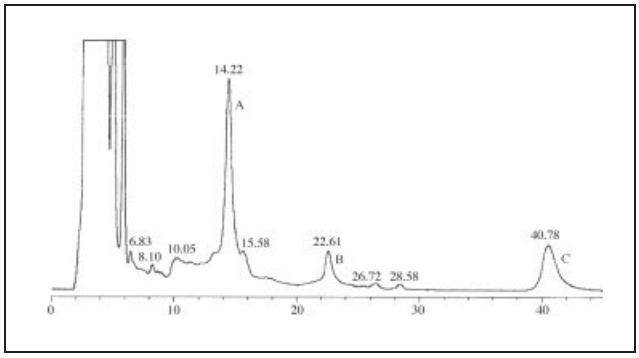


Fig. 2: Typical HPLC elution profile of metabolism in rat urine treated with icariin (120 mg/kg ig as sodium carboxymethyl cellulose suspension). Peaks are A, icariside II; B, desmethylicaritin; and C, icaritin

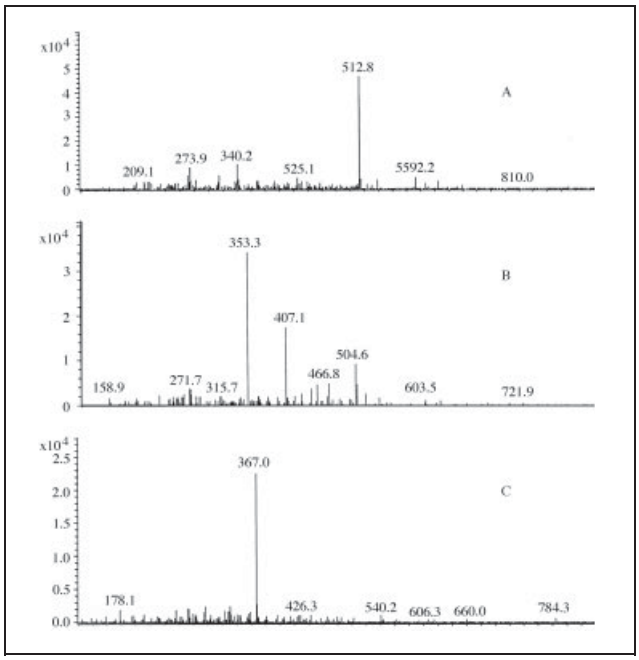


Fig. 3: MS spectra of major metabolites obtained in negative electrospray ionisation mode. A: The peak yielded molecular ion $[M\text{-}H]^-$ of 512.8 corresponding to icariside II (514), B: The peak yielded molecular ion $[M\text{-}H]^-$ of 353.3 corresponding to desmethylicaritin (354), C: The peak yielded molecular ion $[M\text{-}H]^-$ of 367.0 corresponding to icaritin (368)

2.3. Proliferative effects of icariin and its metabolites on MCF-7 cells

The MCF-7 cells are dependent on estrogen for their proliferation in a monolayer culture (Darbre and Daly 1989).

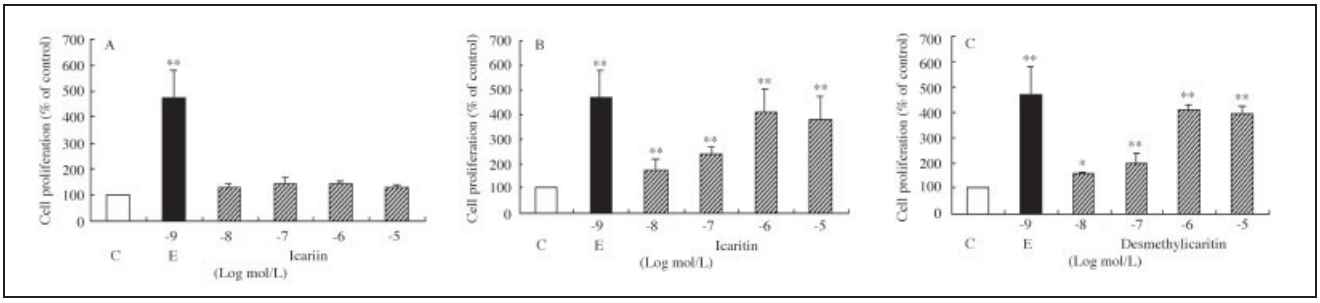


Fig. 4: Effects of icariin (A), icaritin (B), and desmethylicaritin (C) on the growth of MCF-7 cells. The compounds were tested at concentrations ranging from 10^{-8} mol/L (−8) to 10^{-5} (−5) for 6 days. Results are expressed as the mean ± S.D. of three or four determinations. C and E indicated untreated control and 17β-estradiol (10^{-9} mol/L), respectively. *, ** means significantly differ from the control group ($P < 0.05$) and ($P < 0.01$), respectively

