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Liquid chromatography–tandem mass spectrometry analysis of metabolites in rats after administration of prenylflavonoids from *Epimediums*

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ABSTRACT

The metabolites in rats after administration of icariside II, icariin, epimedin C and extracts of four *Epimedium* species were investigated. Feces, bile, plasma and urine samples were detected comprehensively using HPLC-ESI-MS^n method. The structures of metabolites were identified on the basis of their characteristic fragmentations in MS^n experiments. Totally, 54 metabolites were identified in these biosamples. Specific hydrolysis of 7-O glucosides in gut lumen and glucuronic acid conjugation in liver were considered as the main physiologic processes of prenylflavonoids. Icariside II and anhydroicaritin were the major intermediate products in forming of mono- and di-glucuronic acid conjugations *in vivo*. In general, this study revealed the possible metabolite profiles of prenylflavonoids in rats, and might aid the clinical use of different *Epimedium* species.

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1. Introduction

Epimediums (Berberidaceae) are traditionally used as tonic, aphrodisiac and antirheumatic medical herbs for cardiovascular, bone loss and impotence diseases in East Asian countries. Prenylflavonoid is a group of the major active constituents present in *Epimediums*. It has been proved in pharmacology studies [1–5] that prenylflavonoids possess arrays of biological activities such as androgenic, anti-oxidant, antidepressant, penile erection, preventing osteoporosis and anti-tumor effects. Besides, prenylflavonoids have been shown as potent phytoestrogens with selective estrogen receptor-binding activity as well [6]. More specially, Icariside II and icariin derivatives exhibited potent inhibition of human phosphodiesterase-5 with an IC50 very close to that of sildenafil [7]. Meanwhile, as the principal prenylflavonoid in *Epimedium wushanense*, epimedin C enhanced the response of spleen antibody-forming cells significantly [8]. Recent study also revealed its anti-tumor activity against hepatoma SK-Hep-1 cells [9].

These biological activities have stimulated increasing interest in the pharmacokinetic investigations on prenylflavonoids and *Epimediums*. Benefitting from the development of analysis techniques, the *in vivo* quantitative analyses of icariside II, icariin, epimedin C and the total flavonoids were carried out comprehensively by GC-MS and LC-MS [10–12]. Additionally, the low oral bioavailability and first-pass effect of prenylflavonoids have been assessed using human intestinal Caco-2 and the perfused rat intestinal models [13]. In integral animal experiments, the metabolite analyses of icariin in biosamples and pharmaco-metabonomics study of *E. brevicornum* have been performed [14,15]. However, only one prenylflavonoid or species was used in previous investigations, which was not able to clarify the general metabolite profiles of *Epimediums*. The relationship between structures and physiological processes of prenylflavonoids was still unclear and the study of their metabolic pathway should be improved.

Although it is generally believed that, in physiological disposition process, conjugation with glucuronic acid of flavonoids is detoxification and inactivation pathway that leads to increased solubility for excretion [16], the activities of flavonoid conjugations have been reassessed recently. The conjugation position was of immense significance in determining potential biological activity [17]. However, without the reference and credible MS^n fragmentation pathways, the current HPLC-MS/MS method fails to precisely attribute the positions of glucuronic acid and sugar chains at flavonoids in biosamples. On the basis of our previous studies on characteristic MS^n fragmentation behaviors of different types of prenylflavonoids [18], HPLC-ESI-MS^n method was adopted in the present study to elucidate the structures of metabolites existing in feces, bile, plasma and urine samples. The purpose was to illustrate the relationship between the chemical profiles and metabolic processes of *Epimediums*. After oral administration of three pure prenylflavonoids (epimedin C, icariin and icariside II) and extracts of four *Epimedium* species (*E. koreanum*, *E. brevicornum*, *E. sagittatum*, *E. wushanense*) to rats, the biosamples were collected and analyzed by HPLC-ESI-MS^n ($n = 2–5$) experiments. A total number of 28, 8, 9 and 9 metabolites were identified or

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tentatively characterized in feces, bile, blood and urine, respectively. The absorption, metabolism and excretion of orally administered prenylflavonoids have been examined comprehensively. Generally, the possible physiological disposing pathway of prenylflavonoids was proposed according to the analyses of metabolites.

2. Experimental

2.1. General

Pure prenylflavonoids epimedin C, icariin, and icariside II (Fig. 1) were isolated from the aerial parts of *E. koreanum* Nakai by authors and anhydroicaritin was purchased from Yifang Science and Technology Company (Tianjin, China). Their structures were unambiguously identified on the basis of NMR and MS data and purities were about 95% as determined by HPLC analysis.

The powder of four *Epimediums* (*E. koreanum*, *E. brevicornum, E. wushanense, E. sagittatum*) was refluxed for 1.5 h three times with 70% methanol, respectively. The extracts were concentrated under reduced pressure to remove methanol and then dried as powder by vacuum drying. The voucher specimens of these samples were deposited at the Peking University Health Science Center.

E. koreanum was from Jilin Province, *E. brevicornum* was from Shanxi Province, *E. sagittatum* and *E. wushanense* were from Guizhou Province of China. The chemical profiles of these species have been studied by HPLC-ESI-MS/MS in our previous investigation.

HPLC grade acetonitrile (Fisher, Fair Lawn, NJ, U.S.A.) was used for HPLC analysis. Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, U.S.A.). The methanol for extraction was of analytical-reagent grade and purchased from Beijing Chemical Corporation (Beijing, China).

2.2. Instrumentation

This LC system consisted of an Agilent series 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode-array detector, an auto sampler and a column compartment. Chromatography was performed on a Zorbax SB-C18 column (250 mm × 4.6 mm, I.D., 5 μm, Agilent) and monitored at 270 nm with the column temperature at 30 °C. Mobile phase consisted of acetonitrile (A) and water containing 0.2% formic acid (B) and pumped at a flow rate of 1.0 ml/min. Two separate HPLC methods were used for the analyses. For feces samples, a gradient program was used as follows: initial 0–10 min, linear change from A–B (20:80, v/v) to A–B (35:65, v/v); 10–45 min, linear change to A–B (40:60, v/v); 45–50 min, linear change to A–B (50:50, v/v); 50–80 min, linear change to A–B (100:0, v/v). For bile, plasma and urine samples, the other gradient program was as follows: initial 0–20 min, linear change from A–B (15:85, v/v) to A–B (35:65, v/v); 20–60 min, linear change to A–B (60:40, v/v); 60–80 min, linear change to A–B (100:0, v/v).

LC-MS experiments were conducted using a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo *Scientific*, San Jose, CA) via an ESI source in a post-column splitting ratio of 2:1. Ultrahigh pure helium (He) was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. The optimized parameters in the negative ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas (N_2), 50 arbitrary units; auxiliary gas (N_2), 10 U; capillary temperature, 320 °C; capillary voltage, −14 V; tube lens offset voltage −20 V. For full scan MS analyses, the spectra were recorded in the range of *m/z* 100–1500. A data-dependent program was set so that the two most abundant ions in each scan were selected and subjected to tandem mass spectrometry (MS^n, n = 2–5). The collision induced dissociation (CID) energy was adjusted at 25% in LC/MS analysis and the isolation width of precursor ions was 2.0 mass units.

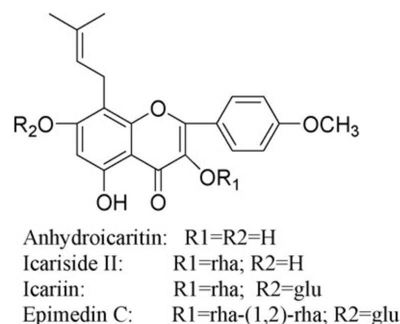


Fig. 1. The structures of anhydroicatin, icariside II, icariin and epimedin C.

2.3. Animals and drug administration

Male Sprague-Dawley rats (250 ± 20 g body weight) were obtained from the Laboratory Animal Center of Peking University Health Science Center, China. Rats used in metabolism studies were housed in an animal room at ambient temperature (22–24 °C) and 60% relative humidity for three days. In the analyses of feces and urine samples, each rat was held in a stainless steel metabolism cage. The animals were fasted 12 h before experiments, but had access to deionized water. Three pure compounds and four extracts were dissolved in 50% propanediol. 80 mg/kg of pure compounds and 800 mg/kg of extracts were set as the dosages for oral administration. Deionized water was administered orally to the rats at a dose of 10 ml/kg body weight for blank groups.

2.4. Collection of samples

Blood samples (500 μl, three as one group) via the orbital sinus were collected into heparinized tubes at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h post dosing to three rats. Plasma was harvested by centrifugation and stored at −80 °C until analysis.

For feces and urine sampling, three rats were housed in metabolic cages with free access to deionized water. Samples were collected at 0–12 h and 12–24 h post-intake and stored at −80 °C until additional extraction and analysis.

For bile sampling, rats were fixed on a wooden plate and anesthetized with 5% urethane. An abdominal incision was made and the common bile duct was cannulated with PE-10 tubing (ID = 0.08 cm, Becton Dickinson, U.S.A.) for the collection of bile samples. Each compound and extract was consciously administered to three animals by oral gavage. Bile samples were collected during 0–12 h and 12–24 h periods and then stored at −80 °C until additional extraction and analysis.

