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Pharmacokinetics of prenylflavonoids and correlations with the dynamics of estrogen action in sera following ingestion of a standardized *Epimedium* extract

Shih Peng Wong[1], Ping Shen[1], L. Lee, J. Li, E.L. Yong*

Department of Obstetrics & Gynecology, National University of Singapore, Yong Loo Lin School of Medicine, National University Hospital, Lower Kent Ridge Road, Singapore 119074, Republic of Singapore

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ABSTRACT

To explore pharmacokinetic properties of prenylflavonoids from the Traditional Chinese Medicinal plant *Epimedium*, three doses of a standardized extract (100, 300 and 600 mg/kg body weight), were administered to ovariectomized rats and serial blood samples were obtained. Serum concentrations of the *Epimedium* prenylflavonoids icariin, icariside I, icariside II, icaritin and desmethylicaritin were determined by LC–MS/MS. Aliquots of sera were also applied to human cell lines that permanently express ERα and ERβ proteins for the *ex vivo* measurement of estrogenic activity. All five prenylflavonoids exhibit non-linear dose-dependent increases in the area under concentration versus time curves. Two distinct pharmacokinetic patterns were evident, an early phase wherein icariin and icariside II reached t_{max} 0.5–1 h, and a late phase wherein icariside I, icaritin and desmethylicaritin peaked at t_{max} 8 h. Total concentrations of icaritin and desmethylicaritin reached C_{max} ~2 μM and ~0.25 μM respectively. Estrogenic activity in *Epimedium*-treated rat sera lagged by several hours compared to animals treated with control drug estradiol benzoate, corresponding to the appearance of bioactive metabolites desmethylicaritin, icaritin and icariside I. Following glucuronidase/sulphatase treatment, prolonged estrogenic activity at higher *Epimedium* doses (300 and 600 mg/kg of body weight) was evident, and correlated with the persistence of micromolar levels of icaritin at the 48–72 h sampling period. The depot effect resulted in time–concentration bioactivity profiles at the three *Epimedium* doses (area under curve 374, 543, and 771 pM E2 h^{-1}) that exceeded that observed for estradiol benzoate (148 pM E2 h^{-1}). Our study correlated the pharmacokinetics of prenylflavonoids with the dynamics of their estrogenic effects and reveals the potential estrogenicity of this *Epimedium* extract. This study may aid the development of prenylflavonoids as drugs for menopause and other conditions requiring estrogenic action.

1. Introduction

Estrogens are small molecules that bind to and activate the estrogen receptors (ERα, ERβ) resulting in a cascade of gene transcription regulating female sexual development and maintaining of the health of reproductive organs, bone, neural and cardiovascular systems. The pleitropic actions of estrogens are shown clearly at menopause where the abrupt cessations of estrogenic action result in hot flashes, osteoporosis and atrophy of reproductive tissues. Estrogenic prodrugs, such as estradiol benzoate, when ingested undergo hepatic metabolism to release the potent estrogenic ligand, estradiol. Although administration of estrogenic drugs can ameliorate some aspects of menopausal syndromes, doubts have emerged about the benefits of estrogen/progesterone replacement therapy because of increased risks for thromboembolism, strokes, cardiovascular disease and breast cancer [1,2]. Attention is now focused on estrogens of botanical origins as alternatives for estrogen replacement therapy [3].

Prenylflavonoids are potent phytoestrogens with selective ER-binding activity. For example the prenylflavone, 8-prenylnaringenin, present in hops and beer has recently been characterized as a potent ERα-selective phytoestrogen [4] and has been shown to exert effects on hot flashes [5], reproductive organs [6] and bone [7] in animal models. Plants of the genus *Epimedium* (Berberidaceae) are traditionally used in East Asian countries to improve bone health and to treat sexual dysfunction [8,9] and are a rich source of prenylflavonoids. Icariin and its derivatives (icariside I, icariside II, icaritin and desmethylicaritin) (Fig. 1) are major constituents of *Epimedium* extracts [10]. Icaritin and desmethylicaritin have estrogenic properties and can stimulate estrogen-driven cell proliferation [11,12], affect osteoblastic/osteoclastic activity [13,14] in bone and exert protective effects on rat neuronal cells [15]. Icariin, the principal *Epimedium* prenylflavonoid, has glucose and

* Corresponding author.
E-mail address: obgyel@nus.edu.sg (E.L. Yong).
[1] Joint first authors.

