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An explanation for fluctuations of icariin content in Epimedium production process

Baohua Meng[1†]*, Yuansong He[1†], Zheyi Wang[7†], Xinqi Deng[2], Chuanzhen Teng[3], Xiao Ke[3], Xiaofeng Hao[3], Yuqing Wang[4], Yujue He[4], Yuan Wang[5] and Chunguo Wang[6]

[1]Sichuan Nursing Vocational College, Chengdu, China
[2]School of Life Sciences, Beijing University of Chinese Medicine, Beijing, China
[3]Chengdu Kanghong Pharmaceutical Co., Ltd, Chengdu, China
[4]School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, China
[5]Wang Jing Hospital of CACMS, Beijing, China
[6]Beijing Research Institute of Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China
[6]Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China

Abstract: Epimedium has beneficial effects in nourishing and building up the body and is widely used in practical production of Epimedium preparations. As one of the major active compounds in Epimedium preparations, icariin is be used as a quality control index of industrial manufacture. However, content of icariin was observed to increase to uncertain extent in pharmaceutical production, which might bring difficulties in quality control. The content fluctuation mainly occurred in high-temperature extraction process. The aim of this study is to investigate what happen to flavonol-glycosides in Epimedium under heating treatment. Ultra-Performance Liquid Chromatography-Linear Ion Trap Mass Spectrometer was applied to profile the transformation rule of flavonol-glycosides in Epimedium and search for an explanation for the increase in icariin content under heating treatment. 56 compounds were found to have significantly changed and their structures were identified, among which 15 flavonol-glycosides were proposed to play a role in icariin content variation. Further studies were conducted based on 8 flavonol-glycosides standard substances to obtain more credible data. Finally, Baohuoside II, 2"-o-rhamnosylicariside II, Epimedin A1, Epimedin A, Epimedin B, Epimedin C, Baohuoside I and Anhydroicaritin were found to transform into icariin during the heating process. This study provides an evidence for the quality control study of Epimedium preparation, as well as reference for chemical researches in natural pharmacy.

Keywords: Epimedium, flavonol-glycosides, icariin, quality control, UPLC-LTQ-Orbitrap MS.

INTRODUCTION

Epimedium is widely applied in practical food and pharmaceutical industries for its outstanding effects on cardiovascular, cerebrovascular, neural, immune, genitourinary and locomotor systems (Chen *et al*., 2014, Li *et al*., 2014a, Li *et al*., 2014b, Li *et al*., 2014c, Wang *et al*., 2018, Zhai *et al*., 2015, Zhang *et al*., 2014, Wang *et al*., 2014). Derivatives of flavonol-glycosides which C-8 substituted by isopentenyl are considered as active compounds of Epimedium. Among which icariin is defined as quality standard marker of medicinal and health products containing Epimedium. However, it had been observed in previous studies that icariin's content significantly fluctuate with thermal processing (Liu *et al*., 2004, Fen *et al*., 2012, Sun *et al*., 2012, Cai *et al*., 2007, Zhong *et al*., 2011, Chen *et al*., 2007b, Li *et al*., 2011). The mechanism which explains for icariin's content variation have not been revealed yet. A systematic study on the transformation process of flavonol-glycosides under heating condition could help revealing why icariin's content fluctuate, as well as providing reference for the improvement of the quality control standard of Epimedium. With high mass resolution and precise molecular weight, high resolution mass spectrometry is potent in obtaining data about the elemental composition of compounds. Orbitrap mass spectrometer is a kind of mass spectrometer with novel structure and breakthrough technology. After differential amplification, the oscillation frequency of each ion is detected with the FT converter, and the mass to charge ratio (m/z) of molecular ions is calculated accordingly. LTQ-Orbitrap is a mass spectrum combination which consist of linear ion trap mass spectrometry (LTQ) and high resolution Orbitrap mass spectrometry. Specifically, LTQ provides structural fragmentation information as a two dimensional linear ion trap; and Orbitrap provides data of elemental composition as high resolution mass spectrometry. In this study, with ultra-performance liquid chromatography coupled with linear ion trap quadrupole-Orbitrap mass spectrometry (UPLC-LTQ-Orbitrap MS) method, we detect the changes in types and concentration of components of Epimedium at different time points under heating condition. Data was processed with principal component analysis (PCA) statistical analysis to screen the candidates which account for icariin's content variation. Finally,

*Corresponding author: e-mail: linxi200344@163.com

Baohuoside II, 2 "-o-rhamnosylicariside II, Epimedin A1, Epimedin A, Epimedin B, Epimedin C, Baohuoside I and Anhydroicaritin were proven to be the major precursors, and pathways by which they transform into icariin was revealed. The results provided explanations for icariin's content variation during thermal processing.