2.5. Sample preparation

Samples of blood (200 μl) and urine (1 ml) were mixed with 500 μl and 2 ml acetonitrile, respectively. Bile samples (500 μl) were extracted with ethyl acetate at the ratio of 1:3 (v/v) for three times. Successively, the mixed samples of blood and urine and the extraction of bile were performed by vortex mixing for 5 min, followed by centrifugation at 9000 rpm for 10 min, respectively. The supernatant of sample was transferred to a clean test tube and dried under a flow of nitrogen gas at 35 °C. The residue was redissolved in 200 μl methanol. After filtering through a membrane (0.45 μm pore size), a 20 μl aliquot was injected into the chromatographic system for analysis.

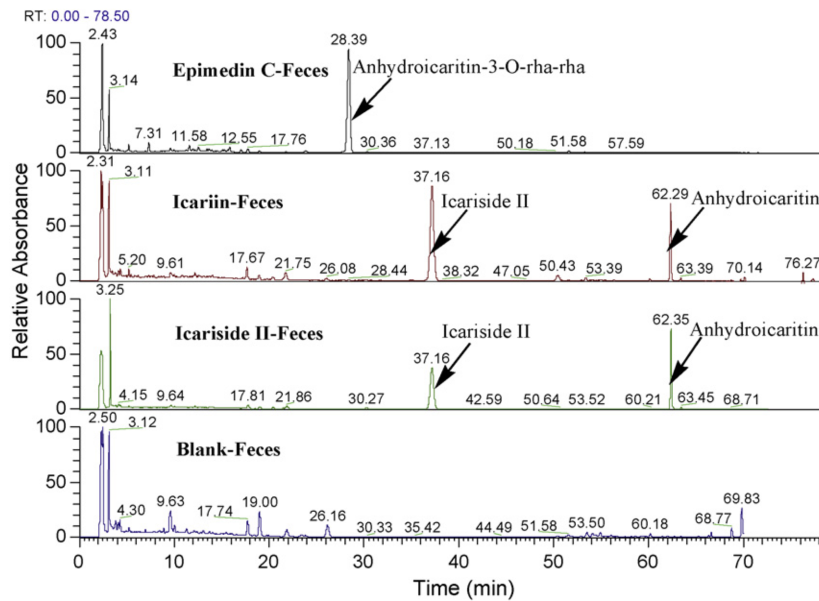


Fig. 2. HPLC/UV chromatograms of rat feces samples after administration of epimedin C, icariin and icariside II.

Feces samples were weighted 0.5 g and supplemented with 5 ml methanol. After ultrasonic extraction for 30 min, the samples were centrifuged at 9000 rpm for 5 min. Then, the supernatant was filtered through a membrane (0.45 μm pore size) and 10 μl aliquot was injected into the chromatographic system for analysis.

3. Results

By comparing the HPLC and MS^n data of biosamples with blanks, changes of herbal components have been occurred in the course of physiological disposition. Relatively abundant metabolites in feces and bile with trace amount in plasma and urine samples were detected. To explore the biotransformation of *Epimediums in vivo*, the structures of metabolites were speculated based on the metabolism rules of drugs and the mass spectra fragmentation pathways of prenylflavonoids deduced before [17]. Firstly, the investigations of three pure compounds were performed and then the extracts of four *Epimediums* were used in experiments. In this paper, the names of sugar moieties were abbreviated as the following: glycoside (gly); rhamnose (rha); glucose (glu); glucuronic acid (gluA). The aglycone ion $[M–H–gly_{(7)}–gly_{(3)}]^-$ and radical aglycone ion $[M–2H–gly_{(7)}–gly_{(3)}]^-$ were labeled as Y_0^- and $[Y_0–H]^-$.

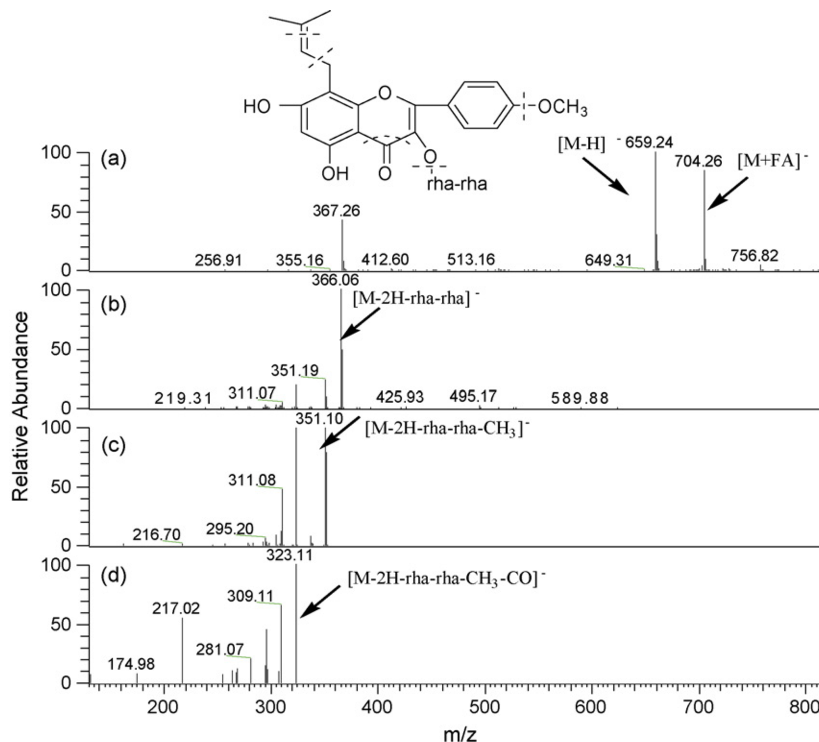
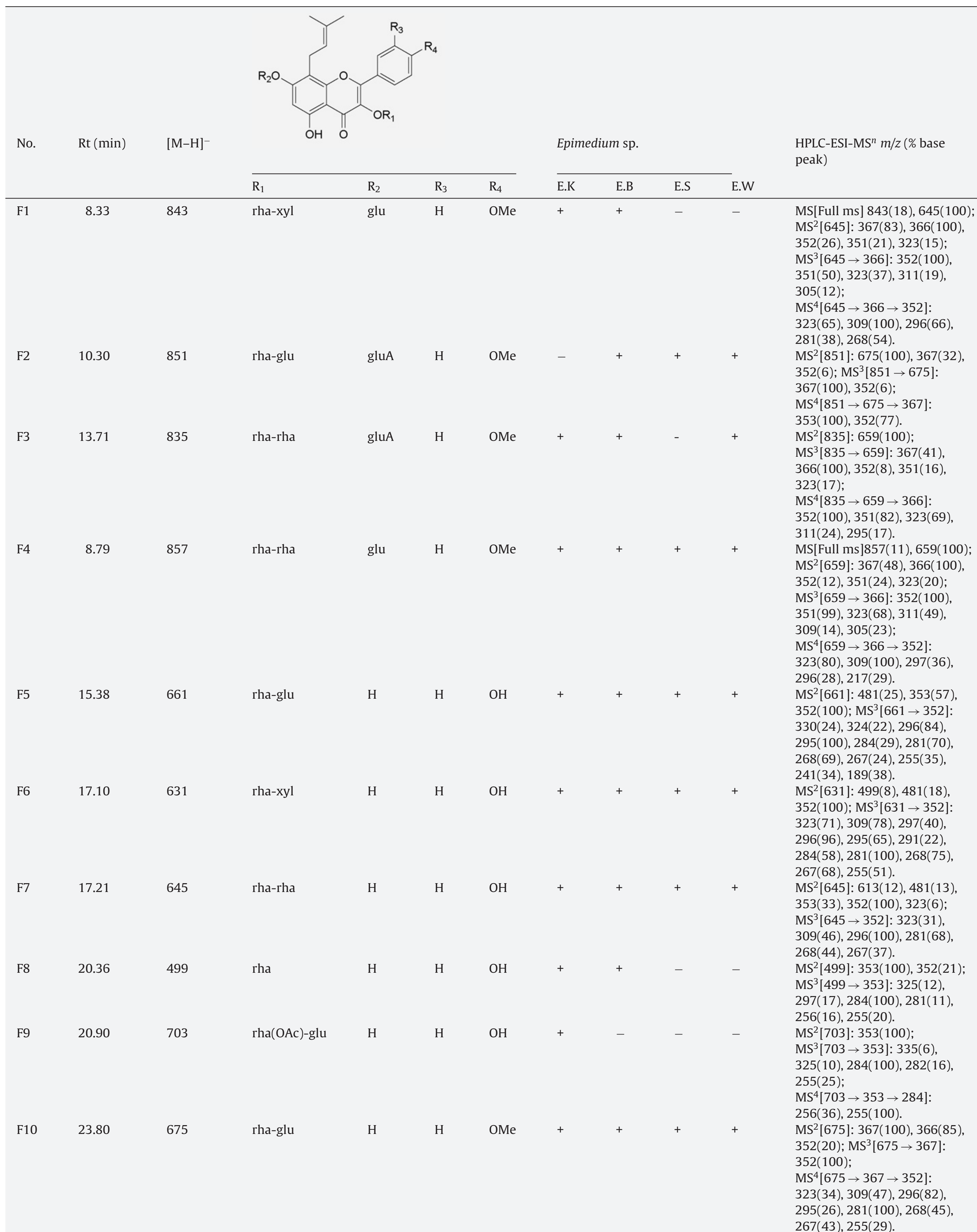