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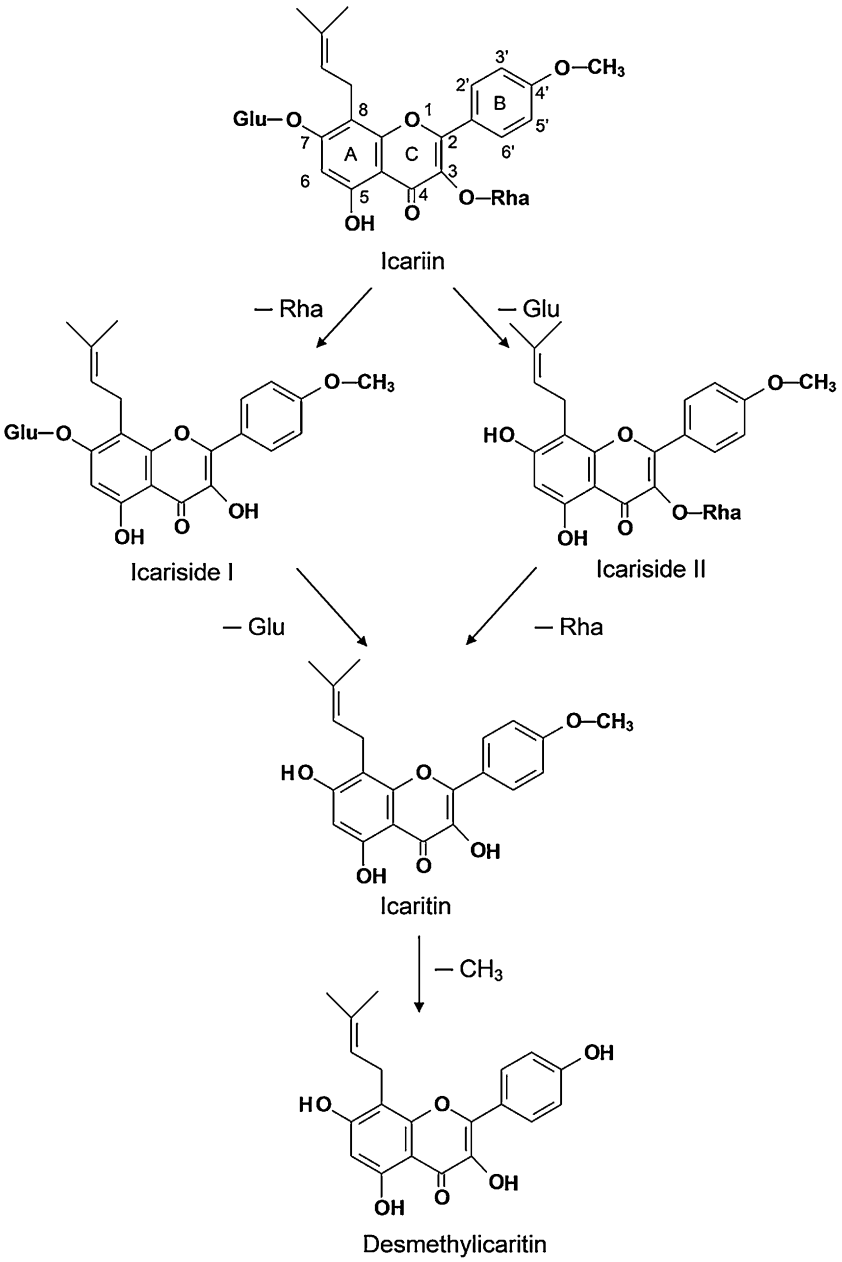

Fig. 1. Structures of icariin and its derivatives. Rha, rhamnose; Glu, glucose.

rhamnose residues on 7-OH of ring A and 3-OH positions of ring C respectively (Fig. 1). Partial deglycosylation of icariin results in icariside I and icariside II (baohuoside 1). Icariin exhibits potent inhibition of human phosphodiesterase-5 [16] and can induce the expression of nitric oxide synthase in the corpus cavernosum smooth muscle [17–19]. In animal studies, administration of icariin and *Epimedium* flavonoids can inhibit bone resorption, stimulate bone formation, prevent osteoporosis in ovariectomized rats [20,21], improve stress-induced behavioral and neuroendocrine alteration [22] and improve erectile function in aged male rats [23]. A randomized double-blind placebo-controlled clinical trial indicates that a preparation containing icariin and the soy phytoestrogens genistein and daidzein can exert significant effects on bone turnover markers in post-menopausal women, compared to placebo [24].

These effects have stimulated increasing interest in utilizing *Epimedium* prenylflavonoids for hormone replacement therapy and erectile dysfunction, as well as therapeutic agents for bone, cardiovascular, neurological and endocrinological diseases [9]. However, development of these compounds and their parent extracts as pharmaceutical-quality drugs are dependent on rigorous pharmacokinetic/pharmacodynamic studies. One crucial problem is that *Epimedium* extracts are composed of many bioactive constituents. Sensitive methods to simultaneously quantify levels of bioactive prenylflavonoids in sera have been developed by us and other laboratories [25–27]. Nevertheless, measurement of actual concentrations of these compounds may not reflect true bioavailability, since prenylflavonoids are rapidly metabolized and are bound to serum proteins [28]. Furthermore prenylflavonoids and their metabolites may exert agonist, partial agonist or antagonistic actions [29]. To measure combinatorial estrogenic effects, we have developed and validated sensitive tools to determine estrogenic activity in serum [29,30]. These *ex vivo* bioassays are based on human cell lines that permanently express ERα and ERβ pro-

teins. Potential estrogenic activity is accurately measured when these cells are exposed to test sera [29]. Estrogenic ligands in sera bind ERα and ERβ. Liganded ER binds to and activates a consensus estrogen response element (ERE) driving a luciferase reporter gene incorporated into the genome. These ultrasensitive bioassays can determine global estrogenic activity of flavonoid mixtures revealing additive, restrictive and enhanced actions in binary and higher order combinations [29].

The aims of this study are to determine the pharmacokinetics of 5 important prenylflavonoids in ovariectomized rats following administration of increasing doses of a standardized *Epimedium* extract, and to correlate pharmacokinetics of these flavonoids with the pharmacodynamics of estrogenic bioactivity in sera measured *ex vivo* by ER-driven bioassays.

2. Experimental methods

2.1. Chemicals

Icariside I, icariside II, icaritin and desmethylicaritin (purity >98%) were provided by Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). Icariin (purity ~98%) was purchased from 3B Scientific Corporation (Libertyville, IL, USA). Coumestrol (purity >99%), estradiol (purity >98%), formic acid (purity ~98%) and ammonium formate (purity ~98%), 4-methylumbelliferone, 4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide were purchased from Sigma (St. Louis, MO, USA). Solvents such as methanol (HPLC grade), ethyl acetate (analytical grade), acetonitrile (HPLC grade) and acetone (analytical grade), were obtained from Merck (Darmstadt, Germany). Stock solutions of compounds were made using methanol, and stored in foiled-wrapped containers under nitrogen gas at −20 °C.