MATERIALS AND METHODS

Chemicals and reagents

A total of 10 batches of medicinal herb Epimedium were collected from the Pharmacopoeia and identified as authentic according to the Pharmacopoeia by Chunguo Wang. Ultrapure water, acetonitrile, formic acid, methanol, and ethanolwere bought from Fisher Scientific, USA and the other reagents were of analytical grade. Icariin, Baohuoside II,2"-O-rhamnosylicariside II, Epimedin A1, Baohuoside I, Epimedin A, Epimedin B, Epimedin C and Anhydroicaritin were sourced from Chengdu Pfeiffer Biotechnology Co., Ltd., the reference HPLC area was normalized to ensure purity of greater than 99%.

Chromatographic and Mass spectrometric conditions

LTQ-Orbitrap XL Mass Spectrometry was purchased from Thermo Scientific, America, and was attached with HESI as well as Xcalibur 2.1 from Thermo Scientific; Dionex Utimate 3000 UHPLC Plus Focused employed binary gradient pump, auto sampler, column oven and DAD detectors using Chromeleon 7, were bought from Thermo Scientific. Millipore Synergy UV was purchased from Millipore, America. While R200D electronic analytical balance (one in 100,000) was purchased from Sartorius, Germany.

Chromatographic conditions: Chromatography column: Agilent Eclipse C_{18} column (4.6 mm×250 mm, 5 μm); mobile phase; 0.2% aqueous formic acid (A), acetonitrile solution (B); gradient elution conditions: 0~10 min (9%~ 9% B), 10~12 min (9%~26% B, 12~33 min (26~26% B), 33~34 min (26%~95% B), 34~40min (95%~95 B), 40~ 42min (95%~9% B), 42~50 min (9%~9% B); rate: 1 mL/min; injection volume: 5μL: column temperature: 30 ℃ and detection wavelength: 270 nm.

Mass spectrometric conditions: Ion source: HESI, mode: ESI(-), ion source temperature: 350°C, ionization source voltage: 3 KV; capillary voltage: 35V; tube lens voltage: 110V, sheath gas and auxiliary gas: high purity nitrogen (purity> 9.99%), sheath gas flow rate: 30 arb and auxilliary gas flow rate: 10 arb. All data was scanned by TF Resolution 3000, data-dependent acquisition $ddMS^2$, using CID fragmentation.

Mass spectrometry data was then imported into Xcalibur 2.2 Software. The structures of component were identified according to the relative retention time, excimer ion peak and fragment ion peak.

Preparation for test solution

About 0.2g Epimedium powder (filtered using No.3 sieve) was obtained, accurately weighed, placed in a conical flask with cover, 20 mL of dilute ethanol was then precisely added and weighed. The mixture was extracted via ultrasound for 1hr and weighed again. The weight loss was adjusted using dilute ethanol. Finally, the solution was shaken, filtered and the extract was collected.

BaohuosideII 8.0mg, 2"-O-rhamnosylicariside II 8.0mg, Epimedin A1 8.0mg, Baohuoside I 5.0 mg, Epimedin A1 5.0mg, Epimedin A 10.0mg, Epimedin B 10.0mg, Epimedin C 10.0mg and Anhydroicaritin 10.0mg were accurately weighed, respectively, dissolved in ethanol and put into a 100 mL volumetric flasks for testing.

HPLC method validation

Standard curve

Precision extraction of icariin reference solution was done using liquid chromatography, 3 parallel tests was done per sample and the average peak area was recorded. The injection curve (X, μg) was plotted on abscissa and the peak area (Y) was plotted on ordinate.

Precision

Precision extraction of icariin reference solution was achieved via 6 continuous injections, to obtain precise data.

Repeatability

Using the same batch of Epimedium sample, 6 test solutions were prepared in parallel, they were then injected into the chromatograph for recording untila reproducible data was obtained

Stability data

The same Epimedium test solution was stored at room temperature and liquid chromatography was performed on 0, 1, 2, 4, 8 and 12hr, respectively, to obtain a stable data.

Sample recovery rate

Six samples of known amounts of Epimedium were accurately weighed and placed in 50 mL volumetric flasks, respectively. An appropriate amount of icariin was added, as per the preparation procedure of the test solution, i.e, the sample solution was recovered by loading. The obtained solution was injected into a chromatograph to obtain the sample recovery test data.

STATISTICAL ANALYSIS

Raw mass spectrum data obtained from UPLC-Orbitrap MS were imported into the Sieve 2.1 software (Thermo Fisher Scientific Inc., San Jose, CA, USA) for peak identification, screening, alignment and noise filtering. After data matrix is normalized, SIMCA-P14.0 software (Umetrics) was employed for PCA and Orthogonal Projections to Latent Structures Discriminant Analysis

(OPLS-DA). 200 times cross validation was used to test the stability and reliability of the discrimination model (Qi *et al*., 2011). The differential components were further identified by using spectral matching method based on Compound Discoverer 3.1 as well as self-built Epimedium component database. Then, hierarchical clustering analysis on differential component data was performed with Mev software (Multi Experiment Viewer, V4.8, TIGR).