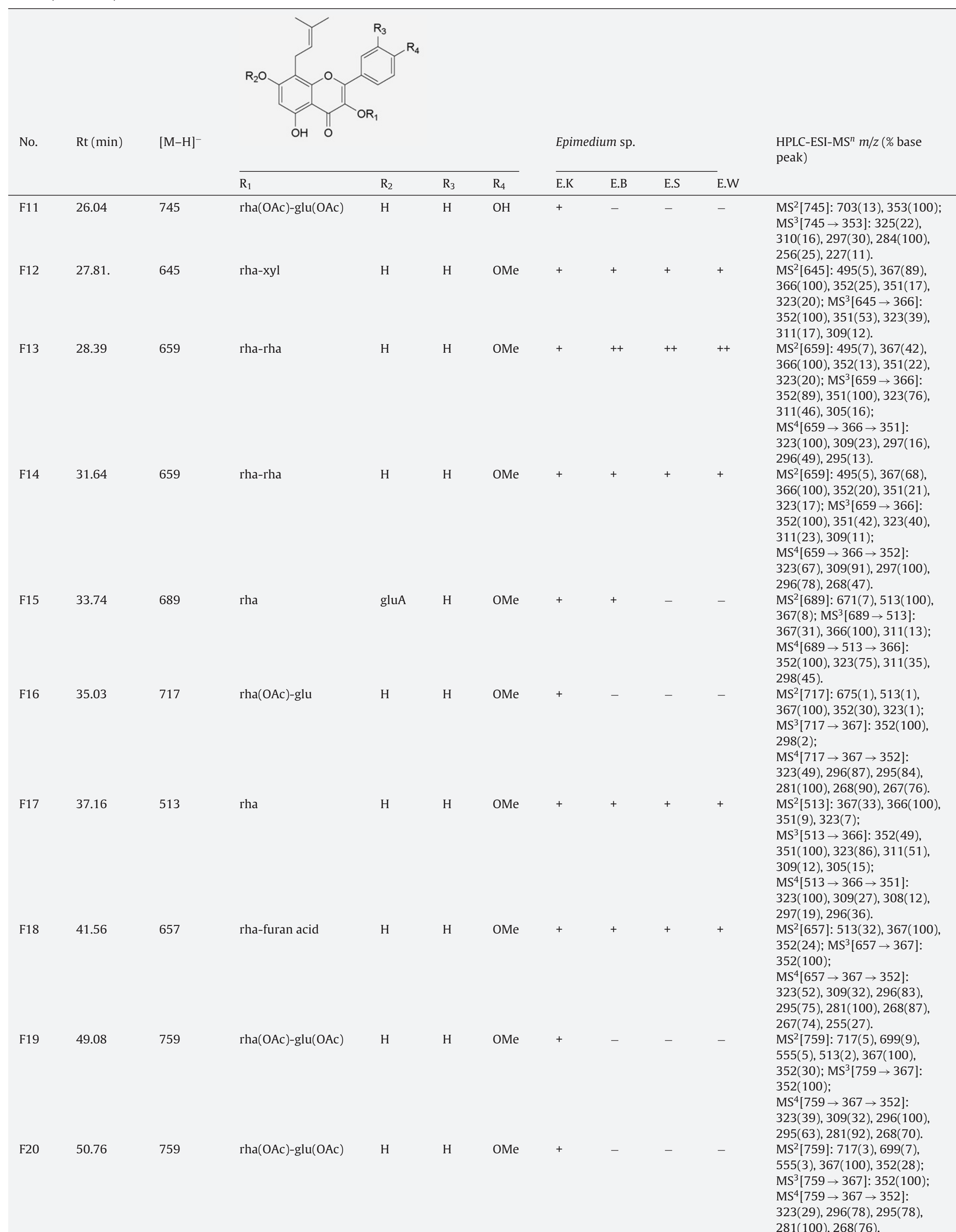
Fig. 3. ESI-MS^n spectra for F13, Anhydroicaritin-3-O-rha-(1,2)rha.

Table 1
HPLC-ESI-MS^n data *in vivo* identification of prenylflavonoids metabolites in rat feces samples.



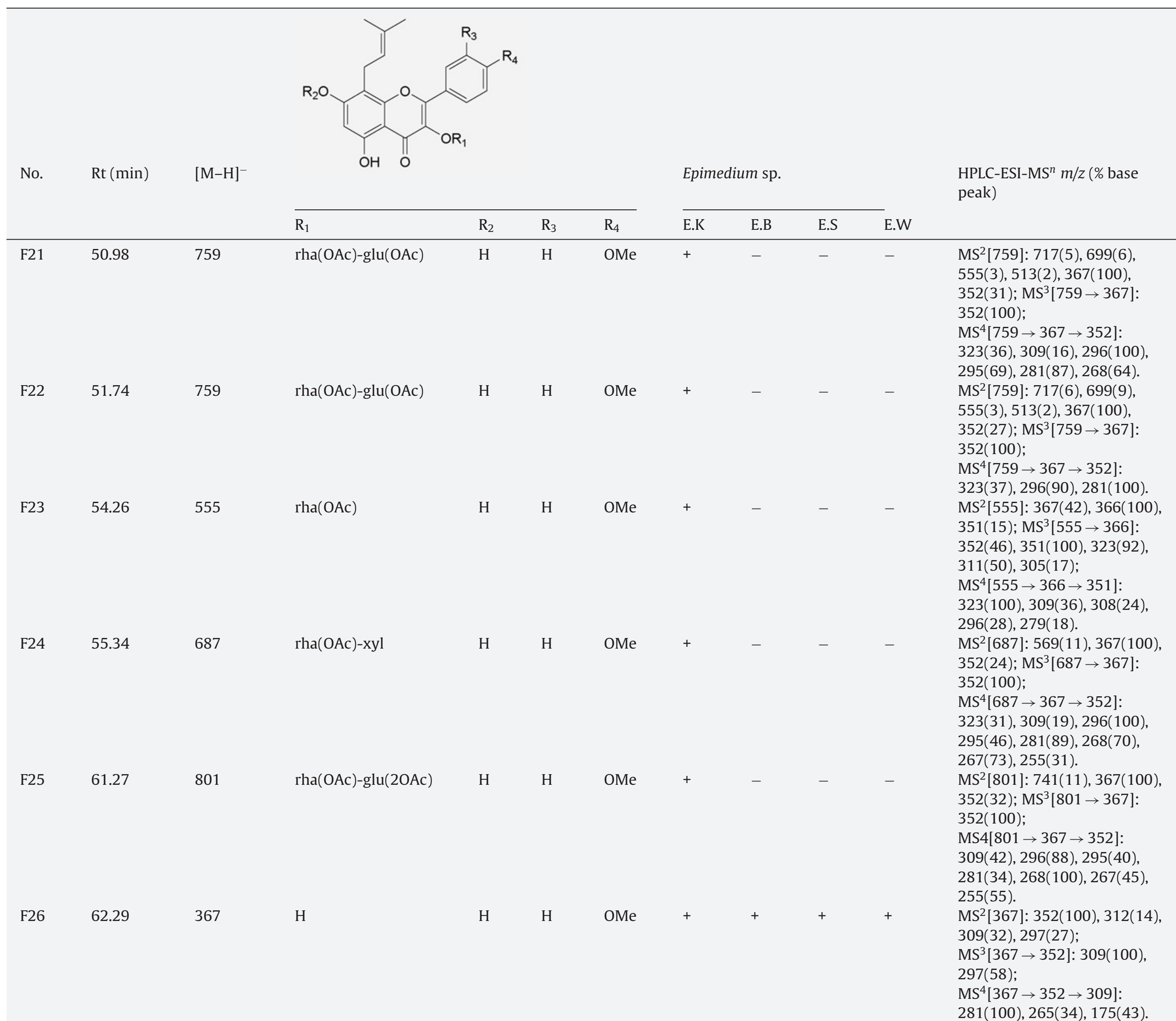
No.	Rt (min)	[M–H]$^-$	R$_1$	R$_2$	R$_3$	R$_4$	*Epimedium* sp. E.K	E.B	E.S	E.W	HPLC-ESI-MS^n *m/z* (% base peak)
F1	8.33	843	rha-xyl	glu	H	OMe	+	+	–	–	MS[Full ms] 843(18), 645(100); MS^2[645]: 367(83), 366(100), 352(26), 351(21), 323(15); MS^3[645 → 366]: 352(100), 351(50), 323(37), 311(19), 305(12); MS^4[645 → 366 → 352]: 323(65), 309(100), 296(66), 281(38), 268(54).
F2	10.30	851	rha-glu	gluA	H	OMe	–	+	+	+	MS^2[851]: 675(100), 367(32), 352(6); MS^3[851 → 675]: 367(100), 352(6); MS^4[851 → 675 → 367]: 353(100), 352(77).
F3	13.71	835	rha-rha	gluA	H	OMe	+	+	-	+	MS^2[835]: 659(100); MS^3[835 → 659]: 367(41), 366(100), 352(8), 351(16), 323(17); MS^4[835 → 659 → 366]: 352(100), 351(82), 323(69), 311(24), 295(17).
F4	8.79	857	rha-rha	glu	H	OMe	+	+	+	+	MS[Full ms]857(11), 659(100); MS^2[659]: 367(48), 366(100), 352(12), 351(24), 323(20); MS^3[659 → 366]: 352(100), 351(99), 323(68), 311(49), 309(14), 305(23); MS^4[659 → 366 → 352]: 323(80), 309(100), 297(36), 296(28), 217(29).
F5	15.38	661	rha-glu	H	H	OH	+	+	+	+	MS^2[661]: 481(25), 353(57), 352(100); MS^3[661 → 352]: 330(24), 324(22), 296(84), 295(100), 284(29), 281(70), 268(69), 267(24), 255(35), 241(34), 189(38).
F6	17.10	631	rha-xyl	H	H	OH	+	+	+	+	MS^2[631]: 499(8), 481(18), 352(100); MS^3[631 → 352]: 323(71), 309(78), 297(40), 296(96), 295(65), 291(22), 284(58), 281(100), 268(75), 267(68), 255(51).
F7	17.21	645	rha-rha	H	H	OH	+	+	+	+	MS^2[645]: 613(12), 481(13), 353(33), 352(100), 323(6); MS^3[645 → 352]: 323(31), 309(46), 296(100), 281(68), 268(44), 267(37).
F8	20.36	499	rha	H	H	OH	+	+	–	–	MS^2[499]: 353(100), 352(21); MS^3[499 → 353]: 325(12), 297(17), 284(100), 281(11), 256(16), 255(20).
F9	20.90	703	rha(OAc)-glu	H	H	OH	+	–	–	–	MS^2[703]: 353(100); MS^3[703 → 353]: 335(6), 325(10), 284(100), 282(16), 255(25); MS^4[703 → 353 → 284]: 256(36), 255(100).
F10	23.80	675	rha-glu	H	H	OMe	+	+	+	+	MS^2[675]: 367(100), 366(85), 352(20); MS^3[675 → 367]: 352(100); MS^4[675 → 367 → 352]: 323(34), 309(47), 296(82), 295(26), 281(100), 268(45), 267(43), 255(29).