Fig. 4 shows the effects of icariin and its metabolites, icaritin and desmethylicaritin, on MCF-7 cell proliferation. The results were calculated in comparison with untreated control cells. Relative to control on day 6, icaritin and desmethylicaritin at 10^{-8} to 10^{-5} mol/L increased cell numbers approximately 1.75 to 4.14-fold and 1.61 to 4.0-fold, respectively. Despite this, icariin at 10^{-8} to 10^{-5} mol/L showed no significant difference ($P > 0.148$) from control. 17β-Estradiol (10^{-9} mol/L), the endogenic estrogen as a positive control, induced proliferation approximately 4.72-fold. The concentration of 17β-estradiol is typical in tests evaluating estrogenicity of the above compounds, and the proliferation induced by it in this study is consistent with previous findings (Soto et al. 1997).

These data suggest that icariin was converted to active metabolites *in vivo* and the estrogenic activity of the compound might be predominantly due to icaritin and desmethylicaritin.

3. Discussion

Icariin was extensively biotransformed in the rat. In the urine extract sample from icariin-treated (ig) rats, both icaritin and desmethylicaritin co-migrated with corresponding peaks intrinsic to the pure compounds. This was combined with the structural analysis by LC/ESI/MS which showed the coelution of the three major metabolites with $[M\text{-}H]^-$ of m/z 512.8, 367, and 353.3, respectively. The results show that icariin was converted to at least three metabolites: icariside II, icaritin, and desmethylicaritin in rat urine. Metabolic experiments with orally administered icariin in rats showed that two metabolites, icariside II and icaritin, were found in both small intestine and urine (Qiu et al. 1999).

According to the major metabolic route of orally administered icariin in the rat, there are at least eight kinds of icariin derived compounds, which can be converted into desmethylicaritin, by the elimination of the sugar moiety, and/or demethylation *in vivo* (Huang et al. 2001). The flora of the gut have an important role in the metabolism of flavonoids. The colonic bacterial flora is known to deconjugate the glycosides to aglycones and remove the methyl groups to form phenolic compounds which are structurally similar to 17β-estradiol (Kelly et al. 1993). After absorption, these also are excreted in urine. This indicated that this novel metabolite, desmethylicaritin, is produced as a result of metabolism of icariin by gut micro-flora, firstly by hydrolysis to icariside II and icaritin, followed by demethylation to form desmethylicaritin. Differential rates of metabolism by gut micro-flora may provide an explanation for the existence of desmethylicaritin in urine. Moreover, this metabolite is consistent with known pathways of flavonoid metabolism by gut microorganisms; biochanin A is metabolized to genistein, and daidzein is produced from formononetin (Bingham et al. 1998).

Both icaritin and desmethylicaritin exhibited a markedly estrogen-like effect on MCF-7 cells, though the effects were not so strong as with 17β-estradiol, whereas icariin failed to show any activity. Furthermore, it has been reported that flavones and isoflavones either cause or notably reinforce estrogenic activities only after hydrolysis of their corresponding glycosides (Miksicek 1995). Other studies have shown that the main structural feature of flavonoids required for estrogenicity is the presence of a mono hydroxyl group in the 4′-position of the B-ring of the flavan nucleus (Vibeke and John 1998). It might be assumed that desmethylicaritin invariably possesses stronger estrogenic activity than icaritin, while icariin definitely has little or no estrogenicity. The present studies demonstrated that icaritin and desmethylicaritin both markedly stimulated cell proliferation and the cell number increased from 1.61 to 4.14 relative to control. Compared with icaritin (4′-methoxy), desmethylicaritin (4′-hydroxy) failed to significantly increase estrogenic activity. It was most likely due to the different cellular model used in the experiment. Bail et al. (1998) have reported that the presence of a 4′-position hydroxy increased bioluminescence (by a transactivation assay using the MVLN cell line). However, they failed to find the same structure-activity relationship between the 4′-position hydroxy and cell proliferation in the MCF-7 cell line.