RESULTS

The change of icariin content under heating condition

The regression equation of icariin was Y=38.05X+0.106 (r=0.999) with good linear relationship at 0.0~0.4 as verified by HPLC. The precision of the instrument and the repeatability of the method were good with a Relative Standard Deviation (RSD)<1% in precision experiment and repetitive experiment. Stability of the sample solution within 48hr was found to be good with RSD<1.5% in stability experiment, and the recovery of icariin was 99.08%, with RSD=1.98%.

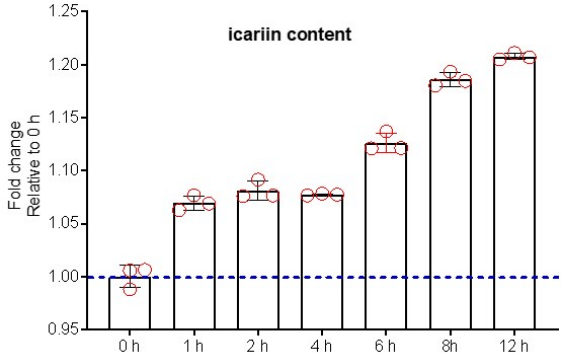

Fig. 1: Variations on icariin content in Epimedium undergoing different heating time (110°C).

The content of icariin in *Epimedium brevicornum* extract heated at 110ºC was tested by HPLC at different time points. The results from this analysis displayed an increase of 21% on the icariin content after 12 hr. This result showed an increasing trend of icariin in Epimedium which is consistent with the results of previous works (Liu *et al*., 2004, Fen *et al*., 2012, Sun *et al*., 2012, Cai *et al*., 2007, Zhong *et al*., 2011, Chen *et al*., 2007b, Li *et al*., 2011). Based on these results, it was hypothesized that some flavonol-glycosides in Epimedium transform into icariin with thermal processing. HPLC-LTQ Orbitrap MS was used to trace the overall profile of changes in the components of Epimedium.

Structure identification of content-changed components in Epimedium during thermal processing

To investigate whether heating condition is a major factor leading to component changes in Epimedium, PCA analysis, a data analysis method based on projection technology, was employed for processing 21×5000 high-throughput mass spectrometry data matrix which obtained from UPLC-MS. The data of 7 groups (0 hr, 1 hr, 2 hr, 4 hr, 6hr, 8hr and 12hr) showed good resolution and separation (R2X> 0.80, Q2> 0.85, Eigen value <1.5) after processing. And the distance to 0 hr group keep increasing as heating time increased (12hr>8hr>6hr> 4hr>2hr>1hr) (fig. 2). This finding indicated significant time dependence of the components proportion variation.

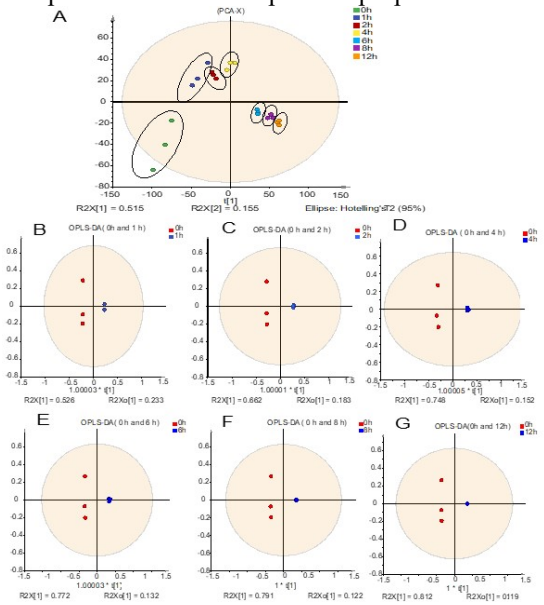

Fig. 2: A. PCA analysis of component variations in *Epimedium* at 0h, 1hr, 2hr, 4hr, 6hr, 8hr, 12hr. B-G. OPLS-DA analysis of component variations in *Epimedium* between different time points.

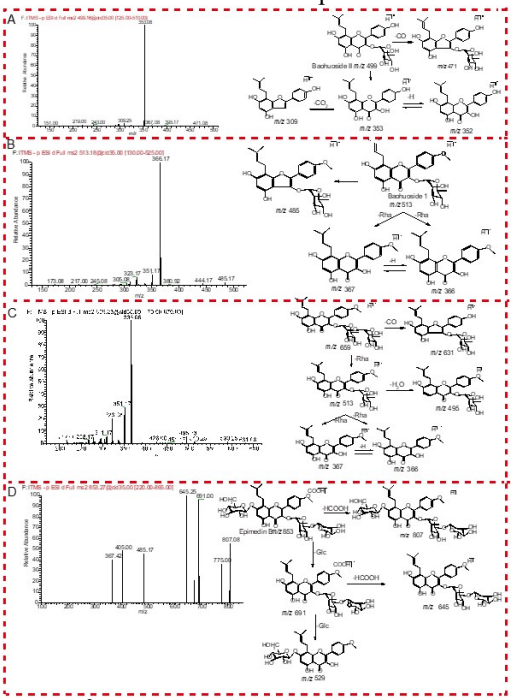

Fig. 3: MS^2 spectrums and cleavage behaviors. A. Component 15; B. Component 16; C. Component 29; D. Component 39 and E. Component 40.