2.2. Animal study protocol

Details of the study protocol have been described [26]. In brief, female Sprague–Dawley rats (Janvier Le Genest, St. Isle, France) were ovariectomized at 6 weeks of age and maintained under standardized environmental conditions (ambient temperature: 21 ± 1 °C; relative humidity: 50–60%; 12 h light/dark cycle) and had *ad libitum* access to phytoestrogen-free food (Altromin, Lage, Germany) and tap water. At 8 weeks of age, rats were treated orally with 100, 300, 600 mg/kg of a standardized *Epimedium* extract by gavage. The *Epimedium* extract (PSC1929/L01-60/B/Wo 06-002-28) was ethanol extracted from dried *Epimedium brevicornu* and contained 143 mg/g of icariin, 0.157 mg/g of icariside I, 18.7 mg/g of icariside II, 9.02 μg/g of icaritin, and 122 μg/g of desmethylicaritin. Sera were obtained at 0, 0.5, 1, 2, 4, 8, 24, 48, and 72 h time points following *Epimedium* administration, and stored at −80 °C. At each time point, 4 rats were sacrificed to obtain sufficient sera for the assays required for PK/PD correlations. Thus 36 animals were used for each of the 4 treatment arms of the study. Animal experiments were performed at the premises of Dr. Willmar Schwabe Pharmaceuticals, Karlsruhe, Germany. Approvals for the study were granted by the veterinary authority of Baden-Wurttemberg, Germany; and the Institutional Animal Care and Use Committee, National University of Singapore.

2.3. LC/MS/MS analysis of icariin, icariside I, icariside II, icaritin and desmethylicaritin

Analysis of icariin, icariside I, icariside II, icaritin and desmethylicaritin was performed using a validated method [26]. All serum samples were thawed at room temperature, spiked with the internal standard (coumestrol, 100 nM) and equilibrated at 37 °C for at least 1 h before extraction. Analytes were extracted together with the internal standard from 0.5 ml serum aliquots thrice via liquid–liquid partition (1 ml ×3) using water-saturated ethyl acetate for 1 min inside a 2 ml polypropylene centrifuge tube. After each round of solvent–solvent partition, the mixture was centrifuged for 1 min at 10,000 × *g* to clearly separate the organic layer from the aqueous layer. The organic layers obtained from three rounds of solvent–solvent partition were combined and dried with a gentle stream of nitrogen gas. The residue was dissolved using 100 μl of methanol by vortexing for 1 min. This was then transferred into a 200 μl borosilicate glass insert placed inside a sample vial for LC/MS/MS analysis.

Flavonoids and internal standard in sera were analyzed with full scan mode to generate the precursor deprotonated molecules $[M-H]^-$ and the most abundant and characteristic product ions were selected for multiple reaction mode analysis. The ion pairs selected for the five flavonoids and IS were 513 → 351 for icariin, 529 → 367 for icariside I, 513 → 351 for icariside II, 367 → 175 for icaritin, 353 → 136 for desmethylicaritin, and 267 → 211 for IS, respectively. Coumestrol was chosen as the internal standard, because it has similar chemical and physical properties to *Epimedium* flavonoids, but is not naturally present in *Epimedium* and the rat diet. The limits of detection and quantification for icariin, icariside I, icariside II, icaritin and desmethylicaritin were <1 nM and 1–2 nM respectively. Inter- and intra-assay variabilities were <15% and accuracies were between 94% and 114% respectively. The linearity of the calibration curves was good ($r^2 > 0.99$) within the concentration range of 0.78–12.5 nM for icariin, icaritin and desmethylicaritin, and 0.78–100 nM for icariside I and II.

2.4. Measurement of conjugated flavonoids in rat sera

To determine the concentration of conjugated flavonoids, sera were exposed to enzyme mixture from *Helix pomatia*, type H5 (Sigma) before LC–MS/MS analysis. Freshly prepared enzyme solution in water with a pH ~ 7 (equivalent to ~2000 U of glucuronidase and ~200 U of sulphatase) was added to 0.5 ml of serum. No buffer was used for enzymatic digestion. The *H. pomatia* enzyme mixture was dissolved in ultrapure water which was then vortexed to mix and finally added to serum to start the enzymatic hydrolysis process. The mixture was vortexed briefly and incubated at 37 °C for 2 h. To determine completeness of enzymatic digestion, 1 μM of 4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide were spiked into commercial rat serum, exposed to enzyme and concentrations of 4-methylumbelliferone and its conjugates were measured by LC–MS/MS. Enzyme hydrolysis was performed in water without buffering reagent and acidifying the rat serum because prenylflavonoids precipitated under acidic conditions (pH 5). The enzymatic digestion efficiencies of the *H. pomatia* enzyme mixture for both 4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide were 100% after incubation at 37 °C for 2 h. The enzyme hydrolysis was terminated by liquid–liquid partition and prenylflavonoid aglycones were found to be stable under the post-preparative conditions [26].

2.5. Measurement of estrogenicity of rat sera

Estrogenicity of flavonoids in mixtures was measured using HeLa cells stably expressing ERα and ERβ proteins as previously described [29]. In this assay, the presence of estrogenic ligands, known and unknown, in complex mixtures can be detected by activation of the permanently incorporated ER-driven luciferase reporter gene, $pERE_4\text{-}Luc_{hygro}$. The assay is specific and displays minimal cross-reactivity to dihydrotestosterone, hydroxycortisone, progesterone, PPARα (WY14643) and PPARγ (rosiglitazone) agonists. For the ERα bioassay, the intra- and inter-assay CVs near the EC_{50} value were about 6% and 14% respectively. For ERβ, the intra-

and inter-assay CVs were 8.1% and 16% respectively. Detection limits for ERα and ERβ were 8.4 and 13.1 pM respectively. Detection limits for both ERα and ERβ assays were set as mean luciferase activity observed with vehicle, plus three SD.

