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HPLC-DAD–MS^n analysis and HPLC quantitation of chemical constituents in Xian-ling-gu-bao capsules

Xiang-Yu Guan [a], Hui-Fang Li [a], Wen-Zhi Yang [a], Chang-Hu Lin [b], Chao Sun [c], Bao-Rong Wang [a], De-An Guo [a], Min Ye [a,*]

[a] The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China
[b] Guizhou Academy of Sciences, Guiyang 550001, China
[c] Institute of Biology, Guizhou Academy of Sciences, Guiyang 550005, China

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ABSTRACT

In this study, a systematic method was established for the global quality control of Xian-ling-gu-bao capsules (XLGB), a popular six-herb Traditional Chinese Patent Medicine (TCPM) for the treatment of osteoporosis. Both qualitative and quantitative analyses were conducted. In qualitative analysis part, a fast and sensitive method based on high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD–MS^n) was established for rapid separation and sensitive identification. Samples were separated on a Waters Symmetry C_{18} column (250 mm × 4.6 mm, 5 μm) by gradient elution using acetonitrile (A) and water–formic acid (B; 0.03%, v/v) as mobile phase at a flow rate of 1.0 ml/min. Based on the mass spectra, UV spectra and retention time, 47 compounds were identified or tentatively characterized, including 27 flavonols (all from Epimedii Herba, the major component herb), 4 coumarins, 3 flavonones, 1 chalcone, 3 isoflavones, 1 coumestrol, 3 triterpenoid saponins, 1 iridoid, 3 steroidal saponins, and 1 phenolic acid. Among them, 18 compounds were confirmed by comparing with reference standards. In quantitative analysis part, 10 major compounds in 18 batches of XLGB were simultaneously determined by HPLC/UV detected at 270 nm. The method was validated with respect to intra- and inter-day precision, repeatability and stability, with RSD less than 1.0%, 1.5%, 2.9% and 1.8%, respectively. All the 10 analytes showed good linearity in wide linear ranges (r^2 = 0.9999), and their average recoveries varied between 97.8% and 104.9%.

1. Introduction

Xian-ling-gu-bao capsule (XLGB) is a popularly used Traditional Chinese Patent Medicine (TCPM) for the treatment of osteoporosis [1–3]. The recipe of XLGB is composed of six herbal medicines, Epimedii Herba (1167 g), Dipsaci Asperoidis Radix (167 g), Psoraleae Fructus (83 g), Anemarrhenae Rhizoma (83 g), Salviae Miltiorrhizae Radix (83 g) and Rehmanniae Radix (83 g) for every 1000 capsules [4]. According to TCM formulation theory, Epimedii Herba is the emperor herb of the formula (the component herb which plays the major therapeutic role).

Although chemical constituents of the six component herbs of XLGB have been intensively studied [5–21], little is known about the chemical composition of XLGB, and few reports are available on its quality control. Li et al. determined the contents of epimedin B, epimedin C and icariin in XLGB [22]. These three flavonoids were all from Epimedii Herba, and could not reflect the quality of the prescription. What makes the quality control of XLGB more challenging is the multi-sourcing of Epimedii Herba. According to the Chinese Pharmacopoeia, this herb is derived from the dry leaves of five *Epimedium* species, i.e. *E. brevicornum* Maxim., *E. sagittatum* Maxim., *E. pubescens* Maxim., *E. wushanense* T.S.Ying, and *E. koreanum* Nakai [23]. In local areas, other species like *E. myrianthum* were also used. Moreover, the underground parts of *Epimedium* plants were medicinally used in some areas [24]. Therefore, to establish rapid, sensitive, and reliable analytical methods for the quality control of XLGB is of significance to guarantee its quality consistency and therapeutic efficacy.

In this study, both qualitative and quantitative methods were established for the comprehensive quality control of XLGB. By high-performance liquid chromatography coupled with diode array detection and electrospray ionization tandem mass spectrometry

Abbreviations: XLGB, Xian-ling-gu-bao capsules; TCPM, Traditional Chinese Patent Medicine; HPLC-DAD–MS^n, high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass spectrometry; t_R, retention time; ESI, electrospray ionization; gly, glycoside; glu, glucose; rha, rhamnose; xyl, xylose; gal, galactose; ara, arabinose; RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantification.

* Corresponding author. Tel.: +86 10 82801516; fax: +86 10 82802700.
E-mail address: yemin@bjmu.edu.com (M. Ye).

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(HPLC-DAD–MS^n), a total of 47 compounds were identified from XLGB, including 27 flavonols, 4 coumarins, 3 flavonones, 1 chalcone, 3 isoflavones, 1 coumestrol, 3 triterpenoid saponins, 1 iridoid, 3 steroidal saponins, and 1 phenolic acid. In addition, 10 compounds (8 flavonols from Epimedii Herba and 2 compounds from Psoraleae Fructus) in 18 batches of XLGB were simultaneously determined by HPLC. This is the first systematic study on the quality control of XLGB.

2. Experimental

2.1. Materials and reagents

Eighteen pure reference standards, epimedoside E (E2), diphylloside A (E3), diphylloside B (E4), epimedin A (E10), epimedin B (E11), epimedin C (E12), icariin (E14), ikarisoside A (E21), sagittatoside A (E22), sagittatoside B (E23), 2″-*O*-rhamosylicariside II (E24) and icariside II (E26) were isolated from *E. wushanense* T.S. Ying by the authors. Psoralen (P3), isopsoralen (P4), bavachin (P7), corylifolinin (P10), asperosaponin VI (D2) and salvianolic acid B (S1) were purchased from Zelang Medical Technology Co., Ltd. (Nanjing, China). All the above compounds showed purities of above 96% by HPLC analysis. HPLC grade acetonitrile and formic acid (J.T. Baker, Phillipsburg, NJ, USA) were used for all analyses. De-ionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Methanol for sample extraction was purchased from Beijing Chemical Corporation (Beijing, China).

Epimedium species were collected in Guizhou Province, China in 2009, and were authenticated by the authors. Psoraleae Fructus, Dipsaci Asperoidis Radix, Anemarrhenae Rhizoma, Salviae Miltiorrhizae Radix and Rehmanniae Radix were purchased from Tianheng Pharmacy, Beijing. The dry crude drug materials were ground into a fine powder (40 mesh) before use. Xian-ling-gu-bao capsules (XLGB) were manufactured by Tongjitang Pharm Co., Ltd. (Guiyang, China).

2.2. Sample preparation

For HPLC-DAD–MS^n qualitative analysis of XLGB, use one capsule each from five batches, mix the powder-like content of the capsules evenly, and weigh 0.2 g of the mixed powder. For HPLC quantitative determination, accurately weigh 0.50 g powder-like content from each batch. The weighted powder was suspended in 10.0 ml of 70% (v/v) methanol and extracted in an ultrasonic water bath for 30 min at 30 °C. The solution was filtered through 0.45 μm membranes prior to use, and a 10 μl aliquot was injected for analysis. For the crude herbal medicines, 0.2 g was used for qualitative analysis.