Table 1: UPLC-LTQ Orbitrap MS for Identification of Significantly Varying Components

No.	tR/min	Molecular formula	Observed mass (m/z)	Observed mass (m/z)	Mass accuracy (ppm)	(-)-ESI-MS/MS (*m/z*)	Identification
1	36.12	$C_{15}H_9O_6$	285.03936	285.03966	-1.052486225	267.01; 257.03; 243.02; 241.03; 217.01; 199.00; 174.95; 150.92; 148.96; 132.96	Luteolin
2	29.81	$C_{15}H_9O_7$	301.03427	301.03407	0.664376185	257.03; 228.98; 178.93; 150.89	Quercetin
3	37.61	$C_{16}H_{11}O_7$	315.049929	315.04932	1.933026939	152.91; 134.95	6-Hydroxyluteolin 4'-methyl ether
4	36.12	$C_{17}H_{13}O_7$	329.065579	329.06587	-0.88432221	311.21; 229.12; 211.09; 171.01	Alfalfa
5	36.98	$C_{20}H_{17}O_5$	337.10705	337.10775	-2.076491726	190.91; 172.96;162.98	Icariin D
6	17.61	$C_{20}H_{17}O_6$	353.101964	353.10116	2.276962696	190.96; 178.96; 172.99; 134.94	Noranhydroicaritin
7	36.21	$C_{22}H_{21}O_7$	397.128179	397.12857	-0.984568763	379; 351; 307; 277; 259; 235	Baohuosu
8	15.35	$C_{27}H_{30}O_{16}$	609.145011	609.14503	-0.031191259	343.07; 301.08; 271.08; 255.12; 178.97	Rutin
9	37.51	$C_{25}H_{25}O_6$	421.164565	421.16496	-0.937875673	421.23; 377.29; 366.26; 351; 323.12	Jayacanol
10	35.70	$C_{25}H_{25}O_6$	421.164565	421.16406	1.199056241	421.23; 377.29; 366.26; 351; 323.12	Yinyanghuo B
11	19.69	$C_{21}H_{19}O_{10}$	431.097273	431.09777	-1.152872057	413.10; 403.19; 285.05	Afzelin
12	17.38	$C_{21}H_{19}O_{11}$	447.092187	447.09268	-1.102680866	429.16; 357.13; 301.15; 285.18	Quercetin 3-O-L-rhamnoside
13	16.42	$C_{21}H_{19}O_{12}$	463.087102	463.08770	-1.291333741	301.11; 271.19; 255.12; 178.96	Hyperoside
14	19.41	$C_{24}H_{29}O_{10}$	477.175523	477.17502	1.05411945	431.09; 301.10; 293.11; 160.99	Icariin A
15	36.90	$C_{26}H_{27}O_{10}$	499.159873	499.15947	0.807356564	367.20; 353.13; 337.14; 309.12	Baohuoside II
16	36.75	$C_{27}H_{29}O_{10}$	513.175523	513.17522	0.590441255	367.2; 351.17; 323.21; 311.13	Baohuoside I
17	36.04	$C_{26}H_{27}O_{11}$	515.154788	515.15498	-0.372703514	379.15; 353.12; 335.12; 178.98	Epimedoside C
18	18.67	$C_{27}H_{29}O_{11}$	529.170438	529.17003	0.771018127	409.23; 367.11	Icariin II
19	36.24	$C_{27}H_{29}O_{11}$	529.170438	529.17073	-0.551807091	459.24; 383.15; 312.20	Wushanicariin/Caohuoside C
20	35.98	$C_{27}H_{31}O_{11}$	531.186088	531.18648	-0.737971134	513.25; 411.17; 339.09; 237.03; 218.97; 193.02	8-pyrene quercetin 4'-methyl ether 3-rhamnoside
21	21.49	$C_{28}H_{29}O_{11}$	541.170438	541.17083	-0.724355901		Epimedium 3-rhamnoside
22	30.49	$C_{28}H_{33}O_{12}$	561.196652	561.19695	-0.531008157	443.21; 441.18; 399.29; 309.16; 279.09; 237.00; 218.97	Caohuoside D
23	15.52	$C_{27}H_{29}O_{14}$	577.155181	577.15508	0.174996263	458.95; 431.10; 284.99	Isoginkgetin
24	19.49	$C_{27}H_{29}O_{14}$	577.155181	577.15568	-0.864585499	431.08; 285.01; 224.93	Kaempferitrin
25	19.69	$C_{29}H_{31}O_{15}$	619.165746	619.16534	0.655721029	473.18; 431.16; 285.02	Kaempferol 3-O-alpha-L-rhamnose pyranosyl-(1->2) -alpha-L-rhamnosepyranoside
26	24.93	$C_{44}H_{55}O_{24}$	967.307229	967.307299	-0.071		Epimedokoreanoside I
27	25.92	$C_{32}H_{37}O_{14}$	645.217782	645.21798	-0.306873129	387.18; 352.18; 323.28	Sagittatoside B