Table 1 (Continued)



No.	Rt (min)	[M–H]⁻	R1	R2	R3	R4	Epimedium sp. E.K	E.B	E.S	E.W	HPLC-ESI-MS^n m/z (% base peak)
F11	26.04	745	rha(OAc)-glu(OAc)	H	H	OH	+	–	–	–	MS^2[745]: 703(13), 353(100); MS^3[745 → 353]: 325(22), 310(16), 297(30), 284(100), 256(25), 227(11).
F12	27.81.	645	rha-xyl	H	H	OMe	+	+	+	+	MS^2[645]: 495(5), 367(89), 366(100), 352(25), 351(17), 323(20); MS^3[645 → 366]: 352(100), 351(53), 323(39), 311(17), 309(12).
F13	28.39	659	rha-rha	H	H	OMe	+	++	++	++	MS^2[659]: 495(7), 367(42), 366(100), 352(13), 351(22), 323(20); MS^3[659 → 366]: 352(89), 351(100), 323(76), 311(46), 305(16); MS^4[659 → 366 → 351]: 323(100), 309(23), 297(16), 296(49), 295(13).
F14	31.64	659	rha-rha	H	H	OMe	+	+	+	+	MS^2[659]: 495(5), 367(68), 366(100), 352(20), 351(21), 323(17); MS^3[659 → 366]: 352(100), 351(42), 323(40), 311(23), 309(11); MS^4[659 → 366 → 352]: 323(67), 309(91), 297(100), 296(78), 268(47).
F15	33.74	689	rha	gluA	H	OMe	+	+	–	–	MS^2[689]: 671(7), 513(100), 367(8); MS^3[689 → 513]: 367(31), 366(100), 311(13); MS^4[689 → 513 → 366]: 352(100), 323(75), 311(35), 298(45).
F16	35.03	717	rha(OAc)-glu	H	H	OMe	+	–	–	–	MS^2[717]: 675(1), 513(1), 367(100), 352(30), 323(1); MS^3[717 → 367]: 352(100), 298(2); MS^4[717 → 367 → 352]: 323(49), 296(87), 295(84), 281(100), 268(90), 267(76).
F17	37.16	513	rha	H	H	OMe	+	+	+	+	MS^2[513]: 367(33), 366(100), 351(9), 323(7); MS^3[513 → 366]: 352(49), 351(100), 323(86), 311(51), 309(12), 305(15); MS^4[513 → 366 → 351]: 323(100), 309(27), 308(12), 297(19), 296(36).
F18	41.56	657	rha-furan acid	H	H	OMe	+	+	+	+	MS^2[657]: 513(32), 367(100), 352(24); MS^3[657 → 367]: 352(100); MS^4[657 → 367 → 352]: 323(52), 309(32), 296(83), 295(75), 281(100), 268(87), 267(74), 255(27).
F19	49.08	759	rha(OAc)-glu(OAc)	H	H	OMe	+	–	–	–	MS^2[759]: 717(5), 699(9), 555(5), 513(2), 367(100), 352(30); MS^3[759 → 367]: 352(100); MS^4[759 → 367 → 352]: 323(39), 309(32), 296(100), 295(63), 281(92), 268(70).
F20	50.76	759	rha(OAc)-glu(OAc)	H	H	OMe	+	–	–	–	MS^2[759]: 717(3), 699(7), 555(3), 367(100), 352(28); MS^3[759 → 367]: 352(100); MS^4[759 → 367 → 352]: 323(29), 296(78), 295(78), 281(100), 268(76).

Table 1 (Continued)



No.	Rt (min)	[M–H]⁻	R_1	R_2	R_3	R_4	*Epimedium* sp. E.K	E.B	E.S	E.W	HPLC-ESI-MS^n m/z (% base peak)
F21	50.98	759	rha(OAc)-glu(OAc)	H	H	OMe	+	–	–	–	MS^2[759]: 717(5), 699(6), 555(3), 513(2), 367(100), 352(31); MS^3[759 → 367]: 352(100); MS^4[759 → 367 → 352]: 323(36), 309(16), 296(100), 295(69), 281(87), 268(64).
F22	51.74	759	rha(OAc)-glu(OAc)	H	H	OMe	+	–	–	–	MS^2[759]: 717(6), 699(9), 555(3), 513(2), 367(100), 352(27); MS^3[759 → 367]: 352(100); MS^4[759 → 367 → 352]: 323(37), 296(90), 281(100).
F23	54.26	555	rha(OAc)	H	H	OMe	+	–	–	–	MS^2[555]: 367(42), 366(100), 351(15); MS^3[555 → 366]: 352(46), 351(100), 323(92), 311(50), 305(17); MS^4[555 → 366 → 351]: 323(100), 309(36), 308(24), 296(28), 279(18).
F24	55.34	687	rha(OAc)-xyl	H	H	OMe	+	–	–	–	MS^2[687]: 569(11), 367(100), 352(24); MS^3[687 → 367]: 352(100); MS^4[687 → 367 → 352]: 323(31), 309(19), 296(100), 295(46), 281(89), 268(70), 267(73), 255(31).
F25	61.27	801	rha(OAc)-glu(2OAc)	H	H	OMe	+	–	–	–	MS^2[801]: 741(11), 367(100), 352(32); MS^3[801 → 367]: 352(100); MS4[801 → 367 → 352]: 309(42), 296(88), 295(40), 281(34), 268(100), 267(45), 255(55).
F26	62.29	367	H	H	H	OMe	+	+	+	+	MS^2[367]: 352(100), 312(14), 309(32), 297(27); MS^3[367 → 352]: 309(100), 297(58); MS^4[367 → 352 → 309]: 281(100), 265(34), 175(43).

3.1. Metabolites in rat feces following administration of *Epimediums*

3.1.1. Administration of three pure compounds (Fig. 2)

For epimedin C (MW = 822 Da), a prominent metabolite F13 (Table 1) was observed at t_R = 28.39 min. In the full mass spectrum, F13 revealed a base peak at *m/z* 659. And then, the [M–H]⁻ ion yielded $[Y_0–H]^-$ ion at *m/z* 366 owing to the elimination of diglycosides in the MS/MS spectrum. Successively, the $[Y_0–H]^-$ ion was selected for further MS^3 analysis to generate a moderate $[Y_0–H–CH_3]^-$ ion at *m/z* 351 due to the cleavage of the C-4′ methoxyl. And as a characteristic ion of prenylflavonoid-3-O-glucosides, *m/z* 323 was one of the highest peaks in the MS^3 and MS^4 spectra of *m/z* 366 and *m/z* 352, respectively. The fragmentation difference between 3-O- and 7-O-substitutes was in the elimination order of the C-4′ methoxyl and isopentenyl and the cleavage styles of ring C as well (Fig. 3(c) and (d)) [17]. Moreover, a significant $[Y_0–CH_3–C_3H_7]^-$ ion at *m/z* 309 was observed clearly in the MS^4 spectrum of *m/z* 351 assigned as the cleavage of isopentenyl at C-8 position. Therefore, this metabolite of epimedin C in feces was identified as anhydroicaritin-3-O-rha-(1,2)-rha (Fig. 3). Apart from this, compared with retention time and MS^n spectra of reference, the other trace metabolite anhydroicaritin (F26, MW = 368 Da, Table 1) was observed in feces .

Similar to early publication indicated [14], in the present study, after administration of icariin (MW = 676 Da), icariside II (F17, MW = 514 Da, Table 1) and anhydroicaritin (F26, MW = 368 Da) were detected in feces as the major two metabolites. However, as the prototype compound, icariin could not be observed at the dosage of 80 mg/kg to rats.

For icariside II group, the unchanged parent drug icariside II and anhydroicaritin were found in feces obviously. Further, the relative content of anhydroicaritin was higher than that in icariin group.