2.3. Preparation of standard stock solutions

For HPLC quantification, a stock solution was prepared by dissolving appropriate amounts of 10 reference compounds in 70% methanol to make the concentrations of 0.62 mg/ml for epimedoside E, 0.32 mg/ml for diphylloside B, 0.26 mg/ml for epimedin A, 0.22 mg/ml for epimedin B, 1.69 mg/ml for epimedin C, 0.75 mg/ml for icariin, 0.14 mg/ml for psoralen, 0.16 mg/ml for ikarisoside A, 0.33 mg/ml for icariside II, and 0.14 mg/ml for bavachin. This stock solution was then diluted accurately to 2%, 5%, 10%, 20%, 50%, and 70% of the original concentration by 70% methanol to obtain seven serial concentrations for the construction of calibration curves. All the working solutions were kept at 4 °C.

2.4. HPLC-DAD–MS^n analysis

HPLC-DAD–MS^n analysis was performed on an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) coupled with an LCQ Advantage ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA) via an electrospray ionization (ESI) interface. The HPLC instrument was equipped with a quaternary pump, a diode-array detector (DAD), an autosampler, and a column compartment. Samples were separated on a Waters Symmetry C_{18} column (250 mm × 4.6 mm I.D., 5 μm) equipped with a Waters Symmetry C_{18} guard column (20 mm × 3.9 mm I.D., 5 μm). The mobile phase consisted of acetonitrile (A) and water containing 0.03% (v/v) formic acid (B). A gradient program was used as follows: 0 min, 19:81 (A:B, v/v); 10 min, 25:75; 22 min, 33:67; 33 min, 55:45; 36 min, 60:40; 38–44 min, 100:0. A 15-min post-run time was set to fully equilibrate the column. The flow rate was 1.0 ml/min. The column temperature was 30 °C. The DAD detector scanned from 190 to 800 nm, and the samples were detected at 270 nm. The sample injection volume was 10 μl. The HPLC eluent was introduced into ESI source of mass spectrometer in a post-column splitting ratio of 5:1. For MS detection, high purity nitrogen (N_2) was used as the nebulizing gas, and ultra-high pure helium (He) as the collision gas. Both negative and positive ion polarity modes were used for compound ionization. The ESI source parameters were optimized by injecting a 5 μl/min flow of icariin, psoralen, asperosaponin VI and salvianolic acid B (0.1 mg/ml in methanol) to obtain maximum intensities of ions. The optimized parameters in the negative ion mode were as follows: source voltage, 4.5 kV; sheath gas (N_2), 40 arbitrary units; auxiliary gas (N_2), 10 units; capillary temperature, 320 °C; capillary voltage, −30 V; tube lens offset voltage, −20 V. In the positive ESI ion mode, the capillary voltage was 15 V, and the tube lens offset voltage was 30 V. For full scan MS analysis, spectra were recorded in the range of m/z 120–1350. The 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, $n = 3$). The isolation width of precursor ions was 2.0 Th. The HPLC-DAD–MS^n system was controlled by Xcalibur™ 1.4 software.

2.5. HPLC quantitation

For HPLC determination, analysis was performed on an Agilent series 1100 HPLC system including a quaternary pump, a UV variable wavelength detector (VWD), an autosampler, and a column compartment. The HPLC conditions were the same as those for HPLC-DAD–MS^n analysis except that the UV detector wavelength was 270 nm under which all the analytes had good absorption. All the data were processed by using Agilent LC B.02.01 ChemStation software.

3. Results and discussion

3.1. Optimization of extraction and analytical conditions

In order to extract the compounds efficiently, variables involved in this procedure such as extraction solvent (30%, 70%, and 100% methanol), solvent volume (10, 20, 50, and 80-fold), extraction method (reflux, ultrasonication, soaking at room temperature), and extraction time (15, 30, 45 and 60 min) were optimized. The finally optimized method was to extract 0.50 g of the powder with 10 ml of 70% methanol in an ultrasonic water bath at 30 °C for 30 min.

The HPLC conditions were optimized, including type of column, column temperature, mobile phase system, flow rate, and detection wavelength of UV. With the optimized conditions, most peaks could be well separated within 44 min. The MS parameters

were optimized with representative pure compounds, including icariin (E14), asperosaponin VI (D2), psoralen (P3), and salvianolic acid B (S1). These compounds were from different herbs, and represented flavonols, saponins, coumarins, and phenolic acids, respectively. Both negative and positive ESI modes were monitored to obtain adequate structural information. Flavonols and saponins were readily to be ionized and fragmented in the negative mode, whilst coumarins and phenolic acids preferred positive mode.

3.2. HPLC-DAD–MS^n qualitative analysis

To reveal the chemical composition of XLGB, HPLC-DAD–MS^n analysis was carried out. Based on MS spectra, UV spectra and retention time (t_R), a total of 47 compounds (Table 1, Fig. 1) were identified or tentatively characterized, and 18 of them were confirmed by reference standards. These compounds included 27 flavonols, 4 coumarins, 3 flavonones, 1 chalcone, 3 isoflavones, 1 coumestrol, 3 triterpenoid saponins, 1 iridoid, 3 steroidal saponins, and 1 phenolic acid. The component herb from which each compound was derived was confirmed by individually analyzing the six herbs of XLGB using the same HPLC-DAD–MS^n method (Fig. 2).

3.2.1. Compounds from Epimedii Herba

A total of 27 XLGB compounds (E1-E27) were from Epimedii Herba. All of the 27 compounds were flavonols. Most of them were glycosides of anhydroicaritin or demethylanhydroicaritin, both bearing an isoprenyl group at C-8 and differentiated by the substituent at C-4′ (–OCH_3 or –OH). The structures were mainly established on the basis of mass spectral information.

In this paper, the names of sugar moieties were abbreviated as follows: gly, glycoside; glu, glucose; rha, rhamnose; xyl, xylose; gal, galactose; ara, arabinose. The nomenclature in literatures was used for fragment ions [25,26]. Take negative mode as example, the ion $[M-H-gly_{(7)}]^-$ and $[M-H-gly_{(3)}]^-$ were labeled as ${}^7Y_0^-$ and ${}^3Y_0^-$, respectively, and the aglycone ion $[M-H-gly_{(7)}-gly_{(3)}]^-$ was labeled as Y_0^-. In positive mode, ions were labeled as ${}^7Y_0^+$, ${}^3Y_0^+$, and Y_0^+ under similar rules.