Pooled rat sera used for the construction of the calibration curve were thawed at room temperature. The thawed sera were treated with dextran-coated charcoal. Norit A charcoal (4C) was mixed with dextran T-70 (Sigma) in a solution containing 0.25 M sucrose, 1.5 mM magnesium chloride, 10 mM HEPES (pH 7.4), to final concentrations of 0.25% and 0.0025%, respectively. The mixture was incubated overnight at 4 °C. After overnight incubation, a volume of the dextran-coated charcoal equal to the volume of serum was added and mixed thoroughly. After 12 h incubation at 4 °C, the mixture was centrifuged at 500 × *g* for 10 min to pellet the charcoal. The supernatant was passed through a 0.22 μm PVDF, radio-sterilized filter (Millipore).

Standards for the construction of calibration curves were obtained by adding increasing doses of estradiol to dextran-coated charcoal-treated pooled rat serum. Calibration standards were equilibrated for 4 h at 37 °C before use. All rat serum samples were diluted to 20% with serum-free media and were tested in duplicate. Estradiol (purity > 98%) was used as the calibration standard. The ranges of concentrations of estradiol for the ERα assay were from 0 to 125 pM (0, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5 and 125 pM) and 0 to 1000 pM (0, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 pM). Calibration curves of luminescence readings against estradiol concentrations were fitted with the regression method which visually best fits the points within the range of the test samples and maximizes the *R*-squared value. Data were expressed as estradiol equivalent activity and obtained by interpolation from calibration curves in each plate. GraphPad Prism was used for curve fitting and interpolation.

For measurement of bioactivity, samples were thawed at room temperature and immediately reconstituted with Eagle's Minimum Essential Medium to a final concentration of 20% sera. These reconstituted samples were then exposed to ERα and ERβ stable cells without further treatment. To measure the bioactivity conferred by conjugated flavonoids and other constituents, serum samples were first hydrolyzed by adding 1 μl of β-glucuronidase from *Escherichia coli* (Sigma) containing 25,000 U of β-glucuronidase and 1 μl of sulphatase from *Aerobacter aerogenes* (Sigma) containing 0.020 U sulphatase into 100 μl of rat serum sample. The mixture was then incubated for 2 h at 37 °C. This enzyme mixture was used instead of glucuronidase from *H. pomatia* (type H5) because the latter has been reported to contain contaminating amounts of estrogenic isoflavones [31]. Isoflavones from *H. pomatia* H5 preparation did not interfere with the measurements of prenylflavone derivatives from *Epimedium*. To ensure complete enzymatic digestion using this method, the hydrolysis of 4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide in rat sera was monitored using liquid chromatography–mass spectrometry (LC–MS/MS). Result of monitoring showed that by 2 h at 37 °C, the two conjugates were completely hydrolyzed and the two conjugates were not detected (results not shown).

ERα or ERβ cells were passaged and plated at a density of 1×10^4 cells per well on a 96-well cell culture plate. Cells were allowed to adhere overnight and culture medium was removed, replaced with medium containing 20% test sera and incubated for 24 h. Calibration standards were constructed by spiking known concentrations of estradiol to 20% dextran-coated, charcoal-stripped rat sera. Medium was decanted after 24 h and cells were washed with phosphate saline buffer and lysed with M-PER® Mammalian Protein Extraction Reagent (Pierce). The firefly luminescence was measured with Promega's Luciferase Assay System (Cat.# E4550) in a GloMax™ 20/20 Luminometer (Promega, USA) following the manufacture's protocols. Estrogenic bioactivity was obtained by extrapolating from calibration standards.