28	36.36	$C_{32}H_{37}O_{14}$	645.217782	645.21748	0.468059016	367.17; 352.18; 323.15; 311.14	Noranhydroicaritin 3-rhamnosyl-(1->2)-rhamnoside
29	37.06	$C_{33}H_{39}O_{14}$	659.233432	659.23373	-0.452040181	513.29; 495.24; 367.18; 366.14; 351.13; 323.13	2″-O-rhamnosylicariside II
30	17.91	$C_{32}H_{37}O_{15}$	661.212696	661.21239	0.462786032	515.24; 499.25; 395.20; 353.15	3-Hydroxyalanine-(1->2)-Sorbitoside or Rhamnosy lIcariin II
31	24.27	$C_{32}H_{37}O_{15}$	661.212696	661.21239	0.462786032	515.24; 499.25; 395.20; 353.15	Ikarisoside B
32	16.56	$C_{32}H_{37}O_{15}$	661.212696	661.21229	0.614023298	515.24; 499.25; 395.20; 353.15	Epimedoside A
33	15.08	$C_{33}H_{39}O_{15}$	675.228346	675.22804	0.453180027	367.13; 352.09; 323.14	Sagittatoside A or Baohuoside VII
34	30.721	$C_{33}H_{39}O_{15}$	675.22834	675.22874	-0.592392197	367.13; 352.09; 323.14	Icariin
35	18.84	$C_{33}H_{39}O_{16}$	691.223261	691.22366	-0.577237519	571.21; 545.22; 529.26; 383.20	Wanepimedoside A
36	29.65	$C_{35}H_{41}O_{16}$	717.238911	717.23851	0.559088463	367.12; 352.17	Sagittatoside C
37	16.87	$C_{37}H_{45}O_{19}$	793.254955	793.25465	0.384491768	775.48; 709.11; 631.33; 593.52	Epimedoside D
38	17.18	$C_{38}H_{47}O_{19}$	807.270605	807.27069	-0.105293069	645.43; 367.20	Diphylloside B
39	25.89	$C_{38}H_{47}O_{19}$	807.270605	807.27067	-0.08051823	645.43; 367.20	Epimedin B
40	27.63	$C_{39}H_{49}O_{19}$	821.286255	821.28645	-0.237432441	659.29; 641.31; 513.27; 367.20; 291.12	Epimedin C
41	16.55	$C_{38}H_{47}O_{20}$	823.26552	823.26582	-0.364402483	661.25; 499.35; 353.20	Ikarisoside C
42	22.91	$C_{39}H_{49}O_{20}$	837.28117	837.28107	0.119434192	367.19; 352.27	Epimedin A1
43	12.82	$C_{39}H_{49}O_{20}$	837.28117	837.28111	0.071660515	367.11; 352.23	Maohuoside B
44	21.68	$C_{45}H_{55}O_{23}$	963.312864	963.31256	0.31557764	367.13; 352.24; 323.21	Epimedin K
45	36.02	$C_{45}H_{55}O_{23}$	963.312864	963.31246	0.419386074	367.13; 352.24; 323.21	Caohuoside B
46	8.11	$C_{16}H_{18}O_9$	353.086708	353.08679	-0.232237573	335.26; 190.94; 178.95; 173.04; 111.00	Chlorogenic Acid
47	14.53	$C_{16}H_{18}O_9$	353.086708	353.08676	-0.147272607	335.26; 190.94; 178.95; 173.04; 111.00	4-Dicaffeoylquinic Acid
48	14.98	$C_{20}H_{23}O_4N$	340.154334	340.15439	-0.164631152	325.14; 281.79; 267.11	Magnoflorine
49	28.43	$C_{41}H_{52}O_{21}$	879.291734	879.29153	0.232004911	366.12; 323.11	Epimedin I
50	36.55	$C_{21}H_{20}O_6$	367.117614	367.11741	0.555680229	352.15; 324.28; 297.02	Anhydroicaritin
51	16.55	$C_{32}H_{38}O_{16}$	677.207611	677.20791	-0.44151896	557.21; 531.25; 515.26; 353.20; 352.10; 311.99; 309.03; 297.03	Hexandraside E
52	20.87	$C_{39}H_{48}O_{20}$	835.26552	835.26532	0.239444818	673.42; 515.56	4, 5-dihydroxyl-8-(3, 3 -dimethylallyl)- flavonol 3-O-[xylopyranosyl (1→3)-4-O-acetylrhamnopyrano side]-7-O-glucopyranoside
53	19.30	$C_{38}H_{45}O_{19}$	805.254406	805.254476	-0.09	661.30; 643.48; 499.17; 353.22	Epimedoside
54	24.93	$C_{44}H_{55}O_{24}$	967.307229	967.307299	-0.071		Epimedokoreanoside I
55	14.83	$C_{45}H_{59}O_{27}$	985.31834	985.31734	1.02		Diphylloside C
56	24.23	$C_{39}H_{49}O_{20}$	837.28117	837.28107	0.119434192	367.19; 352.27	Epimedin A