Here we take epimedin A (E10) as example to illustrate the fragmentation pathways of prenylated flavonols. Epimedin A (E10), or anhydroicariin 3-*O*-glu(2,1)-rha-7-*O*-glucoside, showed maximum UV absorption at 270 nm and 320 nm. The negative ESI spectrum showed an intense ${}^7Y_0^-$ (*m*/*z* 675) instead of $[M-H]^-$ ion. In the MS^2 spectrum, ${}^7Y_0^-$ fragmented into Y_0^- at *m*/*z* 367 due to the loss of 3-*O*-disaccharide moiety, indicating that the aglycone was anhydroicaritin. Besides, the $[{}^7Y_0-104]^-$ ion at *m*/*z* 571, due to $^{0,2}X$ cleavage of the inner rhamnose of 3-*O*-disaccharide chain, indicated that the terminal glucosyl was substituted at C-2 of rhamnose. The Y_0^- ion further yielded an abundant fragment $[Y_0-CH_3]^-$ at *m*/*z* 352, and could also lose 56 Da and 46 Da which were attributed to C_4H_7 and C_3H_7 of the isoprenyl group, respectively. The ion at *m*/*z* 217 derived from RDA fragmentation was also observed. In the positive ion mode, $[M+H]^+$ ion at *m*/*z* 839 and $[M+Na]^+$ ion at *m*/*z* 861 were observed in full scan MS spectrum. In MS^n spectra the $[M+H]^+$ ion gave the MS^n fragments $[M+H-glu]^+$, ${}^3Y_0^+$ and Y_0^+ at *m*/*z* 677, 531 and 369, which were due to the cleavage of external and internal sugar from 3-*O*-disaccharide chain and 7-*O*-glucosyl moiety, respectively.

All the flavonol glycosides showed similar fragmentation patterns as described above for epimedin A. Their structures were established either by comparing with a reference standards (12 compounds, E2–E4, E10–E12, E14, E21–E24, E26) or by analyzing their tandem mass spectra (15 compounds, E1, E5–E9, E13, E15–E20, E25, E27). Most of these compounds had been reported from *Epimedium* species [6,8,10–13,16,17]. Detailed structural information are given in Table 1S.

E6 and E16 had different aglycones from the above compounds, as their $[Y_0-H]^-$ ions were at *m*/*z* 382 and 384, respectively. For E6, it was clear from the comparison of negative mode fragmentation with E14 (icariin) (Fig. 3) that E6 had one more hydroxyl group on ring B, since they both had the fragment ion at *m*/*z* 217, the residue of ring A after the RDA fragmentation of the aglycone, and other fragment ions kept the difference of 16 Da. So E6 was identified as sagittasin C (3′-hydroxyicariin-3-*O*-rha-7-*O*-glucoside) [8]. Similarly, E16 was identified as icaritin 3-*O*-rhamnoside [14], which had one molecule of H_2O added on the isopentenyl group of E26 (icariside II) (Fig. 4).

E1 was also a flavonol derived from Epimedii Herba though it does not have isopentenyl group. It showed $[M+H]^+$, $[M+Na]^+$, and $[M+H-162]^+$ ions at *m*/*z* 465, 487, and 303 in positive mode, and gave $[M-H]^-$ and $[M-H-162]^-$ ions at *m*/*z* 463 and 301 in negative mode. The $[M-H-162]^-$ ion further yielded ions at *m*/*z* 255 and 271. The above data were in good accordance with those reported for hyperoside (quercetin 3-*O*-galactoside) [13]. Thus, E1 was identified as hyperoside, which had been isolated from several *Epimedium* species [6].

3.2.2. Compounds from Psoraleae Fructus

Twelve compounds from Psoraleae Fructus (P1-P12) were identified in XLGB, including four coumarins, three flavonones, one chalcone, three isoflavones, and one coumestrol. Among them, P3 (psoralen), P4 (isopsoralen), P7 (bavachin), and P10 (corylifolinin) were confirmed by reference compounds. The structural identification was mainly based on UV spectra and MS behaviors (Table 1S). The tandem mass spectrometry fragmentation behaviors of *Psoralea* compounds reported previously were referred to in this study [5,20].

3.2.3. Compounds from other component herbs

Eight compounds derived from the other component herbs were identified from XLGB, including three triterpene saponins (D2–D4) and one iridoid (D1) from Dipsaci Asperoidis Radix, three steroidal saponins (A1–A3) from Anemarrhenae Rhizoma, and one phenolic acid (S1) from Salviae Miltiorrhizae Radix. The structural identification of these compounds was mainly based on the MS^n fragmentation behaviors because most of these compounds had no significant UV absorption [7,9,15,18,19] (Table 3S). Among them, D2 (asperosaponin VI) and S1 (salvianolic acid B) were identified by comparing with reference standards. D1 (cantleyoside) was an iridoid from Dipsaci Asperoidis Radix. Its MS^n fragmentation pathway was proposed for the first time as shown in Fig. 5.

Rehmanniae Radix was the only drug from which no compound was detected in HPLC-DAD–MS^n analysis. The major constituents of Rehmanniae Radix are iridoids [21] which are unstable, and they were probably destroyed during the procedures of manufacture. Thus, even though no compound was detected, it is not fair to say that Rehmanniae Radix made no contribution to the pharmacological effect of XLGB.

3.3. HPLC quantitation

3.3.1. Calibration curves

A stock solution containing ten reference standards was prepared and diluted by 70% methanol to seven concentrations, as described under Section 2.3, and the stock solutions of each concentration were analyzed twice for the construction of calibration curves. Peak areas (x) of serial working solutions of each analyte were plotted against the concentrations (y, mg/g). All the calibration curves showed good linearity with correlation coefficients (r^2) no less than 0.9999. The regression equations are given in Table 2.

Table 1
Identification of chemical constituents of XLGB by HPLC-DAD–MS^n.