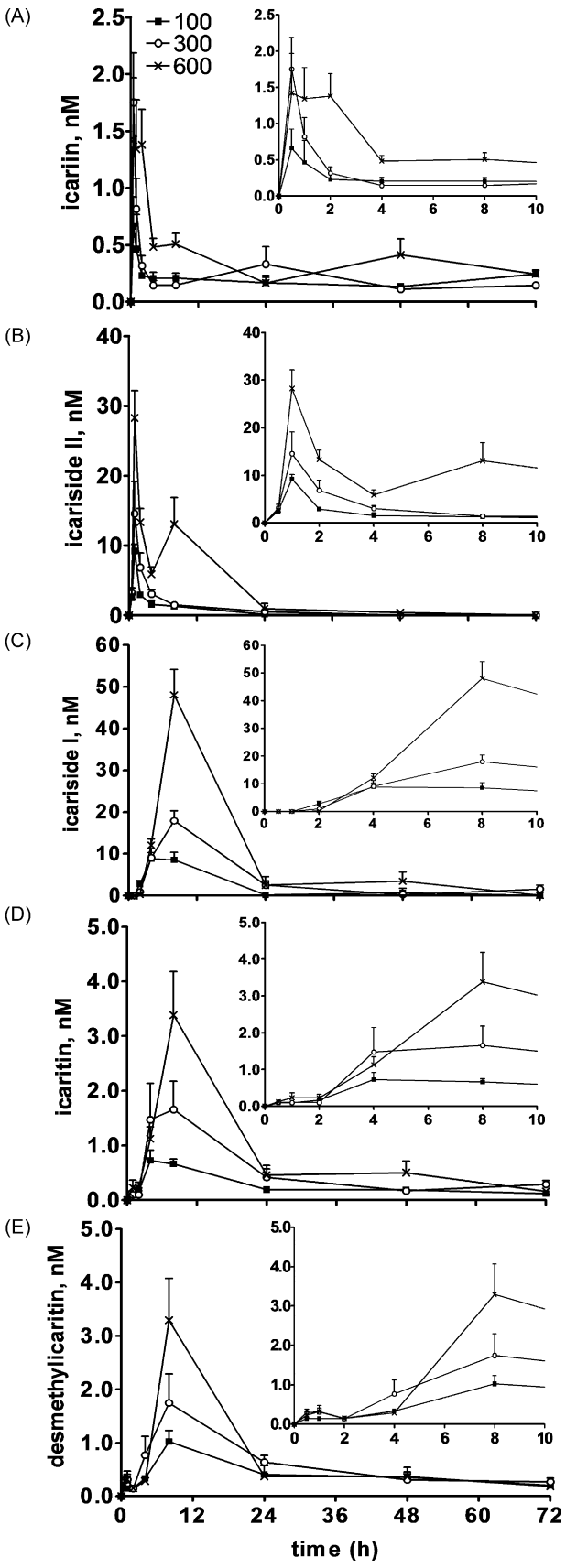

Fig. 2. Concentration–time profiles of free icariin (A), icariside II (B), icariside I (C), icaritin (D) and desmethylicaritin (E) in rat sera following oral administration of 100 mg/kg (–■–), 300 mg/kg (–○–) or 600 mg/kg (–×–) of an *Epimedium brevicornu* extract. Sera were sampled at the indicated time points, and concentrations of prenylflavonoids were determined using LC–MS/MS. Data points ($n = 4$) were the means ± SEM.

2.6. Statistical methods

The area under the concentration–time and bioactivity–time curves (AUC) of flavonoids was calculated by the trapezoidal method. Only the 0–24 h period was used for AUC calculations in order to reduce variability due to long sampling intervals after this time period. The maximum plasma concentration (C_{max}), maximum estrogenic effect (E_{max}) and the time to reach these maxima (t_{max}) were obtained by visual inspection of the experimental data. Estrogenic activity in sera was reported as estradiol equivalents (pM E2) based on calibration curves constructed with spiked estradiol. Values below the detection limit of the bioassay were scaled to 0. Comparison of estrogenic effects between treatments was performed by calculating AUC and these were reported as pM E2 h^{-1}.

3. Results and discussion

3.1. Flavonoid content in rat sera following administration of *Epimedium*

Female ovariectomized Sprague–Dawley rats were administered increasing doses of a standardized extract of *Epimedium* flavonoids by gavage. Blood samples were obtained at sequential time points over a 72-h period. Serum concentrations of icariin, icariside I, icariside II, icaritin and desmethylicaritin (Fig. 1) were measured using a sensitive LC–MS/MS method recently reported [26].

After administration of the extract, the diglycoside icariin was the first flavonoid to be detected in the serial samples (Fig. 2A). Icariin increased in a non-linear dose-dependent manner with AUC of 5.0, 6.5 and 11.6 nM h^{-1} following oral administration of 100, 300 and 600 mg/kg of *Epimedium* extract respectively (Table 1A). The early icariin peak at t_{max} 0.5 h (Fig. 2A) was similar to that observed when icariin was administered as a pure compound [32] and consistent with direct absorption [33]. Although icariin was the main flavonoid glycoside in the extract (14% by weight), peak concentrations of this glycoside in serum were in the low nanomolar range, suggesting poor bioavailability of the intact molecule. In comparison, the monoglycoside icariside II was detected at several-fold higher concentrations, with AUCs of 31.5, 50.9 and 198 nM h^{-1} with increasing *Epimedium* dosing (Table 1A). Concentrations of icariside II in serum were higher than icariin despite its lower concentration in the extract (icariin:icariside II, 7.8:1.0; w:w). Compared to icariin, icariside II lacks a glucose moiety at position 7 on ring A (Fig. 1) and this may make the latter more bioavailable. There is evidence from the rat intestinal model that apical to basolateral permeability of monoglycosides can be more than 2-fold greater than prenylflavonoids with 2 or more sugar moieties due to higher absorptive permeability and carrier-mediated transport in the intestine [34]. When icariin was administered alone, icariside II can be rapidly derived from icariin by first-pass deglycosylation and displays an early $t_{max} \sim 1$ h [33], similar to that observed in our study (Fig. 2B). Thus the early peak of icariside II with t_{max} 1 h is likely to be the sum of absorption of icariside II in the extract and metabolism of icariin.

At the highest *Epimedium* dose, a secondary delayed icariside II peak with t_{max} of 8 h was observed. This delayed peak of icariside II was not seen when it was derived from icariin administered alone [33], but was reminiscent of the delayed re-entry peak observed when biochanin A was administered as a mixture with quercetin and (−)-epigallocatechin-3-gallate [35]. Remarkably, icariside I, icaritin and desmethylicaritin also exhibited a major peak at t_{max} 8 h (Fig. 2C–E). At t_{max} 8 h, the most abundant flavonoid was icariside I with an AUC of 538 nM h^{-1} at the highest drug dose. In comparison, AUC of the aglycones icaritin and desmethylicaritin were an order of magnitude lower.

Thus, two distinct pharmacokinetic patterns for prenylflavonoids can be discerned following administration of *Epimedium* extract. An early phase during which icariin and icariside II reached peak levels in 0.5–1 h (Fig. 2A and B), and a late phase wherein icariside I, icaritin and desmethylicaritin peaked at 8 h (Fig. 2C–E). Treatment with *Epimedium* extract resulted in non-linear increases in AUC and delayed peaks.