3.1.2. Administration of four extracts (Fig. 4)

Following oral administration of four *Epimediums* extracts, 28 metabolites (F1–F28) were identified or tentatively characterized by mass spectra in rat feces (Tables 1 and 2). As the common metabolites in four groups, the appearance peaks at 23.80 min

Table 2
HPLC-ESI-MS^n data *in vivo* identification of other metabolites in rat feces samples.

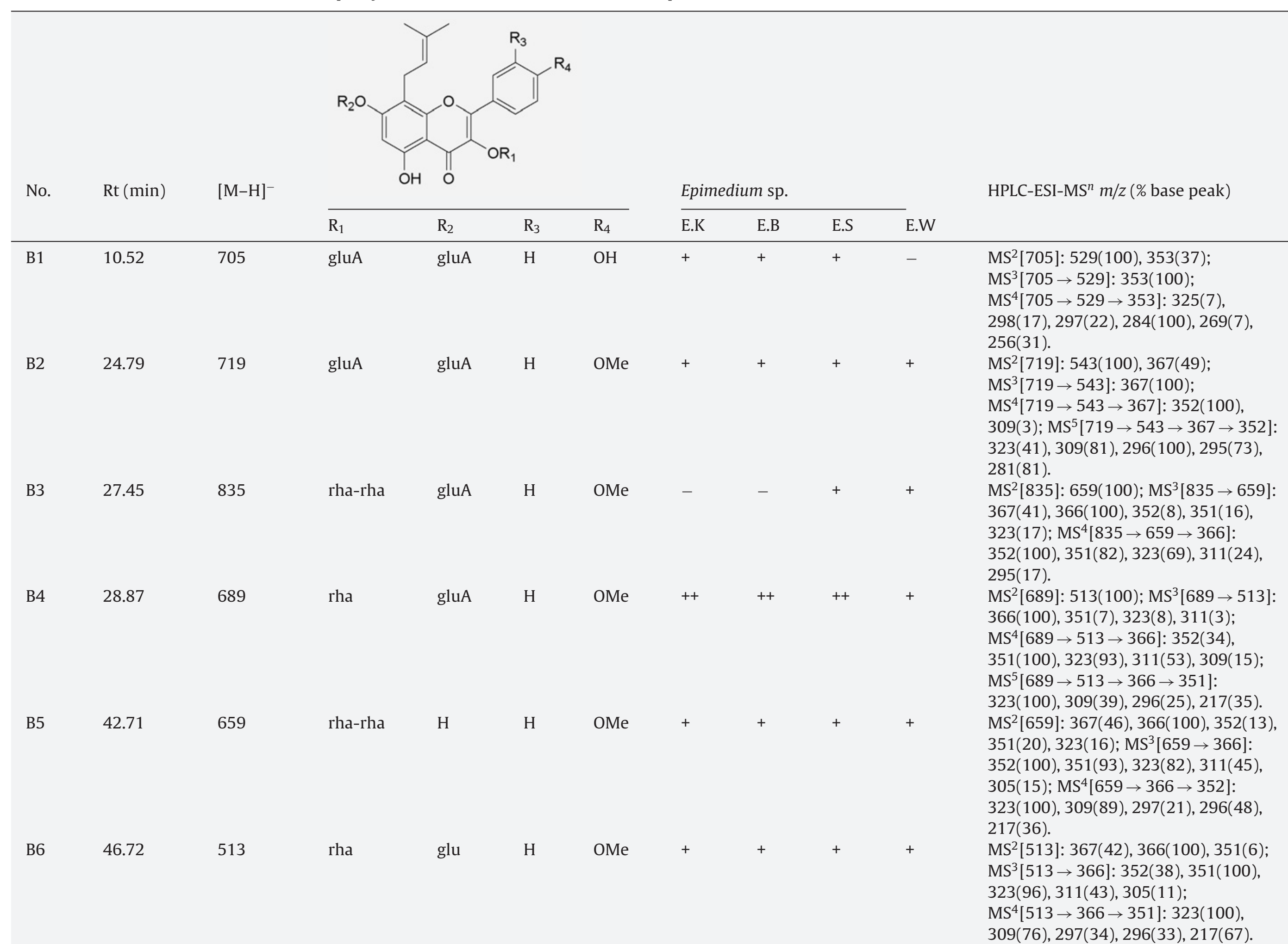
No.	Rt (min)	$[M–H]^–$	Compounds	*Epimedium* sp.				HPLC-ESI-MS^n *m/z* (% base peak)
				E.K	E.B	E.S	E.W	
F27	6.48	463	Hyperin	+	+	+	+	MS^2[463]: 301(100), 300(49), 299(6); MS^3[463 → 301]: 271(100), 255(38), 179(63), 178(14), 151(27).
F28	7.32	301	Quercetin	+	+	+	+	MS^2[301]: 283(100), 265(11); MS3[301 → 283]: 265(100), 239(27), 221(44), 171(9), 153(29), 97(13).

(F10, Table 1), 27.81 min (F12, Table 1), 28.48 min (F13, Table 1) and 31.64 min (F14, Table 1) revealed four metabolites $[M–H]^–$ ions at *m/z* 675, 645, 659, 659, respectively. In their MS/MS spectra, the first elimination of diglycosides yielded $Y_0^–$ ion at *m/z* 367 or $[Y_0–H]^–$ ion at *m/z* 366 with the characteristic ion at *m/z* 323, which indicated that the linkage of diglycosides was at C-3 position of anhydroicaritin aglycone. Considering the retention time and the contents of their prototypes in *Epimediums*, F10 and F12 were tentatively assigned as anhydroicaritin-3-O-rha-(1,2)-glu and anhydroicaritin-3-O-rha-(1,2)-xyl, respectively. However, with the identical fragmentation behaviors, F13 and F14 were distinguished only by MS^n data. Comparing with the metabolite in rat feces after dosing epimedin C, F13 was identified as anhydroicaritin-3-O-rha-(1,2)-rha. And F14 was attributed as anhydroicaritin-3-O-rha-(1,3)-rha in reference of literature [18]. These four metabolites were considered as the hydrolysis products of epimedin A, B, anhydroicaritin-3-O-rha-(1,3) -rha-7-O-glu and epimedin C, respectively.

As another important common metabolite in feces of four groups, F18 (Table 1) revealed $[M–H]^–$ ion at *m/z* 657 in full mass spectrum and yielded the low $[M–H–144]^–$ ion at *m/z* 513 and prominent $[Y_0–H]^–$ ion at *m/z* 367 in MS^2 fragmentation. The characteristic ion at *m/z* 323 indicated that the substitute chain of rhamnose–dideoxyfuranose was at C-3 position. Considering its specific structure [19] and the content of its parent compound in *Epimediums*, this metabolite was deduced as anhydroicaritin-3-O-rha-(1,2)-dideoxyfuranose (F18, MW = 658 Da).

From 49.08 min to 51.74 min in chromatography, four isomers (F19–F22, Table 1) with $[M–H]^–$ ions at *m/z* 759 were detected in feces after dosing the extract of *E. koreanum*. Their MS fragmentation behaviors were almost identical to each other as follows: $Y_0^–$ ion at *m/z* 367 was observed in their MS/MS spectra.

Table 3
HPLC-ESI-MS^n data *in vivo* identification of prenylflavonoids metabolites in rat bile samples.



No.	Rt (min)	$[M–H]^–$	R_1	R_2	R_3	R_4	*Epimedium* sp. E.K	E.B	E.S	E.W	HPLC-ESI-MS^n *m/z* (% base peak)
B1	10.52	705	gluA	gluA	H	OH	+	+	+	–	MS^2[705]: 529(100), 353(37); MS^3[705 → 529]: 353(100); MS^4[705 → 529 → 353]: 325(7), 298(17), 297(22), 284(100), 269(7), 256(31).
B2	24.79	719	gluA	gluA	H	OMe	+	+	+	+	MS^2[719]: 543(100), 367(49); MS^3[719 → 543]: 367(100); MS^4[719 → 543 → 367]: 352(100), 309(3); MS^5[719 → 543 → 367 → 352]: 323(41), 309(81), 296(100), 295(73), 281(81).
B3	27.45	835	rha-rha	gluA	H	OMe	–	–	+	+	MS^2[835]: 659(100); MS^3[835 → 659]: 367(41), 366(100), 352(8), 351(16), 323(17); MS^4[835 → 659 → 366]: 352(100), 351(82), 323(69), 311(24), 295(17).
B4	28.87	689	rha	gluA	H	OMe	++	++	++	+	MS^2[689]: 513(100); MS^3[689 → 513]: 366(100), 351(7), 323(8), 311(3); MS^4[689 → 513 → 366]: 352(34), 351(100), 323(93), 311(53), 309(15); MS^5[689 → 513 → 366 → 351]: 323(100), 309(39), 296(25), 217(35).
B5	42.71	659	rha-rha	H	H	OMe	+	+	+	+	MS^2[659]: 367(46), 366(100), 352(13), 351(20), 323(16); MS^3[659 → 366]: 352(100), 351(93), 323(82), 311(45), 305(15); MS^4[659 → 366 → 352]: 323(100), 309(89), 297(21), 296(48), 217(36).
B6	46.72	513	rha	glu	H	OMe	+	+	+	+	MS^2[513]: 367(42), 366(100), 351(6); MS^3[513 → 366]: 352(38), 351(100), 323(96), 311(43), 305(11); MS^4[513 → 366 → 351]: 323(100), 309(76), 297(34), 296(33), 217(67).