Icaritin and desmethylicaritin have been found to be more potent in terms of *in vitro* estrogenicity than their parent compound, suggesting that metabolites, if present at adequate concentrations, might contribute to the observed estrogenic response. It is thus likely that these metabolites might play an important role in mediating the *in vivo* estrogenic response, and contribute to the estrogenic response in metabolic competent cell lines, such as MCF-7 cells. The pivotal role of gut micro-flora in flavanoid metabolism in rats is likely to have a significant effect on biological activities.

The estrogenicity of desmethylicaritin can be understood in view of the superficial and comformational similarity between the compound and the dihydroxystilbene estrogens (diethyl stilbestrol and hexestrol). Structural parallels have been drawn between the estrogenic flavones (genistein and daidzein) and the principal physiological estrogen (17β-estradiol). These similarities include a planar ring system that contains a parahydroxy substituted A-ring and a second in-plane hydroxy group located at a distance of approximately 12Å from the first (Thomas and Keenam 1986). All these features are also preserved in the structure of desmethylicaritin.

Based on these results and findings, it seems that free icaritin and desmethylicaritin may be acting as a xeno-estrogen to target cells in reproductive organs. Further study by *in vivo* uterotrophic assay will be necessary to estimate the estrogenic activity derived from icariin and its metabolites in immature or ovariectomized rats.

In conclusion, the present study shows that in rat urine, icariin is converted to three major compounds, icariside II, icaritin, and desmethylicaritin. However, the effect of icaritin and desmethylicaritin on MCF-7 cell proliferation was only weakly estrogenic compared that of 17β-estradiol, whereas the parent compound was essentially inactive at the concentrations used.

4. Experimental

4.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), red-free DMEM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were supplied by Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Biological Products Co., Ltd. (Hangzhou, Zhejiang, China). Adult male human serum (HuS) was provided from Zhejiang Central Blood Bank (Hangzhou, Zhejiang, China). Other chemicals were obtained from various sources in the highest available quality.

4.2. Reference compounds

Icariin was purchased from the National Institute for the Control of Pharmaceuticals and Biological Products, Beijing, China. Icaritin and desmethylicaritin were not commercially available in pure form and were pre-

pared and purified from icariin (purity 98.9 and 98.6%, respectively). Carbamazepine (as internal standard, IS) was provided by Zhejiang Hisun Pharmaceutical Co.Ltd. 17β-Estradiol (E_2) was supplied by Sigma (St. Louis, MO, USA).

4.3. Animals and treatment

Male Sprague-Dawley (SD) rats (200–220 g) were provided by the Experimental Animals Center of Zhejiang University (Hangzhou, Zhejiang, China). Animals were fasted for 18–24 h with free access to water before experimenting. The study was approved by the local animal ethics committee. Icariin (120 mg/kg ig as sodium carboxymethyl cellulose suspension) was administered to 6 rats. Rats were then immediately kept in individual metabolic cages for collection of urine. Urine samples were collected just before dosing and every 24 h up to 96 h. The urine samples from six rats were combined and the overall volume was measured immediately after collection and the urine sample was stored frozen at −20 °C until analysis.

4.4. Isolation of urinary metabolites

The urine sample was allowed to stand and warm to room temperature, then filtered. 10 ml of centrifuged urine was extracted twice with 40 ml ethyl acetate. The combined ethyl acetate extract was centrifuged at 3500 rpm for 10 min and the organic layer was evaporated to dryness at 45 °C with nitrogen. The dry residue was redissolved in methanol-water-glacial acetic acid (75 : 25 : 0.1, v/v/v).

4.5. CZE analysis

The CZE analysis was performed on a BioFocus® 3000 automated Capillary Electrophoresis system (Bio-Rad, Hercules, CA) equipped with UV detection at 270 nm. BioFocus® 3000 software was used for instrumental control, data acquisition and data analysis. An untreated fused-silica capillary tube (effective length 43.6 cm × 50 μm I.D.) (Hebei Yongnian Photoconductive Fibre Factory, Hebei, China) was used for separation. Sodium tetraborate (30 mM), monobasic sodium phosphate (50 mM)-acetonitrile (50 : 50, v/v) (pH 10.0) was chosen as the running buffer. The electrophoresis buffer was passed through a 0.45 μm filter and then degassed ultrasonically for 5 min before use. The typical analytical conditions were: voltage, 12.0 kV; injection, 10 psi × s; capillary and carousel temperatures were 25 °C and 10 °C, respectively. Analysis was conducted in the following manner: The capillary tube was rinsed first with sodium hydroxide (0.1 M) for 2 min and then with distilled water for 2 min, and was conditioned with the electrophoresis buffer for 3 min between each injection.