3.2. Flavonoid content in rat sera after digestion with glucuronidase/sulphatase

To measure the amounts of conjugated flavonoids in test sera, analyses were repeated after digestion by β-glucuronidase and sulphatase. To test digestion efficiency, 1 μM of 4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide were exposed to enzymes and analyzed for the presence of conjugates. No conjugates were detected after enzyme digestion under the described conditions. Different dosages of icariin, icariside I and icariside II, up to 1 mM, were digested under the same conditions. Up to the maximal dose of 1 nM, enzymatic digestion efficiency was 100%, as icariin and icariside I were not observed in the LC/MS/MS chromatograms. Under these conditions, icariin and icariside I were deglycosylated completely to icariside II and icaritin respectively, and were not detected in enzyme-treated sera (data not shown). However, icariside II was stable because its rhamnose moiety was resistant to enzyme digestion. Therefore, only three compounds, icariside II, icaritin and desmethylicaritin, could be detected in rat serum samples after enzyme hydrolysis. Here, icariside II peaked at 0.5 h (Fig. 3A) and is consistent with rapid absorption and conjugation [33]. As expected, AUC values of icariside II after digestion were

Table 1
Concentration–time and ERα bioactivity–time AUC profiles of prenylflavonoids. Rats were administered the indicated interventions and blood sampled as in Section 2. Concentrations of prenylflavonoids were determined by LC–MS/MS. Estrogen bioactivity was measured with an ER-driven luciferase reporter gene in HeLa cells stably expressing ERα. Sera were examined before (A), and after (B), treatment with glucuronidase/sulphatase.

(A)	AUC (concentration) nM h^{-1}					AUC (ERα) pM E2 h^{-1}
Free	Icariin	Icariside I	Icariside II	Icaritin	Desmethylicaritin	ERα
E2 benzoate (2 mg/kg)	–	–	–	–	–	67.2
Epimedium (100 mg/kg)	5.00	118	31.5	10.7	14.9	0
Epimedium (300 mg/kg)	6.50	229	50.9	24.6	25.5	0
Epimedium (600 mg/kg)	11.6	538	198	41.5	37.3	104.4

(B)	AUC (concentration) nM h^{-1}			AUC (ERα) pM E2 h^{-1}
Total	Icariside II	Icaritin	Desmethylicaritin	ER-α
E2 benzoate (2 mg/kg)	–	–	–	148.7
Epimedium (100 mg/kg)	71.8	11.12×10^3	3.12×10^3	374.6
Epimedium (300 mg/kg)	181	34.61×10^3	5.07×10^3	543.9
Epimedium (600 mg/kg)	734	33.96×10^3	7.69×10^3	771.5

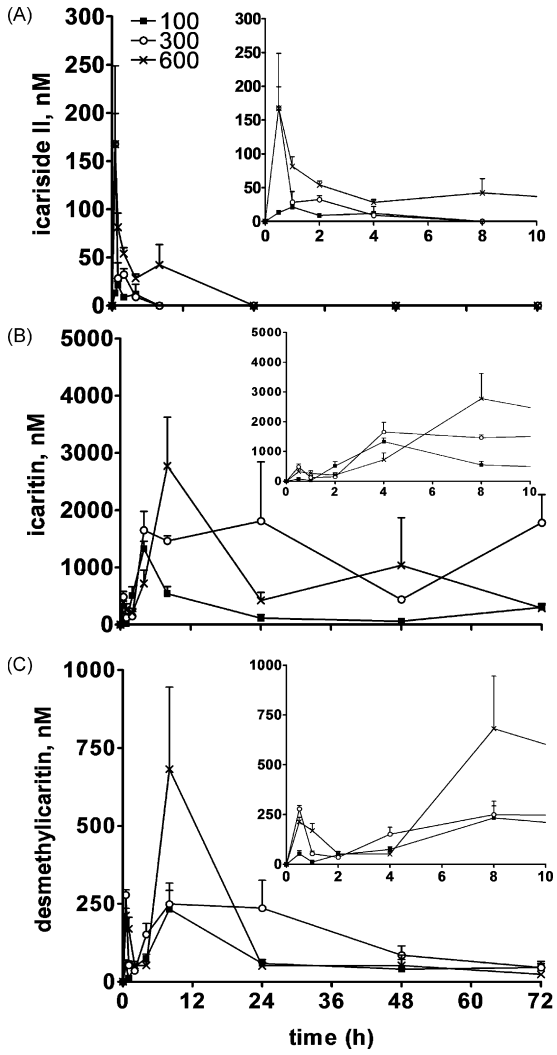

Fig. 3. Concentration–time profiles of total icariside II (A), icaritin (B) and desmethylicaritin (C) in rat sera following digestion with *Helix pomatia* β-glucuronidase. Rats were administered with 100 mg/kg (–■–), 300 mg/kg (–○–) or 600 mg/kg (–×–) of an *Epimedium* extract and serum samples obtained at the indicated time points. Data points were the means ± SEM.

several orders of magnitude higher than non-digested samples (Table 1), the increase reflecting the sum of deconjugated icariside II and deglycosylated icariin. Desmethylicaritin and icaritin exhibited two peaks, a minor one at 0.5 h and a more prominent one at 8 h (Fig. 3B and C). Remarkably, levels of desmethylicaritin and icaritin after treatment with glucuronidase were two to three orders of magnitude higher than before exposure to enzyme (Table 1, comparing A and B), suggesting that the greater proportion of these compounds and their precursors existed in conjugated forms. This was consistent with data with the flavanone, naringenin, whereby >95% of the compound exists in the conjugated form [28].

The C_{max} of icaritin and desmethylicaritin reached ~2 and ~0.25 μM respectively, both occurring at 8 h. Non-linear dose-dependent AUC increases were observed. Saturation effects at higher *Epimedium* doses were evident for icaritin, possibly due to either reduced absorption or increased metabolism. Interestingly, the isoflavone, genistein, also displays non-linear pharmacokinetics, attributed to reduced absorption at high doses [36]. Remarkably, conjugated icaritin can be detected at micromolar levels up to 72 h after administration of the high dose *Epimedium* extract. This has not been observed when genistein alone was administered, where conjugated genistein reached baseline levels after 36 h [36].