The results showed that the components proportion changed significantly with time. OPLS-DA was applied on the groups between 0-1hr, 0-2hr, 0-4hr, 0-6hr, 0-8hr and 0-12hr to explore the major components responsible for the difference. The results showed that 829 components had Variable Importance of Projection (VIP) Score>1 and Oneway ANOVA p<0.05.

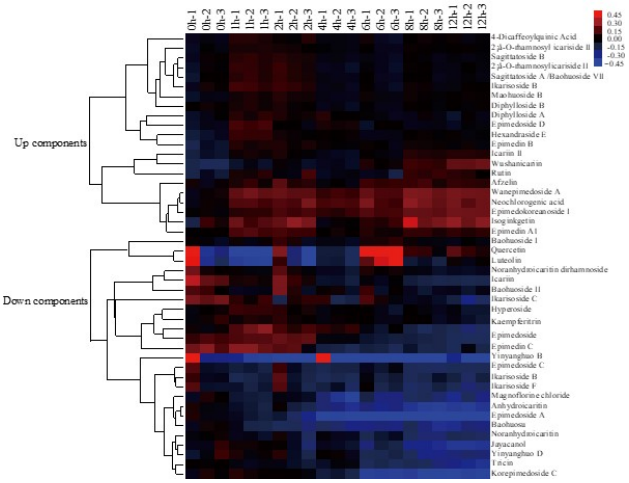

Fig. 4: Hierarchical clustering results of major different components.

56 structures were identified from these 829 components (table 1) via multistage mass spectrometry (MS^n) with retention time (Rt), high mass resolution and accurate mass measurements, as previously described. The specific identification methods were illustrated on the basis of several components as follow; Compound-15 showed molecular ion peak at m/z 499.15947($[M+H]^-$, Mass accuracy=0.81 ppm) with a retention time of 36.90 min. Characteristic fragments ions were shown at m/z 471.25, m/z 430.25, m/z 395.17, m/z 367.25, m/z 353.16, m/z 352.17, m/z 323.25, m/z 309.17, m/z 291.25, m/z 243.00, and m/z 219 in LTQ-Orbitrap MS. The strongest being m/z 353.16 and was different from the molecular ion peak by one molecule of rhamnose (146 Da). The parent structure was speculated to be a flavone, based on typical ions and fragmentation patterns such as m/z 323.25, m/z 309.17 and m/z 291.25 formed by the loss of CO and CO_2 as well as m/z 149.00 and m/z 193.98 formed by RDA cleavage (fig. 3A). It was deduced that compound-15 was a flavonoid glycoside containing one molecule of rhamnose and ultimately identified as Baohuoside II as previously reported (Sun *et al*., 2018, Feng *et al*., 2018).

Compound-16 showed molecular ion peak at m/z 513.17522 ($[M+H]^-$, Mass accuracy=0.59ppm) with a retention time of 36.75 min. Characteristic fragment ions were shown at m/z 485.17, m/z 444.16, m/z 409.23, m/z 391.32, m/z 367.14, m/z 366.18, m/z 351.20, m/z 323.18 and m/z 311.08 in LTQ-Orbitrap MS (fig. 3B). The strongest being m/z 367.16 and was also different from the molecular ion peak by one molecule of rhamnose (146 Da). With fragmentation patterns and characteristic ions, includingm/z 323.25, m/z311.17, m/z279.23 and m/z175.25, which were similar to those of compound-29 (fig. 3C), it was deduced that compound-16 has a flavones parent structure and was identified as Baohuoside I based on previous studies (Chen *et al*. 2007; Polat *et al*. 2018).