Compound	t_R	Component Herb	UV	(−)-ESI		(+)-ESI			Identification
				$[M-H]^-$	MS/MS (m/z, relative intensity%)	$[M+H]^+$	MS/MS of $[M+H]^+$ (m/z, relative intensity%)	$[M+Na]^+$	
P1	5.8	P	246, 294	365	n.a.	n.a.	n.a.	389	Psoralenoside [5]
P2	6.2	P	248, 300	365	n.a.	n.a.	n.a.	389	Isopsoralenoside [5]
E1	7.3	E	256, 316	463	301	465	303	n.a.	Hyperoside [6]
D1	10.4	D	n.a.	745	583(100), 513(20), 459(20)	n.a.	n.a.	769	Cantleyoside [7]
E2[a]	10.9	E	270, 320	823	661	825	663(40), 517(100), 355(40)	847	Epimedoside E
E3[a]	11.5	E	270, 320	793	631	795	663(100), 517(70), 355(20)	817	Diphylloside A
E4[a]	12.2	E	270, 320	807	645	809	663(60), 517(100), 355(40)	831	Diphylloside B
E5	12.7	E	270, 320	661	515(30), 499(80), 353(100)	663	517(100), 355(20)	685	Epimedoside A [6]
E6	13.8	E	270, 320	691	529(100), 383(20)	693	n.a.	715	Sagittasin C [8]
A1	15.3	A	n.a.	919	757	n.a.	n.a.	943	Timosaponin B-II [9]
E7	16.2	E	270, 320	835	673	837	705(100), 517(70)	859	Sempervirenoside B [10]
E8	17.7	E	270, 320	n.a.	367(100), 366(50)	839	677(70), 531(100), 369(50)	861	Hexandraside F [8,11]
E9	18.0	E	270, 320	983	659	985	839(50), 693(60), 531(80), 369(100)	1007	Acuminatoside [12]
E10[a]	18.7	E	270, 320	n.a.	n.a.	839	677(70), 531(100), 369(50)	861	Epimedin A
E11[a]	19.2	E	270, 320	n.a.	n.a.	809	677(100), 531(80), 369(40)	831	Epimedin B
E12[a]	20.1	E	270, 320	n.a.	n.a.	823	677(100), 531(80), 369(50)	845	Epimedin C
E13	20.4	E	270, 320	n.a.	n.a.	823	677(100), 531(80), 369(50)	845	Anhydroicariin 3-*O*-rha-(4,1)-rha-7-*O*-glucoside [13]
E14[a]	21.1	E	270, 320	n.a.	n.a.	677	531(100), 369(20)	699	Icariin
S1[a]	21.5	S	n.a.	717	n.a.	n.a.	n.a.	n.a.	Salvianolic acid B
E15	23.1	E	270, 320	515	353(100), 352(50)	517	355	539	Epimedoside C [11]
E16	23.3	E	270, 320	531	384	533	n.a.	555	Icaritin 3-*O*-rhamnoside [14]
D2[a]	24.5	D	n.a.	927	603(80), 323(100)	n.a.	n.a.	951	Asperosaponin VI
A2	25.3	A	n.a.	901	739	903	n.a.	n.a.	Timosaponin B-III [15]
P3[a]	26.2	P	242, 282	n.a.	n.a.	187	159(20), 143(100), 131(20), 115(20)	n.a.	Psoralen
E17	27.2	E	270, 320	661	481(20), 353(50), 352(100)	663	n.a.	685	Ikarisoside B [16]
P4[a]	27.4	P	242, 280	n.a.	n.a.	187	159(90), 143(100), 131(70), 115(20)	n.a.	Isopsoralen
E18	27.8	E	270, 320	819	657(70), 367(100)	821	677(100), 531(90)	843	Anhydroicariin 3-*O*-rha-dideoxyfuranose-7-*O*-glucoside [13]
E19	28.3	E	270, 320	631	481(20), 353(50), 352(100)	633	n.a.	655	Ikarisoside F [17]
E20	28.4	E	270, 320	645	481(20), 353(40), 352(100)	647	501(100), 483(40), 465(30)	669	2″-*O*-Rhamnosylikarisoside A [16]
D3	28.8	D	n.a.	969	645	n.a.	n.a.	993	4′-*O*-Acetyl-akebia saponin D [18]
D4	29.3	D	n.a.	795	471	n.a.	n.a.	819	Dipsacussaponin A [19]
E21[a]	29.8	E	270, 320	499	353	501	355	523	Ikarisoside A
E22[a]	31.3	E	270, 320	675	367(100), 352(20)	677	515(100), 369(60)	699	Sagittatoside A
E23[a]	32.2	E	270, 320	645	367(90), 366(100), 352(30)	647	515(100), 479(30)	669	Sagittatoside B
E24[a]	32.5	E	270, 320	659	366(100), 367(50)	661	515(100), 497(40)	683	2″-*O*-Rhamosylicariside II
E25	32.8	E	270, 320	529	367	531	369	553	Icariside I [16]
P5	34.0	P	280, 318	323	221(40), 203(100)	325	269(100), 205(20)	n.a.	Isobavachin [20]
E26[a]	34.3	E	270, 320	513	366(100), 367(50)	515	369	537	Icariside II
E27	35.0	E	270, 320	687	367(100), 352(30)	689	557(40), 539(20), 479(30), 369(100)	711	Anhydroicariin 3-*O*-rha(OAc)-xyloside
P6	35.4	P	250, 306	321	n.a.	323	267(100), 255(10)	n.a.	Neobavaisoflavone [20]
P7[a]	36.8	P	276, 322	323	221(90), 203(100)	325	269(100), 167(30)	n.a.	Bavachin
A3	38.5	A	n.a.	739	577	741	n.a.	763	Timosaponin A-III [15]
P8	38.7	P	250, 306	319	n.a.	321	303(100), 279(40), 211(40), 137(80)	n.a.	Corylin [20]
P9	39.0	P	346	335	n.a.	337	281(100), 309(30)	n.a.	Psoralidin [20]
P10[a]	40.5	P	368	323	221(20), 203(100)	325	269(100), 205(30)	n.a.	Corylifolinin
P11	40.8	P	274, 320	n.a.	n.a.	339	283(100), 271(90)	n.a.	Bavachinin [20]
P12	40.9	P	250, 294	389	n.a.	391	335(20), 267(100)	n.a.	Corylifol A [20]

[a] Compounds identified by comparing with reference standards. t_R, retention time; E, Epimedii Herba; P, Psoraleae Fructus; D, Dipsaci Asperoidis Radix; A, Anemarrhenae Rhizoma; S, Salviae Miltiorrhizae Radix; n.a., not available.

No.	R_1	R_2	R_3	No.	R_1	R_2	R_3
E2	-rha(2,1)-glu	-glu	H	E15	H	-glu	H
E3	-rha(2,1)-xyl	-glu	H	E17	-rha(2,1)-glu	H	H
E4	-rha(2,1)-rha	-glu	H	E18	-rha-furan acid	-glu	Me
E5	-rha	-glu	H	E19	-rha(2,1)-glu	H	H
E7	-rha(4,1)OAC-(3,1)xyl	-glu	H	E20	-rha(2,1)-rha	H	H
E8	-rha(3,1)-glu	-glu	Me	E21	-rha	H	H
E9	-rha(2,1)-rha	-glu(2,1)-glu	Me	E22	-rha(2,1)-glu	H	Me
E10	-rha(2,1)-glu	-glu	Me	E23	-rha(2,1)-xyl	H	Me
E11	-rha(2,1)-xyl	-glu	Me	E24	-rha(2,1)-rha	H	Me
E12	-rha(2,1)-rha	-glu	Me	E25	H	-glu	Me
E13	-rha(4,1)-rha	-glu	Me	E26	-rha	H	Me
E14	-rha	-glu	Me	E27	-rha(OAc)-xyl	H	Me