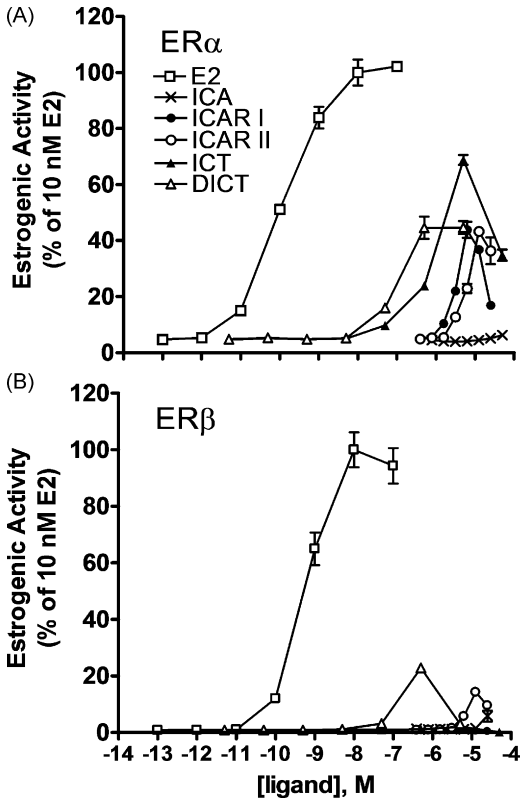

Fig. 4. Estrogenic activity of *Epimedium* prenylflavonoids *in vitro*. HeLa cells stably expressing ERα (A) and ERβ (B) proteins and an ER-driven luciferase reporter gene, $pERE_4$-Luc_{hygro} were exposed to increasing concentrations of icariin (ICA), icariside II (ICAR II), icariside I (ICAR I), icaritin (ICT) and desmethylicaritin (DICT) *in vitro*. Estrogenic activity of ligands was expressed as a percentage of maximal effect observed with 10 nM estradiol (E2).

3.3. Measurement of estrogenic effects in rat sera using ERα and ERβ bioassays

To evaluate their potential estrogenic effects, we constructed dose–response curves of these prenylflavonoids *in vitro,* using HeLa cell lines stably expressing ERα (or ERβ) and an estrogen-responsive reporter gene (Fig. 4). Icariin did not exhibit either ERα or ERβ activity. Desmethylicaritin, icaritin, icariside I, and icariside II induced maximal ERα activity (E_{max}) that was about 45% of the peak activity observed for estradiol (Fig. 4A, Table 2), indicating that they were partial agonists. The potency of the compounds was in the order desmethylicaritin > icaritin > icariside I > icariside II with their EC_{50} being 0.07, 0.83, 5.45 and 13.1 μM respectively (Table 2). Icaritin and icariside I were ERα-selective as these com-

Table 2
Estrogenic effects of *Epimedium* prenylflavonoids measured *in vitro* with ER-driven luciferase reporter genes in HeLa cells stably expressing ERα or ERβ.

Compound	EC_{50} (μM)		E_{max} (% of 10 nM E2)	
	ERα	ERβ	ERα	ERβ
Estradiol	1.19×10^{-4}	6.48×10^{-4}	100	100
Icariin	NSA	NSA	NSA	NSA
Icariside I	5.45	NSA	43.9	NSA
Icariside II	13.1	6.78	43.3	22.8
Icaritin	0.839	NSA	68.8	NSA
Desmethylicaritin	0.0706	0.0789	44.7	14.3

EC_{50}: half-maximal activity extrapolated from dose–response curves depicted in Fig. 4, E_{max}: maximum estrogenic activity attained in the dose–response curve of each compound (depicted in Fig. 4) compared to estradiol (E2). NSA: no significant activity.

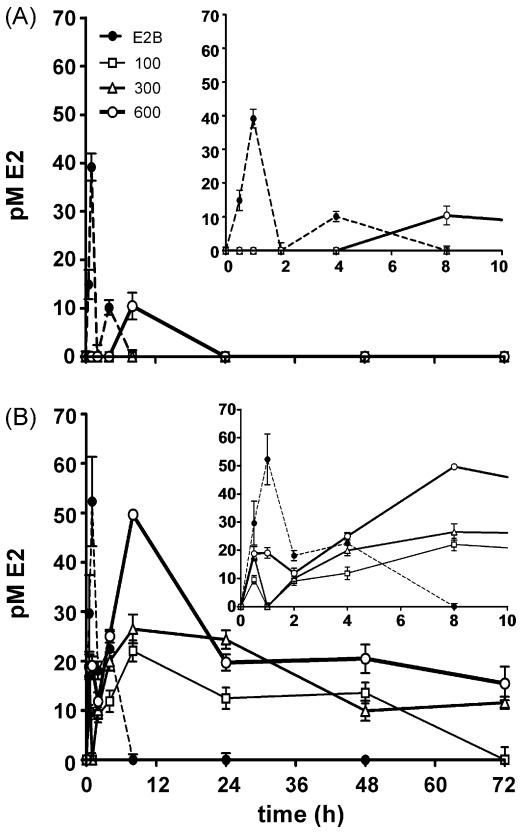

Fig. 5. Dynamics of estrogenic activity in rat sera. Aliquots of sera from rats administered estradiol benzoate (E2B, –●–), 100 mg/kg (–□–), 300 mg/kg (–△–) or 600 mg/kg (–○–) *Epimedium* extract were examined *ex vivo* for ERα bioactivities before (A), and after (B), glucuronidase/sulphatase treatment. Data points ($n = 4$) were the means ± SEM and expressed as pM estradiol (E2) equivalent.

pounds did not stimulate ERβ activity (Fig. 4B). Icariside II and desmethylicaritin exhibited slight ERβ activity corresponding to ≤20% of that observed with estradiol. Since the prenylflavonoids examined exhibited weak ERβ activity and were predominantly ERα-selective, subsequent bioassays were focused on ERα pharmacodynamics.