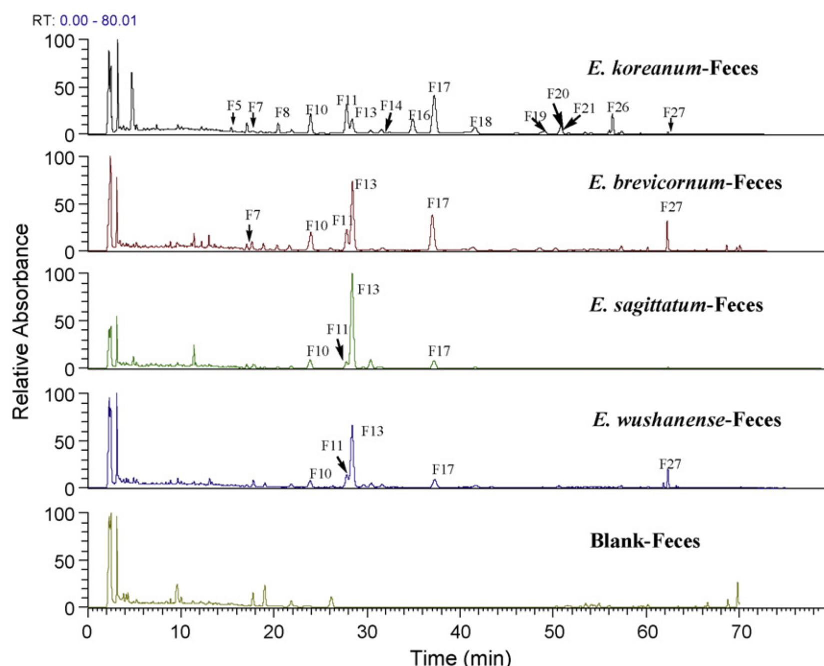


Fig. 4. HPLC/UV chromatograms of rat feces samples after administration of four *Epimedium* species.

Meanwhile, low abundance [M–H–OAc]$^-$, [M–H–glu(OAc)]$^-$ and [M–H–glu(OAc)–(OAc)]$^-$ ions at *m/z* 699, 555, 513 were clearly found. As the characteristic metabolites in *E. koreanum* group, these four flavonoids with acetyl were deduced as the isomers of anhydroicaritin-rha(1,4-OAc)-(1,3)glu(OAc). According to their prototype compounds isolated from *E. koreanum* [19], we believed that the structural difference existed in the attached positions of acetyl group at the outer glucose.

In general, the metabolites in feces were closely related to the chemical profiles of *Epimedium* species. For instance, the high levels of epimedin C in *E. wushanense* and *E. sagittatum* led to the relatively high contents of anhydroicaritin-3-O-rha-(1,2)-rha in feces samples. For the same reason, as the major metabolite of icariin, icariside II in *E. koreanum* and *E. brevicornum* was almost 10-fold higher than that in the other two groups.

Besides, two trace prototype triglycosides of prenylflavonoids with 7-O-glu (F1 and F4, Table 1) were observed in feces, which was probably caused by the high dose of extracts (800 mg/g) to rats in experiments. This phenomenon suggested that the first-pass biotransformation in alimentary canal might be saturated while the concentration of prenylflanonoids exceeded the utmost limit.

3.2. Metabolites in rat bile following administration of Epimediums

3.2.1. Administration of three pure compounds

For epimedin C group, a low quantity di-glucuronic acid conjugation was eluted at 24.79 min (B2, Table 3). In the MS/MS spectrum, [M–H]$^-$ ion at *m/z* 719 yielded a significant ion at *m/z* 543 and a moderate ion at *m/z* 367. Because of the higher proton affinity of 7-O-glycoside than that of 3-O-glycoside [20] and the relative abundance of fragments [17], the first elimination position of gluA was attributed at C-7 of anhydroicaritin. Further, the Y_0^- ion at *m/z* 367 from *m/z* 543 in MS^3 experiment should be assigned as the second gluA loss at C-3 position. The 5-OH did not appear to be a site for conjugation on account of the intra-molecular hydrogen bond with oxygen at C-4 carbonyl group [21]. Additionally, the successive MS^4 and MS^5 data also supported the fact the structure of B2 was anhydroicaritin-3-O-gluA-7-O-gluA (Fig. 5).

Except for anhydroicaritin-3-O-gluA-7-O-gluA, an abundant metabolite peak was observed at 27.45 min (B3). Full mass spectrum of B3 (Table 3) revealed [M–H]$^-$ ion at *m/z* 835. This spectra presented strong [M–gluA–H]$^-$ ion at *m/z* 659 in the MS/MS spectrum. This indicated the gluA at C-7 position of aglycone. And then, the obtained [M–gluA–H]$^-$ ion was triggered by the loss of rha-rha group in the MS^3 spectrum and yielded $[Y_0–H]^-$ ion at *m/z* 366. Meanwhile, the characteristic ion at *m/z* 323 was observed. This indicated that the diglycosides (rha-rha) was attached to C-3 position. Further, in the MS^4 spectrum, the diagnostic ions at

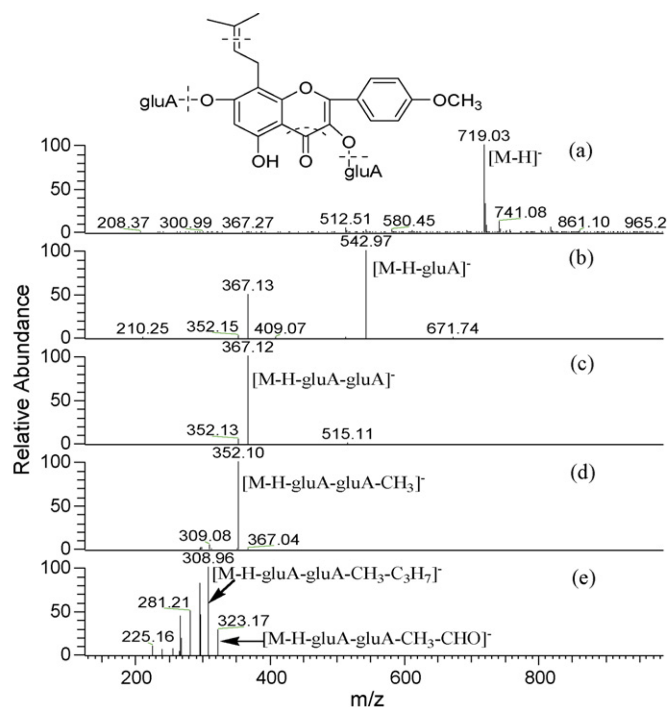


Fig. 5. ESI-MS^n spectra for B2, Anhydroicaritin-3-O-gluA-7-O-gluA.

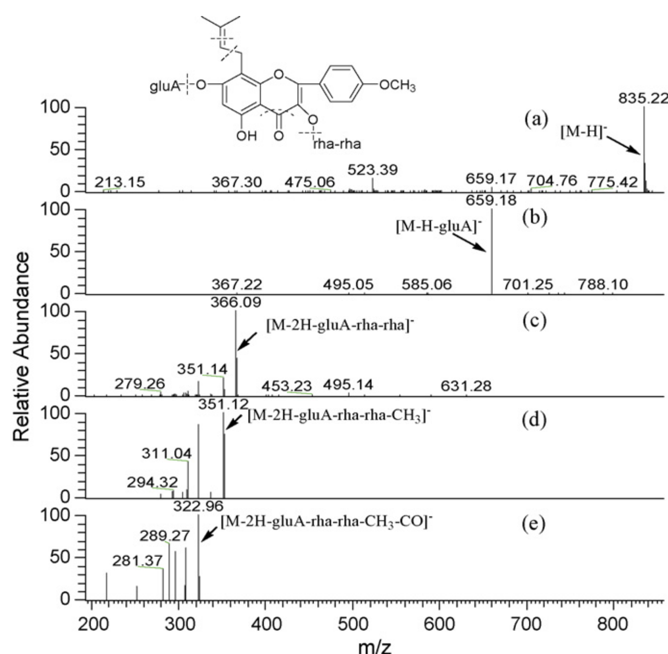


Fig. 6. ESI-MS^n spectra for B3, Anhydroicaritin-3-O-rha-rha-7-O-gluA.

m/z 352, 323 and 311 were also found in the low *m/z* region. All the aforementioned information indicated the structure of B3 as anhydroicaritin-3-O-rha-(1,2)-rha-7-O-gluA (Fig. 6). Additionally, the other metabolite anhydroicaritin-3-O-rha-(1,2)-rha (B5, MW = 660 Da, Table 3) was found in bile sample as well.

For icariin group, a principal peak showed $[M-H]^-$ ion at *m/z* 689 in bile sample at 28.87 min (B4, Table 3). In its MS/MS spectrum, $[M-H]^-$ ion yielded the prominent $[M-gluA-H]^-$ ion at *m/z* 513. Because of the easier loss of C-7 substitutions compared with that of C-3 position for prenylflavonoids 3,7-di-O- glucosides in negative ion mode of mass spectra, gluA was deduced at C-7 position. And the obtained $[M-gluA-H]^-$ ion produced $[Y_0-H]^-$ at *m/z* 366 in successive fragmentation. Simultaneously, the characteristic ion at *m/z* 323 indicated the attachment of rhamnose at C-3 position. Therefore, this metabolite was identified as anhydroicaritin-3-O-rha-7-O-gluA, which has been isolated from the rat bile and identified by NMR experiment before [14]. Additionally, the low content of icarisid II (B6, MW = 514 Da, Table 3) was observed in this sample.