Compound-29 exhibited molecular ion peak at m/z 659.23373 ($[M+H]^-$, Mass accuracy=-0.45ppm) with a retention time of 37.06 min. Characteristic fragments ions appeared at m/z 631.25, m/z 590.25, m/z 513.25, m/z 495.17, m/z 367.16, m/z 366.08, m/z 351.25, m/z 323.17 and m/z 311.08 in LTQ-Orbitrap MS. The strongest one was m/z 367.16 and differed from the molecular ion peak by two molecules of rhamnose (292 Da). The parent structure was speculated as a flavone, according to fragmentation patterns and characteristic ions such as m/z 323.25, m/z 311.17, m/z 291.25 and m/z 175.25 (fig. 3C). Therefore, it was deduced that compound-29 was a flavonoid glycoside containing two molecules of rhamnose and ultimately identified as 2-O-rhamnosylicariside II with reference to previous works (Chen *et al*., 2007a).

Compound-39 showed molecular ion peak at m/z 853.27643 ($[M+H]^-$, Mass accuracy=-0.08ppm) with a retention time of 25.89 min. Characteristic fragments appeared at m/z 806.81, m/z 691.29, m/z 645.40, m/z 529.43 and m/z 483.52 in LTQ-Orbitrap MS (fig. 3D). The second strongest signal was m/z691.29, which was also different from the molecular ion peak and the fragment ion at m/z 529.43, respectively, by one molecule of glucose (162 Da). With reference to earlier reports (Chen *et al*. 2007), it was deduced that compound-39 contains two molecules of glucose and was ultimately identified as Epimedin B .

Identification of precursor molecules of icariin

It was speculated that significant increase of icariin is mainly derived from components with similar parent structure as well as from decreased content during the heating process. The identified components were hierarchical clustered into two groups according to content variations (fig. 4). Among which the down-regulated ones were considered as the probable precursors of icariin. 15 components with down-regulated content (fig. 5) were considered to be precursor molecules of icariin among the 56 distinctly modified components.

To further identify components from which the extra icariin is derived, relative standard substances were used for transformation studies. After 4hr of heat treatment of single standard substance of assumed precursor at 170°C, transformations into icariin were observed (fig. 6). The peak area of Epimedin A decreased by 19% and icariin appeared as a conversion product (fig. 6A).

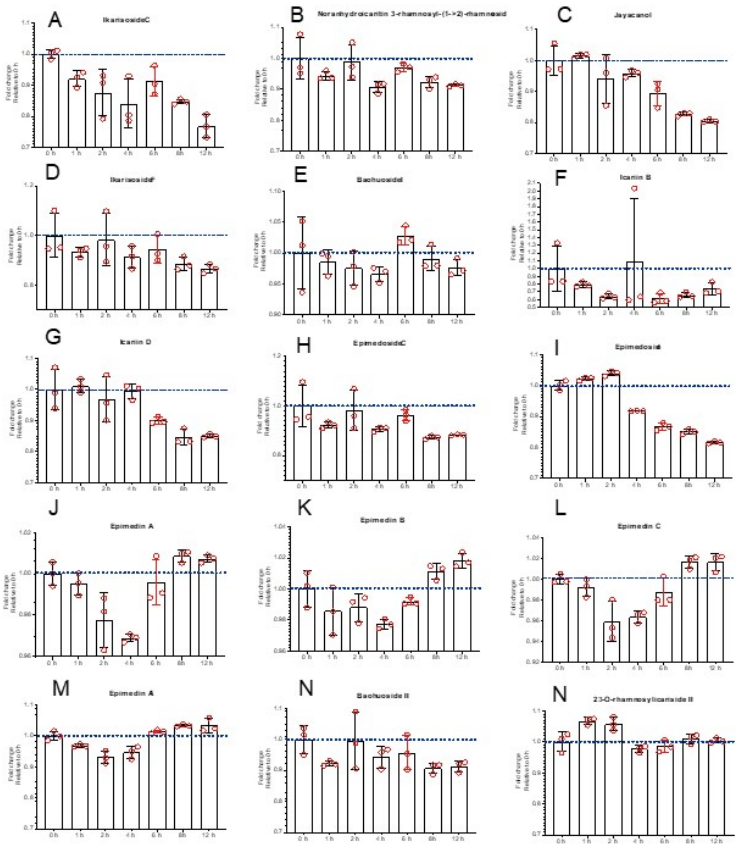

Fig. 5: Assumed precursor molecules of icariin.