Rat sera were examined *ex vivo* for ERα bioactivity (Fig. 5). Control rats administered estradiol benzoate displayed a major peak of ERα activity at t_{max} 1 h with E_{max1} 67 pM E2 equivalent, and a minor peak at 4 h, with E_{max2} 40 pM E2 equivalent (Fig. 5A). Rats administered with low doses of *Epimedium* did not display any significant ERα activity (Fig. 5A). However, at the highest *Epimedium* dose (600 mg/kg), ER bioactivity was observed at the 8 h timepoint. This peak was comparable in size to that of the minor peak after estradiol benzoate administration. Thus, estrogenic activity in sera after ingestion of *Epimedium* lagged by several hours compared to estradiol benzoate, and corresponded to the appearance of the bioactive metabolites desmethylicaritin, icaritin and icariside I at 8 h (Fig. 2C–E).

3.4. Estrogenic activity in sera after digestion with glucuronidase/sulphatase

To determine bioactivity that may potentially be exerted by the conjugated *Epimedium* constituents, sera were exposed to glucuronidase/sulphatase and re-analyzed for estrogenic activity. Control animals fed estradiol benzoate exhibited a temporal pattern of estrogenic activity (Fig. 5B) similar to that observed before exposure to enzyme (Fig. 5A), except that the peaks were higher, contributing to an increased AUC of 148 pM E2 h^{-1} compared to 67 pM E2 h^{-1} before enzyme treatment (Table 1). This increase in AUC value probably reflects conjugated estradiol rendered bioactive again through enzyme treatment. Remarkably, sera from rats fed *Epimedium* exhibited strong ERα bioactivity throughout most of the 72 h study period (Fig. 5B). Dose-dependent increases in ERα activity were observed with AUC of 374, 543 and 771 pM E2 h^{-1} with increasing doses of *Epimedium* (Table 1B). The peaks of ERα activity for *Epimedium* were biphasic with a small first peak at 0.5–1 h and larger second peak at 8 h (Fig. 5B). This biphasic pattern reflected the appearance of icaritin, desmethylicaritin, and icariside II at 0.5 and 8 h (Fig. 3). Concentrations of desmethylicaritin and icaritin reached 0.2 and 0.4 μM respectively at 0.5 h, corresponding values at the 8 h time-point being 0.6 and 1.5 μM. Since the EC_{50} of desmethylicaritin and icaritin was 0.07 and 0.83 μM respectively (Table 2), it is plausible that estrogenic activity of *Epimedium* is contributed by desmethylicaritin and icaritin. Another unique feature of *Epimedium* is its prolonged effect, with strong estrogenic activity (40% of peak activity) being detected up to 72 h (Fig. 5B). Prolonged estrogenic activity especially at higher *Epimedium* doses correlated with the persistence of micromolar levels of icaritin at the 48–72 h sampling period (Fig. 3B). The depot effect resulted in time–concentration bioactivity profiles (374, 543, and 771 pM E2 h^{-1}) following administration of increasing *Epimedium* doses to exceed that observed for estradiol benzoate (148 pM E2 h^{-1}) (Table 1B). It is relevant to note that pharmacological doses of flavonoids were administered in this study. At the level of the enterocyte, cycling of flavonoid conjugates back to the intestinal lumen at high doses may be markedly decreased, reflecting a saturation of the intestinal conjugation pathways [37]. The long elimination time for the highest dose of icaritin may also be due to differences in the type of Phase II enzymes that are activated. The possibility exists that sulfotransferases, rather than glucuronosyltransferases, may be preferentially recruited at higher doses, resulting in the production of very hydrophilic metabolites which may be less efficiently eliminated by the kidney [38], thereby contributing to the prolonged presence of icaritin in sera even 72 h after dosing. Since intestinal and hepatic metabolism of flavonoids at high doses may differ from more physiological doses, our results may not be directly applicable to all doses of flavonoids. Nevertheless, similar depot effects have been observed in ERE-luc transgenic mice, whereby a single physiological dose of the isoflavone was able to induce significant ER/ERE-driven activity in diverse target organs such as liver, cerebral cortex and testis for over 24 h [39]. Such delayed effects may persist for at least 2 weeks, were unmasked by fasting and are thought to be due to bioaccumulation in the intestines, liver and reproductive tissues [40].

4. Conclusion

Our study correlated the pharmacokinetics of prenylflavonoids with the dynamics of their estrogenic effects and reveal new insights into the pharmacokinetic/pharmacodynamic interactions underlying the biological effects of a complex mixture like *Epimedium*. The appearance of bioactive prenylflavonoids in sera corresponded broadly with estrogenic activity. Non-linear pharmacokinetics and prolonged effects for up to 72 h following administration of a single dose of *Epimedium* extract were observed. The coupled processes of enteric and enterohepatic recycling may allow different polyphenols to be reabsorbed and results in longer than expected apparent plasma half-lifes for some *Epimedium* compounds and their conjugates. The vast majority of prenylflavonoids was conjugated and relatively non-estrogenic. However, these concentrations of flavonoids in sera may potentially be bioactive if deconjugated. Besides the intestine and liver, glucuronidase activity has long been known to be present in many tissues such as bone

[41], brain [42] and mammary glands [43]. One challenge for the future would be to use these bioassays to determine if *Epimedium* prenylflavonoids in target tissues can be deconjugated and become bioactive in target tissues such as bone and breast. In this regard, it is interesting to note that administration of 8-prenylnaringenin can exert effects on the hypothalamic–pituitary–uterine axis in rats [44] and post-menopausal women [45]. These data have implications for the development of prenylflavonoids as drugs for menopause and other conditions requiring estrogenic action.

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