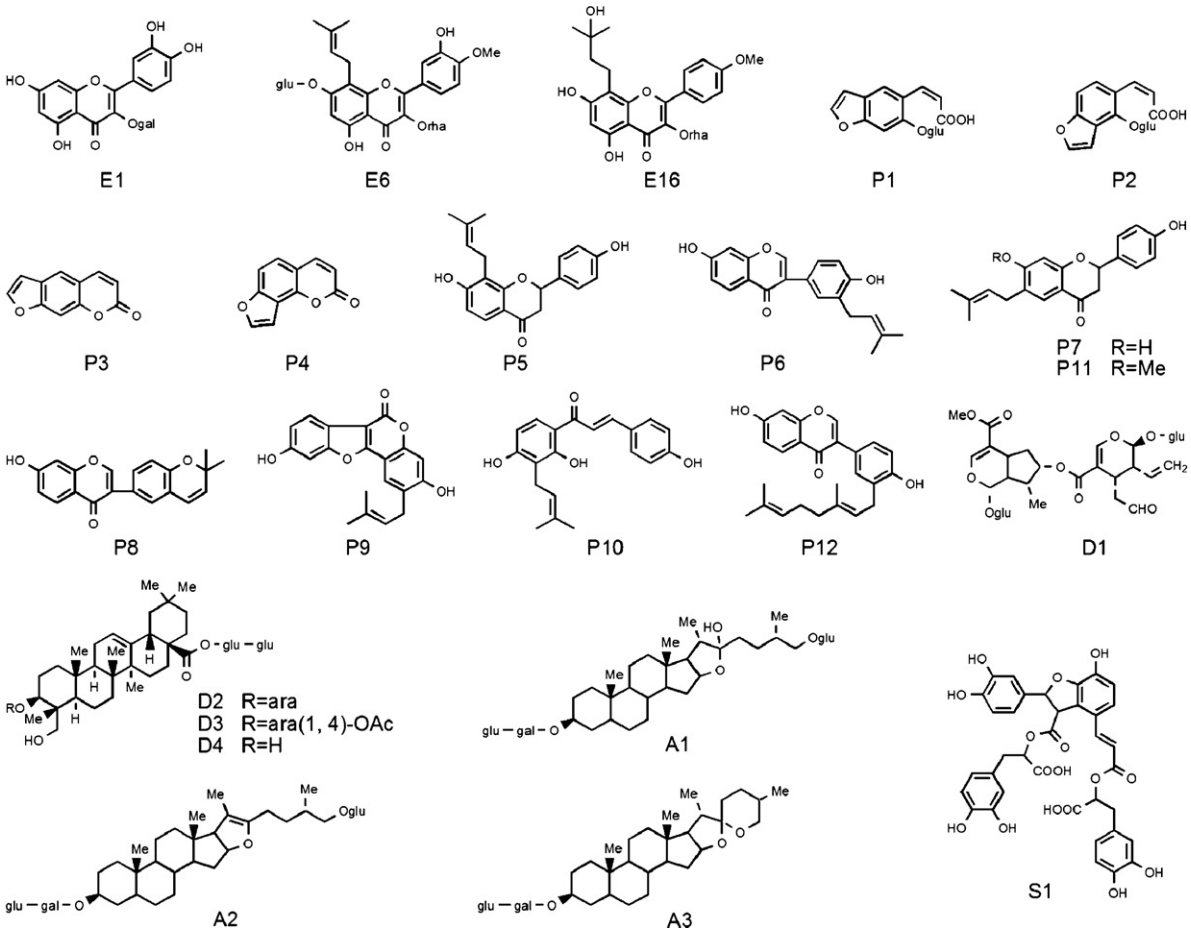

Fig. 1. Chemical structures of compounds identified in XLGB. glu, glucose; rha, rhamnose; xyl, xylose; gal, galactose; ara, arabinose.

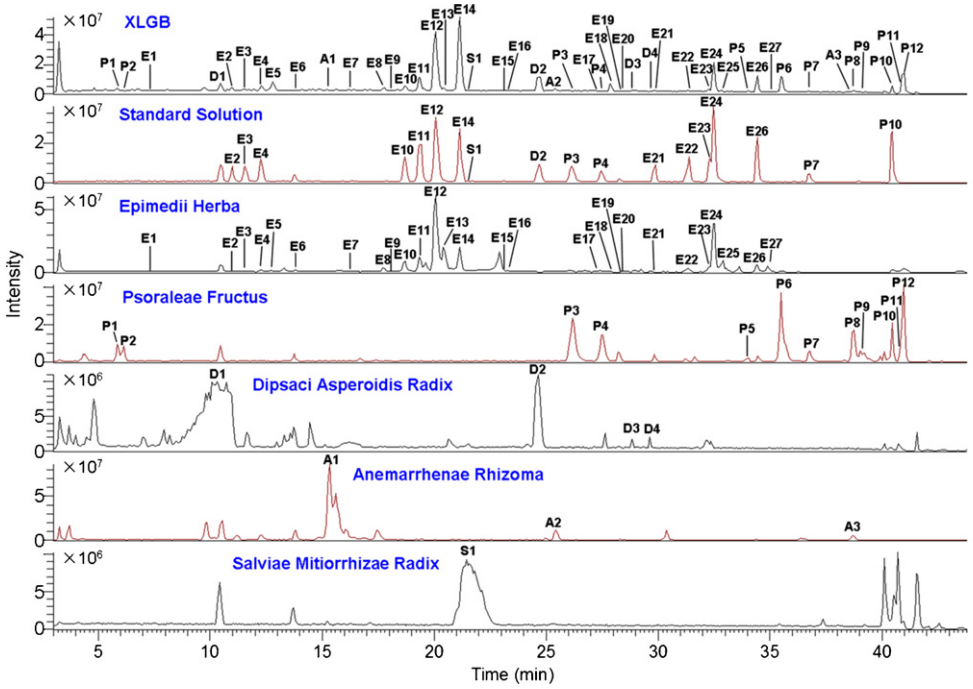

Fig. 2. Base peak chromatograms of XLGB and its component herbs by HPLC-DAD–ESI-MS^n in the positive ion mode.