For icariside II group, similar to icariin, anhydroicaritin-3-O-rha-7-O-gluA (B4, MW = 690 Da) and the prototype compound were observed in bile sample. Icariside II was speculated as the intermediate product in forming of anhydroicaritin-3-O-rha-7-O-gluA.

3.2.2. Administration of four extracts

Eight metabolites were detected in bile samples after administration of four *Epimediums* extracts, including six mono- and di-glucuronic acid conjugates (Fig. 7 and Table 3).

Anhydroicaritin-3-O-gluA-7-O-gluA (B2, MW = 720 Da), anhydroicaritin-3-O-rha-7-O-gluA (B4, MW = 690 Da), anhydroicaritin-3-O-rha-rha (B5, MW = 659 Da) and icariside II (B6, MW = 514 Da) were observed in bile samples as common metabolites in four groups. Among these, B4 was the principal one due to its high level in plants.

Apart from this, two mono-glucuronic acid conjugates of quercetin and kaempferol were detected (Table 4). As stated in literatures, UDP-glucuronosyltransferase (UGT) exhibited the highest apparent V_{max} for the C-7 position for quercetin and 7-O-gluA conjugation should be the predominant form in liver [22]. Besides, glucuronidation at the 7-OH position on the A-ring has been proved as a major metabolic pathway of kaempferol in hepatic cells [23,24]. Therefore, these two metabolites were tentatively identified as quercetin-7-O-gluA (B7, MW = 478 Da, Table 4) and kaempferol-7-O-gluA (B8, MW = 462 Da, Table 4).

3.3. Metabolites in rat plasma following administration of *Epimediums*

Under UV conditions, plasma revealed almost identical to blank samples in both pure compounds and extracts' groups at the dosage mentioned before. Using LC-MS method, nine metabolites were detected (Table 5). Although the determination of epimedin C in rat plasma was reported by HPLC-UV method before, the dosage to rats was very higher than that in clinical use [11].

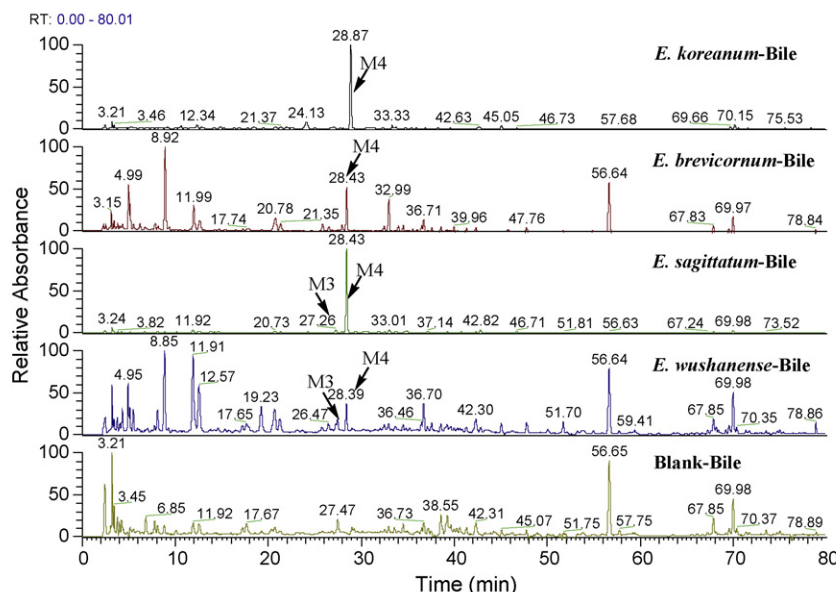


Fig. 7. HPLC/UV chromatograms of rat bile samples after administration of four *Epimedium* species.

Table 4
HPLC-ESI-MS^n data *in vivo* identification of other metabolites in rat bile samples.

No.	Rt (min)	[M–H]$^-$	Compounds	*Epimedium* sp.				HPLC-ESI-MS^n *m/z* (% base peak)
				E.K	E.B	E.S	E.W	
B7	4.99	477	Quercetin-7-O-gluA	+	–	+	–	MS^2[477]: 301(100), 273(31); MS3[477 → 301]: 286(6), 273(100), 181(6); MS^4[477 → 301 → 273]: 258(27), 255(100), 175(28), 139(72).
B8	5.62	461	Kaempferol-7-O-gluA	+	–	+	–	MS^2[461]: 285(100); MS^3[461 → 285]: 267(27), 241(100).

3.3.1. Administration of three pure compounds

After oral lavage of epimedin C for 30 min, anhydroicaritin-3-O-rha-rha-7-O-gluA (M5, MW = 836 Da, Table 5) and anhydroicaritin-3-O-gluA-7-O-gluA (M4, MW = 720 Da, Table 5) were detected in plasma sample. M4 was observed until 12 h later after administration.

For icariin group, anhydroicaritin-3-O-gluA-7-O-gluA (M4, MW = 720 Da) and anhydroicaritin-3-O-rha-7-O-gluA (M6, MW = 690 Da, Table 5) were observed since 15 min in plasma samples. Nevertheless, almost no metabolites were found in 8 h sample. For icariside II group, anhydroicaritin-3-O-gluA-7-O-gluA (M4, MW = 720 Da), anhydroicaritin-3-O-rha-7-O-gluA (M6, MW = 690 Da) and icariside II (MW = 514 Da) were detected clearly. It was noted that these constituents were not detected 8 h later after dosing.

In comparison, the metabolites of epimedin C (triglycoside) remained much longer in plasma than those of icariin (diglycoside) and icariside II (monoglycoside). We speculated that the duration diversity due to the different absorption positions in gut lumen and the different hepato-enteral circulation patterns probably. Additionally, the result indicated that the rates of conjugated reactions of prenylflavonoids were closely related to the number of 3-O-sugar moieties as well.

3.3.2. Administration of four extracts

Anhydroicaritin-3-O-gluA-7-O-gluA (M4, MW = 720 Da), anhydroicaritin-3-O-rha-7-O-gluA (M6, MW = 690 Da), anhydroicaritin-3-O-rha-rha (M7, MW = 660 Da, Table 5) and kaempferol-3-O-gluA-7-O-gluA (M2, MW = 638 Da, data not shown) were detected in plasma samples as the major common metabolites of four *Epimediums* groups.

Besides, as a metabolite of dihydroflavonoid, M3 (MW = 532 Da, data not shown) was observed in *E. koreanum* and *E. brevicornum* groups. [M–H]$^-$ ion at *m/z* 531 of M3 yielded the [M–H–gluA]$^-$ ion at *m/z* 355 in the MS/MS spectrum. And the product ions at *m/z* 337, 327 and 311 were observed in the MS^3 spectrum of *m/z* 355. The fragmentation pattern was similar to that of dihydrodemethylicaritin-7-O-glu [17]. Thus, this metabolite was deduced as dihydrodemethylicariine-7-O-gluA.

In *E. sagittatum* group, a specific metabolite M10 displayed [M–H]$^-$ ion at *m/z* 597 in full scan mass spectrum. In the MS/MS spectrum, the base peak at *m/z* 421 from the loss of gluA was detected. And the ion at *m/z* 366 was found owing to the loss of isopentenyl from the aglycone in the MS^3 spectrum. Comparing with the fragmentation of epimedokoreanin B-7-O-glu reported before [17], the structure of this metabolite was identified as epimedokoreanin B-7-O-gluA (M10, MW = 598, data not shown).

Meanwhile, anhydroicaritin-3-O-rha-rha-7-O-gluA (M5, MW = 836 Da) was detected specifically in plasma sample after administration of *E. wushanense* because of its high level of epimedin C in plant.

In comparison of metabolite and chemical profiles of four *Epimediums* [17,25], the amount variance of metabolites in plasma was caused by the difference of parent compounds in plants directly. For example, epimedokoreanin B-7-O-gluA was only observed in *E. sagittatum* because of the absence of epimedokoreanin B in other species.

3.4. Metabolites in rat urine following administration of Epimediums

In accord with plasma samples, eight trace mono- and di-glucuronic acid conjugations were detected in urine samples (Table 5).

3.4.1. Administration of three pure compounds

In addition to anhydroicaritin-3-O-gluA-7-O-gluA (M4, MW = 720 Da), a minor metabolite with mono-glucuronic acid M8 was found at t_R = 38.67 min. M8 revealed [M–H]$^-$ ion at *m/z* 543 in full scan mass spectrum. This obtained ion produced [M–H–gluA]$^-$ ion at *m/z* 367. Then the Y_0^- ion was selected for further MS^3 analysis to generate a significant $[Y_0–CH_3]^-$ ion at *m/z* 352, a low abundance $[Y_0–CH_3–C_3H_7]^-$ ion at *m/z* 309, which were assigned as the cleavage of the C-4 methoxyl and the isopentenyl at the C-8 position, respectively. Furthermore, the diagnostic ion [M-2H-glu-CH_3-CO]$^-$ of prenylflavonoid 3-O-glycoside at *m/z* 323 was observed in the MS^4 spectrum of *m/z* 352. The fragmentation behavior of its aglycone was similar to that of icariside II

Table 5
HPLC-ESI-MS^n data *in vivo* identification of metabolites in rat plasma and urine samples.