4.6. HPLC conditions

HPLC was carried out using a Shimadzu LC-10ATvp gradient-controlled HPLC system (Kyoto, Japan) with a Shimadzu SPD-10AVvp UV-VIS detector (270 nm), a Shimadzu CTO-10ASvp column oven (25 °C). A C18 Dikma Diamonsil ODS reversed-phase column (5 μm; 250 × 4.6 mm) was used. Methanol-water-glacial acetic acid (75 : 25 : 0.1, v/v/v) was used as mobile phase at a flow rate 1.0 ml/min. The chromatographic data were collected and processed on an Upper chromatopac station version (Zhejiang University, Hangzhou, China).

4.7. LC-ESI-MS assay

An Esquire-LC 00075 quadrupole mass spectrometer detection system (Billerica, MA, USA) was used. The instrument was operated in negative electrospray ionization (ESI) mode. The capillary voltage was fixed at −106.7 V, and the skimmer was set at −33.9 V. The HPLC fluid was nebulized using N_2 and its temperature was 300 °C. Scanning began at 100 m/z and ended at 1200 m/z. Liquid chromatography was performed with an HP 1100 solvent delivery system (Hewlett Packard, Palo Alto, USA). Samples were separated on an Agilent Hypersil ODS column (particle size 5 μm, 2.1mm × 100 mm ID, Agilent, Palo Alto, California, USA). The mobile phase consisted of methanol-water-glacial acetic acid (75 : 25 : 0.1, v/v/v) at a flow rate of 0.5 $mL \cdot min^{-1}$.

4.8. Cell culture, cell media, and supplements

Estrogen receptor-positive human breast cancer MCF-7 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). For routine maintenance, cells were grown as a monolayer culture in DMEM at 37 °C in an atmosphere of 5% CO_2/95% air under saturated humidity. The culture medium was supplemented with 5% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml) and $NaHCO_3$ (3.7 g/l). Cells were grown to confluence and passaged with trypsin-EDTA (0.05/0.02%).

2.9. Determination of estrogenic activity in MCF-7 cells (E-Screen Assay)

The proliferation test (E-Screen assay) was carried out according to the principle of a previously described method (Soto et al. 1995). Prior to initiating experiments, one 70% confluent 25 cm^2 flask of MCF-7 cells was seeded and put in 24-well plates at a density of 8×10^3 cells per well in 1 ml DMEM medium supplemented with 5% FBS. After 48 h the cells were washed with 1 ml PBS. The medium was replaced with 1 ml estrogen-free medium (phenol red-free DMEM with estrogen-free HuS). After culture for another 48 h with the fresh estrogen-free medium, the cells were exposed to E_2 (10^{-9} mol/l) or the chemicals being tested ($10^{-8} \sim 10^{-5}$ mol/l). The chemicals were dissolved in DMSO (final concentration in the culture medium did not exceed 0.1%; the cell growth was not affected). The medium was changed every 48 h and the chemicals were replenished with each change. Estrogen-free HuS was prepared using the dextrancharcoal procedure (Soto and Sonnenschein 1985). Phenol red-free DMEM was chosen as it has been shown that phenol red is itself estrogenic (Berthois et al. 1986).

Cell proliferation was assessed on the sixth day using MTT assay as described before (Carmichael et al. 1987). The assay was started by incubating the cells for 4 hrs at 37 °C with 50 μl (5 mg/ml) MTT medium per well. The yellow tetrazolium salt is reduced in the cells to the blue insoluble MTT-formazan. After removal of the MTT medium the formazan was dissolved in 500 μl DMSO per well and the optical density (OD) at 570 nm was measured using a microplate photometer (Elx 800, BIO-TEK, USA).

2.10. Statistical procedures

All results are presented as mean ± standard deviation (S.D.). Statistical analysis was performed using the one-way analysis of variance (ANOVA).

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