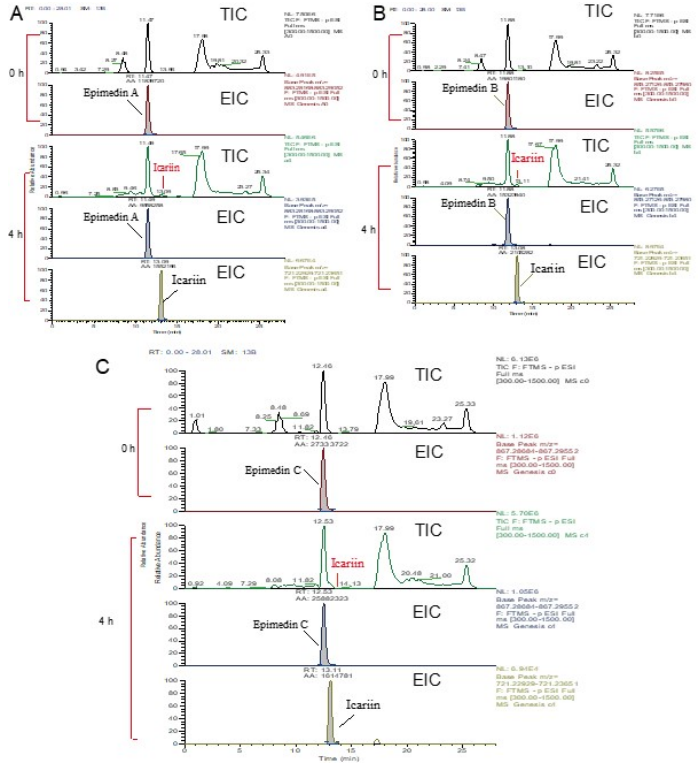

Fig. 6: Total Ion Current map (TIC) and Extracted Ion Chromatography (EIC) at 0hr and 4hr at 170°C. A. transformations of Epimedin A to icariin, B. transformations of Epimedin B to icariin, C. transformations of Epimedin C to icariin.

Similarly, the peak area of Epimedin B decreased by 22.6% and icariin appeared as a conversion product (fig. 6B). Further, the peak area of Epimedin C decreased by 5.3% and icariin appeared as a conversion product (fig. 6C). These 3 standard substances were confirmed to be direct precursors of icariin. Moreover, a heat treatment of mixed standards of assumed precursor molecules at 170°C for 1hr, 2hr, 4hr and 6hr, resulted in degradation of the mixed components. Peak areas of Baohuoside II, 2-O-rhamnosylicariside II, Epimedin A1, Epimedin A, Epimedin B, Epimedin C, Baohuoside I and Anhydroicaritin were decreased and the content of icariin increased gradually from zero and plateaued at 4hr (fig. 7A, 7B).

DISCUSSION

Icariin, being a component of great significance in clinical use, is also an indicator of quality for Epimedium (Ma *et al*., 2011, Xie *et al*., 2010). However, icariin is unstable with unstable content, making it difficult to maintain quality of Epimedium. Obvious increase in content of icariin is a ubiquitous trend in heat-treated Epimedium, which is controversial in production process. Chen Yan *et al*. believe that degradation of Epimedin C leads to increased icariin (Chen *et al*., 2007a), Li Dongxue's study revealed that the decrease of Epimedin A, B and C along with heating process is less than the increase of icariin, indicating that other degradations may contribute to the generation of icariin (Li *et al*., 2017). Therefore, a comprehensive and systematical investigation was hereby conducted to provide a reference for the re-establishment of quality control standard of Epimedium. Nowadays, ultra high-performance liquid chromatography coupled with high-resolution mass spectrometry technique were widely applied in herbal component study. With higher resolution and superior selectivity, UPLC-LTQ-Orbitrap MS is one of the outstanding approaches providing reliable data in both qualitation and quantitation of herbal complex chemical system (Koprivica *et al*., 2018, Zhang *et al*., 2021, Zhou *et al*., 2020).

In this study, HPLC data showed that icariin content of Epimedium increased by 21% in 12hr under heating. Further, HPLC-LTQ Orbitrap MS was used to show the content variation profile of components in Epimedium at different time points under heating condition. Among the 829 significantly changed components, 56 were identified via a multistage mass spectrometry with retention time, high mass resolution and accurate mass measurements. PCA and OPLS-DA were applied for data analysis and differential factors screening. By applying hierarchical clustering of heat maps using the Pearson correlation distance metric, 15 drastic changed isopentenyl flavonoid molecules, which have similar structural characteristic to that of icariin, were screened and deduced to be major precursors of icariin. Next, 8 available standard substances were employed for validation experiments under 170°C. Transformations of standard substances to icariin were observed in both single and mixed samples of Baohuoside II, 2"-o-rhamnosylicariside II, Epimedin A1, Epimedin A, Epimedin B, Epimedin C, Baohuoside I and Anhydroicaritin. Taken together, degradations of components with similar parent structure as icariin's in Epimedium account for increased icariin content during production process. This study provided reference for the quality control and pharmacodynamics study in preparations of Epimedium and suggests a demand for new quality control standard for Epimedium.

CONCLUSION

Icariin, the indicator of quality for Epimedium, was observed to be increased during heating process. A systematic illustration about the precursor molecules of icariin, as well as an explanation of the fluctuation of icariin content in pharmaceutical production was provided with UPLC-LTQ-Orbitrap-MS in this study. This paper provided reference for trouble shooting in quality control of Epimedium preparation, as well as chemical researches in natural pharmacy.

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