No.	[M–H]$^-$	Compounds	Blood samples				Urine samples			
			E.K	E.B	E.S	E.W	E.K	E.B	E.S	E.W
M1	705	Demethylanhydroicaritin-3-O-gluA-7-O-gluA	+	+	+	–	+	–	–	–
M2	637	Kaempferol-3-O-gluA-7-O-gluA	+	+	+	+	–	–	–	–
M3	531	Dihydrodemethylicariine-7-O-gluA	+	+	–	–	+	–	–	–
M4	719	Anhydroicaritin-3-O-gluA-7-O-gluA	+	+	+	+	+	+	+	+
M5	835	Anhydroicaritin-3-O-rha-rha-7-O-gluA	–	–	–	+	+	–	–	+
M6	689	Anhydroicaritin-3-O-rha-7-O-gluA	+	+	+	+	+	+	+	+
M7	659	Anhydroicaritin-3-O-rha-rha	+	+	+	+	+	+	+	+
M8	543	Anhydroicaritin-3-O-gluA	–	–	–	–	+	+	+	+
M9	513	Icariside II	+	+	+	–	+	+	+	+
M10	597	Epimedokoreanin B-7-O-gluA	–	–	+	–	–	–	–	–

Table 6
Major metabolites in rat feces, bile, plasma and urine samples after administration of three pure prenylflavonoids.

Metabolites	Epimedin C			Icariin				Icariside II				
	Feces	Bile	Plasma	Urine	Feces	Bile	Plasma	Urine	Feces	Bile	Plasma	Urine
Anhydroicaritin-3-O-gluA -7-O-gluA	–	+	+	+	–	+	+	+	–	+	+	+
Anhydroicaritin-3-O-rha -7-O-gluA	–	–	–	–	–	+	+	+	–	+	+	+
Anhydroicaritin-3-O-rha-rha -7-O-gluA	–	++	+	–	–	–	–	–	–	–	–	–
Anhydroicaritin-3-O-gluA	–	–	–	+	–	–	–	+	–	–	–	+
Icariside II	–	–	–	–	++	+	+	+	++	+	+	+
Icariin	–	–	–	–	–	–	+	–	–	–	–	–
Anhydroicaritin-3-O-rha-rha	++	++	+	+	–	–	–	–	–	–	–	–
Anhydroicaritin	+	–	–	–	++	+	+	+	++	+	+	+

++ means maximum constituents in biosamples. Abbreviations: *E. koreanum* Nakai (E.K); *E. brevicornum* Maxim (E.B); *E. sagittatum* Maxim (E.S); *E. wushanense* T. S. Ying (E.W).

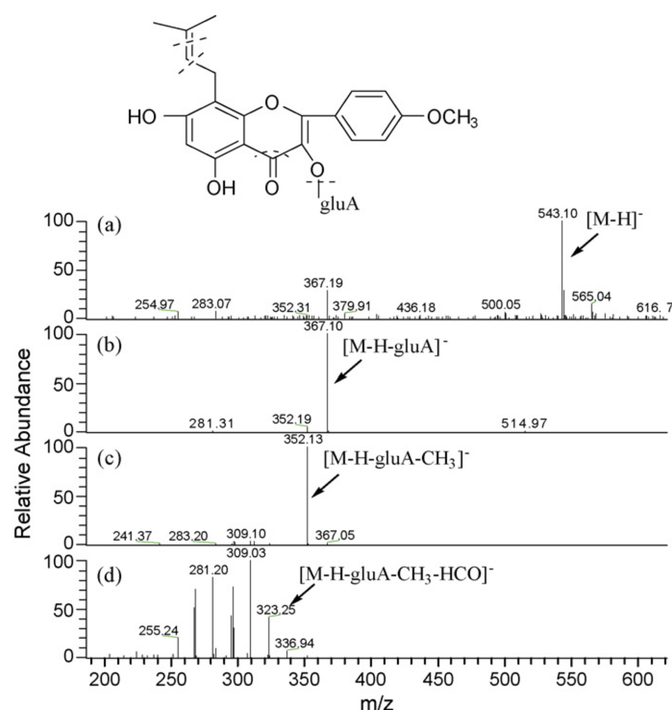


Fig. 8. ESI-MS^n spectra for M8, Anhydroicaritin-3-O-gluA.

(anhydroicatin-3-O-rha) [17]. Thus, this metabolite was deduced as anhydroicaritin-3-O-gluA (M8, MW = 544) tentatively (Fig. 8). Remarkably, this metabolite was only detected in urine samples.

For icariin and icariside II, anhydroicaritin-3-O-rha-7-O-gluA (MW = 690 Da) was observed significantly besides M4 and M8.

3.4.2. Administration of four extracts

Anhydroicaritin-3-O-gluA-7-O-gluA (M4, MW = 720 Da), anhydroicaritin-7-O-gluA-3-O-rha (M6, MW = 690 Da), anhydroicaritin-3-O-rha-rha (M7, MW = 660 Da) and anhydroicaritin-3-O-gluA (M8, MW = 544 Da) were considered as the common metabolites in urine of four *Epimediums* groups. Additionally, demethylanhydroicaritin-3-O-gluA-7-O-gluA (M1, MW = 706 Da, data not shown) and dihydrodemethylicaritin-7-O-gluA (M3, MW = 532 Da) were observed in the urine of *E. koreanum* group.

4. Discussion

Based on the previous investigation of credible MS^n fragmentation behaviors of various types of prenylflavonoids in *Epimediums*, the structures of most metabolites were deduced in the present paper, clarifying the general metabolism of *Epimediums in vivo*.

First of all, hydrolysis was considered as the initial biotransformation step of prenylflavonoids *in vivo*. This biotransformation made the glucosides easier for diffusion and absorption in intestine. Plentiful partial hydrolysis reactions of 7-O-glucosides were observed. Remarkably, for F19–F22, even though the unstable acetyl groups were still attached to the sugar, the loss of 7-O-glu was detected. And then, as the metabolites of hydrolysis, prenylflavonoids-3-O-glycosides were partly absorbed intact, which was conjugated with glucuronic acid in liver or further deglycosylated by indogenous broad-specificity cytosolic β-glucosidase in enterocyte or liver as aglycone form before excretion [26]. Finally, the secondary metabolites were excreted from bile and urine in the shape of mono- and di-glucuronic acid conjugations. The difference of metabolite amounts between urinary and biliary excretions illustrated that prenylflavonoids should be mostly excreted from the rat via bile mostly.

Additionally, it was reported that the hydrolysis of certain flavonoid glycosides could be accomplished in the whole alimentary canal including oral cavity and stomach [27], however, small intestine and colon were presumed as the major organs of prenylflavonoid 7-O-glu deglycosylation. The reason lays in the comparison of conjugations contents in bile and feces samples. As the major metabolites in bile, only two trace conjugations with 7-O-gluA were detected in feces, which implicated the hydrolysis position was at least below stomach. The lactase phlorizin hydrolase (LPH) and the microbial in colon should be responsible for the deglycosylation within gut lumen [28–31]. Apart from this, the mono-glucuronic acid conjugation anhydroicaritin-3-O-gluA (M8, MW = 544) was only detected in urine samples after dosing prenylflavonoids. It was probably caused by the hydrolysis of anhydroicaritin-3-O-gluA-7-O-gluA by β-glucuronidase located in the kidney [35].

To our knowledge, the metabolism processes of flavonoid glycosides were predicted to be closely dependent on their structures, particularly the number of sugar moiety groups [32]. Flavonoid glycosides were thought to delay intestinal absorption until the release of hydrolysis products by colonic microflora in large intestine [33]. In this study, the appearance time in plasma of their deglucosylation products (icariside II > icariin > epimedin C) also supported this idea. Although the absolute contents of these metabolites were not determined in our investigation, the trend showed that the increased number sugars on 3-O-glucoside results in reduced prenylflavonoids absorption rate. It also indicated that the up-taken position of flavonoid glycoside was correspondingly lowered down in gut lumen. Even 12 h after dosing of epimedin C, the glucuronic acid conjugation anhydroicaritin-3-O-gluA-7-O-gluA was detected in plasma sample, which was consistent with the delayed hydroly-

sis of epimedin C in intestine. This result was supported by Caco-2 experiments of icariside II, icariin and epimedin C as well [34]. Actually, this phenomenon was a benefit for keeping drugs at effective concentrations in plasma, the various anhydroicaritin glycosides in *Epimediums* acted as a natural "sustained-release flavonoids preparations". Meanwhile, the multiple metabolic rates also indicated the distinguished use of different *Epimedium* species in clinical application.

The metabolism of icariin has been investigated in previous research [14] and icariside II was considered as the intermediated product in forming of anhydroicaritin -3-O-rha-7-O-gluA, which was found in bile, plasma and urine samples. In the present paper, anhydroicaritin was speculated to form the other important conjugation anhydroicaritin-3-O-gluA-7-O-gluA in the physiological process on the grounds of the absence of Icariside II and anhydroicaritin-3-O-rha-7-O-gluA in biosamples of epimedin C group (Table 6). The result also suggested that two sugars of prenylflavonoids 3-O-di-glycosides were hydrolyzed simultaneously and completely *in vivo*.

5. Conclusions

In this study, a HPLC-ESI-MS^n method was developed for the metabolites analyses of rat feces, bile, plasma and urine after oral administration of three pure compounds and four *Epimediums* extracts. The metabolite and chemical profiles of *Epimediums* have been correlated closely. And the physiological process of prenylflavonoids in *Epimediums* has been demonstrated generally. In brief, this investigation provided scientific evidence to clarify the disposing process of prenylflavonoids *in vivo*, which was valuable and dependable for the further study of the metabolism of *Epimediums*.

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