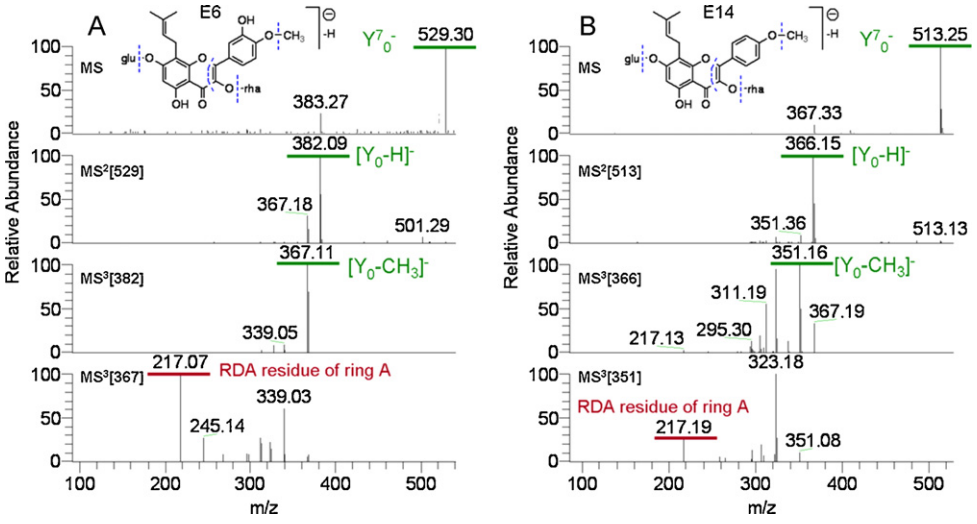

Fig. 3. (−)-ESI-MS^n spectra of E6 (A, sagittasin C) and E14 (B, icariin).

Table 2
The regression equations of 10 analytes.

Analytes	Linear range (mg/g)	Regressive equation	r^2	LOD (μg/g)	LOQ (μg/g)
Epimedoside E	0.25–12.4	$y = 1.39 \times 10^{-3}x + 0.044$	0.9999	3.9	15.5
Diphylloside B	0.13–6.4	$y = 1.35 \times 10^{-3}x + 0.029$	0.9999	4.0	16.0
Epimedin A	0.10–5.2	$y = 1.31 \times 10^{-3}x + 0.025$	0.9999	6.5	13.0
Epimedin B	0.08–4.4	$y = 1.11 \times 10^{-3}x + 0.024$	0.9999	2.8	11.0
Epimedin C	0.67–23.6	$y = 1.22 \times 10^{-3}x + 0.024$	0.9999	1.3	10.6
Icariin	0.30–15.0	$y = 9.34 \times 10^{-4}x + 0.005$	0.9999	2.3	9.4
Psoralen	0.06–2.8	$y = 1.27 \times 10^{-3}x + 0.016$	0.9999	3.5	14.0
Ikarisoside A	0.06–3.2	$y = 6.62 \times 10^{-4}x + 0.013$	0.9999	2.0	4.0
Icariside II	0.13–6.6	$y = 7.47 \times 10^{-4}x + 0.012$	0.9999	4.1	8.3
Corylifolin	0.06–2.8	$y = 8.95 \times 10^{-4}x + 0.013$	0.9999	1.8	7.0

Note: In the regression equation, x is the peak area, y the concentration of each analyte (mg/g), and r the correlation coefficient. LOD: limit of detection. LOQ: limit of quantification.

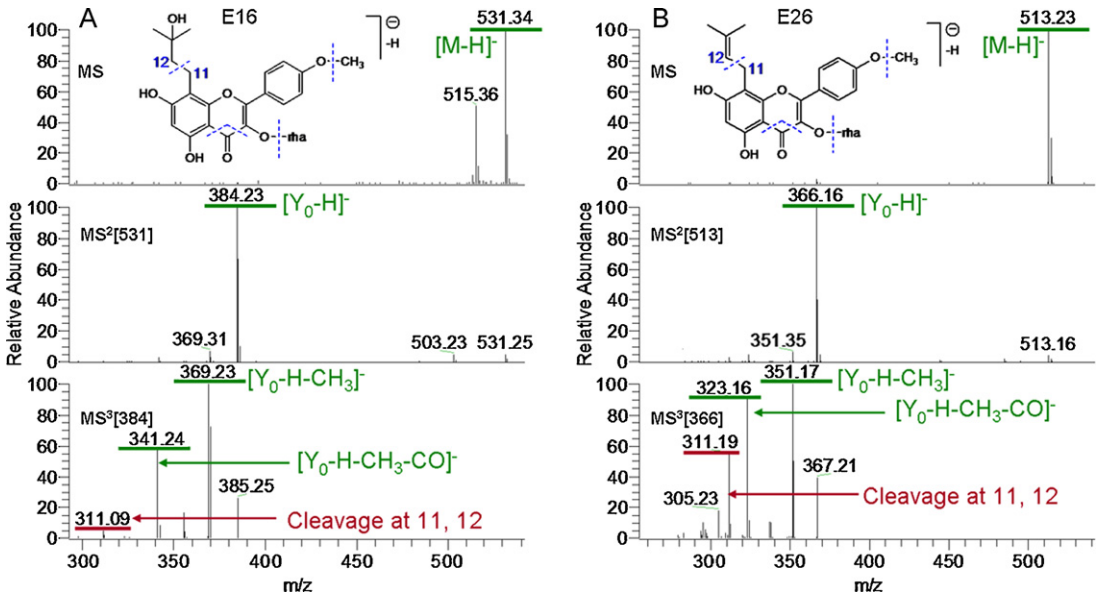

Fig. 4. (−)-ESI-MS^n spectra of E16 (A, icaritin 3-*O*-rhamnoside) and E26 (B, icariside II).

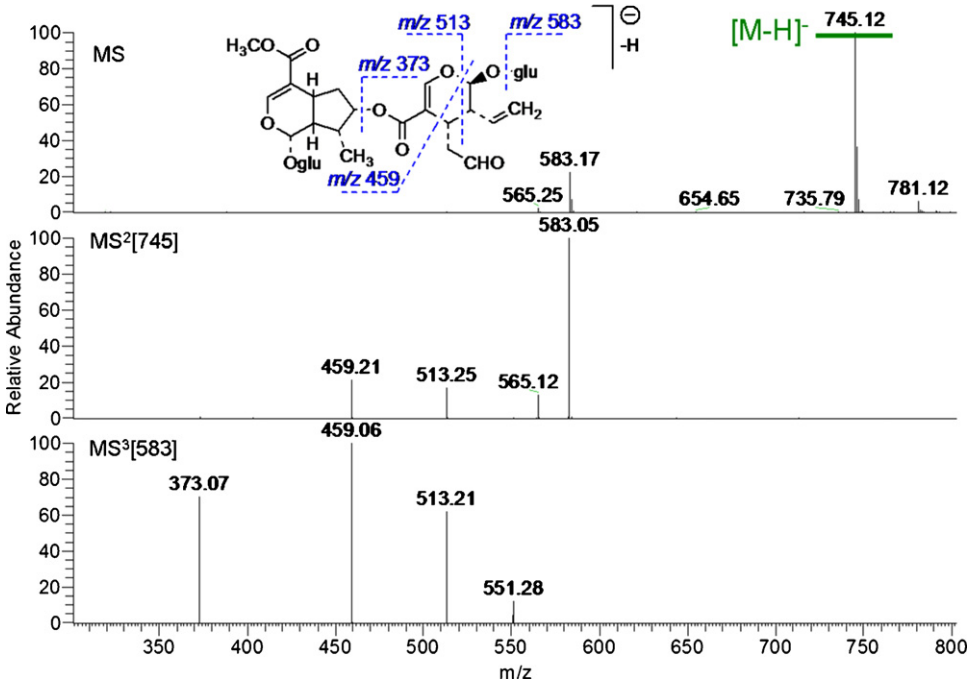

Fig. 5. (−)-ESI-MS^n spectra of D1 (cantleyoside).

Table 3
Precision, repeatability, and stability tests (RSD %).

Analytes	Intra-day precision (n = 6)	Inter-day precision (n = 3)	Repeatability (n = 6)	Stability (n = 6)
Epimedoside E	0.26	0.82	2.84	0.73
Diphylloside B	0.40	0.96	1.34	0.91
Epimedin A	0.22	0.50	0.48	0.66
Epimedin B	0.39	1.36	0.79	1.24
Epimedin C	0.27	0.66	0.51	0.58
Icariin	0.30	0.55	0.71	0.57
Psoralen	0.70	0.73	0.41	0.95
Icarisoside A	0.44	0.43	1.59	0.42
Baohuoside I	0.39	1.11	1.03	0.88
Corylifolin	0.91	1.45	0.62	1.72

Table 4
Recoveries of 10 compounds in XLGB ($n = 3$).

Analytes	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)	RSD (%)
Epimedoside E	1.52	1.35	2.91	103.1	0.53
Diphylloside B	0.46	0.58	1.06	104.9	0.37
Epimedin A	0.38	0.45	0.85	104.1	0.28
Epimedin B	0.49	0.55	1.06	102.7	1.37
Epimedin C	2.83	2.23	5.13	103.3	0.72
Icariin	1.19	1.13	2.36	103.4	0.80
Psoralen	0.23	0.30	0.54	103.6	1.46
Ikarisoside A	0.09	0.07	0.16	103.8	1.34
Icariside II	0.12	0.13	0.25	102.7	0.73
Corylifolin	0.05	0.06	0.11	97.8	1.43

Note: Recovery (%) = 100 × (amount found − original amount)/amount spiked.

Table 5
Contents of 10 analytes in 18 batches of XLGB ($n = 2$).

Batches	Contents (mg/g)									
	E2	E4	E10	E11	E12	E14	P3	E21	E26	P7
1	1.33	1.02	1.63	2.12	12.73	6.63	0.54	0.23	0.83	0.25
2	1.18	1.26	1.19	1.73	13.56	5.20	0.47	0.25	0.77	0.26
3	0.57	0.95	1.17	1.87	13.78	4.29	0.78	0.21	0.54	0.17
4	0.46	1.03	1.06	1.72	14.67	4.04	0.86	0.23	0.58	0.20
5	0.62	1.11	0.94	1.69	13.75	4.00	0.84	0.23	0.65	0.18
6	0.73	1.32	1.40	2.01	14.45	5.67	0.83	0.23	0.58	0.20
7	1.72	1.38	1.06	1.67	11.96	4.72	0.79	0.31	0.88	0.15
8	1.30	1.04	1.44	2.15	12.19	5.80	0.75	0.22	0.83	0.23
9	0.62	0.86	1.32	1.96	11.30	5.77	0.82	0.22	0.72	0.20
10	0.67	1.35	1.12	1.82	13.83	5.16	0.82	0.22	0.69	0.19
11	0.86	1.05	1.23	1.84	11.94	5.29	0.81	0.21	0.58	0.19
12	5.60	1.71	1.31	1.78	10.65	4.37	0.75	0.35	0.43	0.18
13	6.09	1.85	1.53	1.96	11.32	4.77	0.93	0.38	0.47	0.22
14	1.67	0.88	1.12	1.74	9.30	4.16	0.85	0.21	0.50	0.16
15	1.12	0.96	1.24	0.94	11.36	4.90	0.85	0.21	0.53	0.17
16	0.70	0.89	1.49	2.17	12.68	6.22	0.95	0.19	0.61	0.19
17	0.64	0.86	1.49	2.16	12.61	6.26	0.96	0.19	0.61	0.19
18	0.83	1.10	1.32	1.97	12.68	5.74	0.87	0.21	0.59	0.20
Average	1.48	1.15	1.28	1.85	12.49	5.17	0.80	0.24	0.63	0.20
RSD (%)	110.04	24.84	14.76	15.35	11.19	15.76	15.53	21.88	20.57	14.83

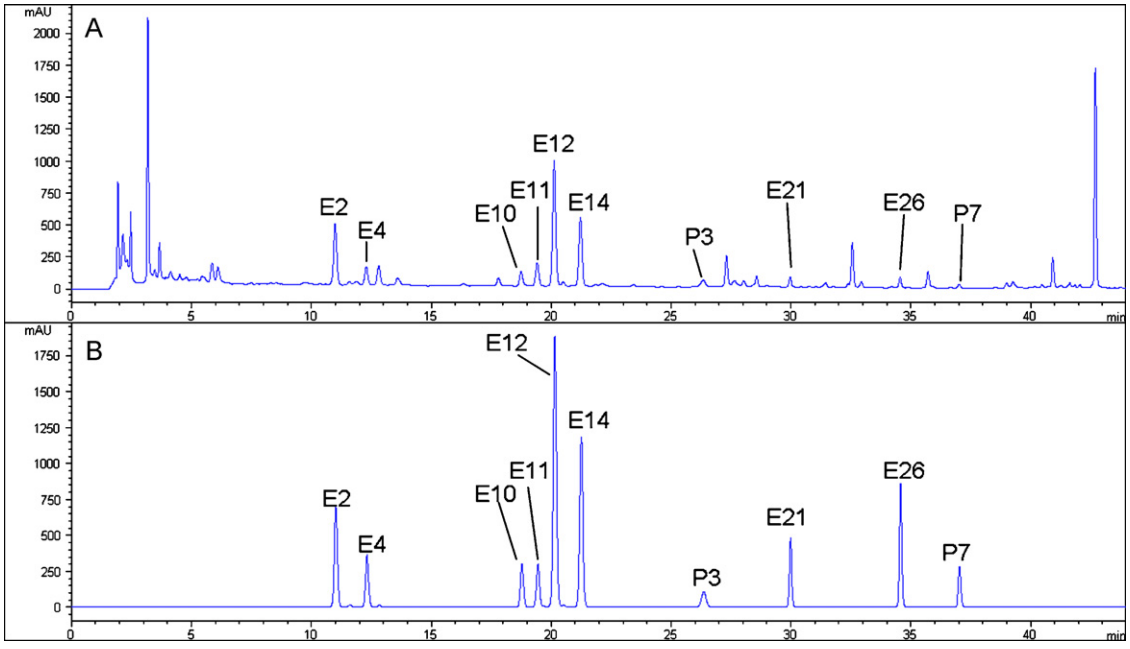

Fig. 6. HPLC chromatograms of XLGB (A) and mixed standards (B) for quantitative analysis.

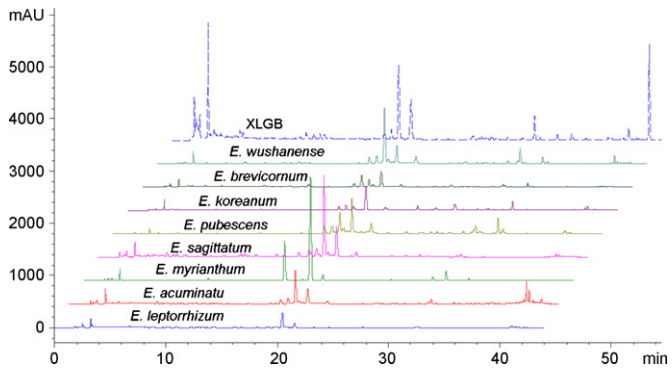

Fig. 7. HPLC chromatograms of XLGB and *Epimedium* species.

3.3.2. Limits of detection and quantification

The stock solution mentioned above was diluted to a series of appropriate concentrations with 70% methanol, and an aliquot of the diluted solutions were injected into HPLC for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of around 3 and 10, respectively. As shown in Table 2, LOD and LOQ of the analytes were no more than 6.5 μg/g and 16.0 μg/g, respectively.

3.3.3. Precision, repeatability and stability

Precision, repeatability and stability of the method were also validated for each analyte. The analysis was repeated using the same XLGB sample for 6 times in the same day and additionally on 3 consecutive days to determine intra- and inter-day precision, respectively. Six XLGB samples from the same batch were extracted and analyzed to measure the method repeatability. The same sample was stored at 25 °C, and analyzed at 0, 2, 4, 6, 12 and 24 h for stability test. Relative standard deviations (RSD) of intra- and inter-day precision, repeatability and stability were less than 1.0%, 1.5%, 2.9% and 1.8% respectively, indicating good precision, repeatability and sample stability of the method (Table 3).

3.3.4. Recovery

The recovery was performed by adding a known amount of each individual standard into a certain amount (0.25 g) of XLGB content. The mixture was extracted and analyzed following the procedure described under Section 2.2. Three replicates were performed for the test. As shown in Table 4, the developed analytical method was accurate with recoveries of 97.8–104.9% (RSD < 1.5%).

3.3.5. Determination of 10 compounds in XLGB

The HPLC method described under Section 2.5 was applied to the determination of 10 analytes in 18 batches of XLGB samples. As shown in Fig. 6, all the analytes were well separated. The concentrations were calculated by external standard method, and the results are summarized in Table 5. According to the national quality standard for XLGB [4], the content of icariin (E14) should be no less than 3.0 mg/g. Our results showed that all the 18 batches complied with this standard. However, we still discovered that the HPLC profiles of different batches varied significantly. This variation might be due to the quality of Epimedii Herba.

As Epimedii Herba has multiple botanical sources, the chemical composition of XLGB could also change when different *Epimedium* species are used. In all the 18 batches of XLGB we tested, the Epimedii Herba was most likely derived from *E. wushanense* because the HPLC fingerprints of XLGB showed high similarity with those of *E. wushanense* rather than the other species (Fig. 7). Coincidentally, XLGB were manufactured in Guizhou province, which is the major growing area of *E. wushanense* [24].

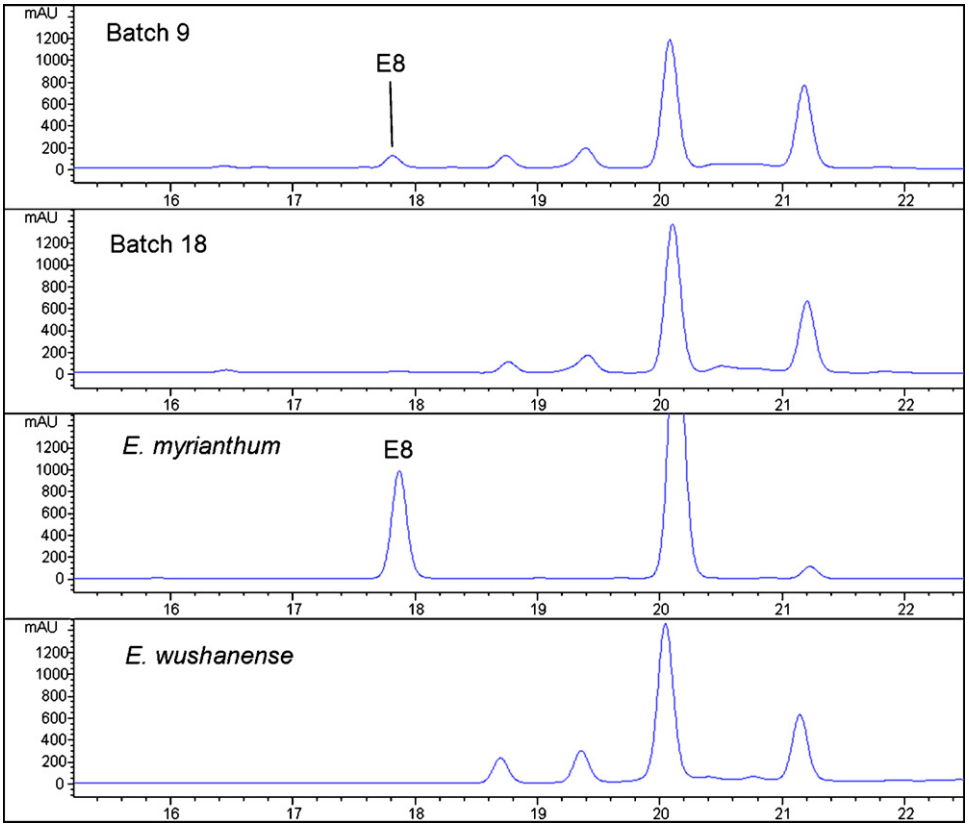

Fig. 8. HPLC chromatograms of XLGB (batch 9 and batch 18) and the leaves of *E. myrianthum* and *E. wushanense*.

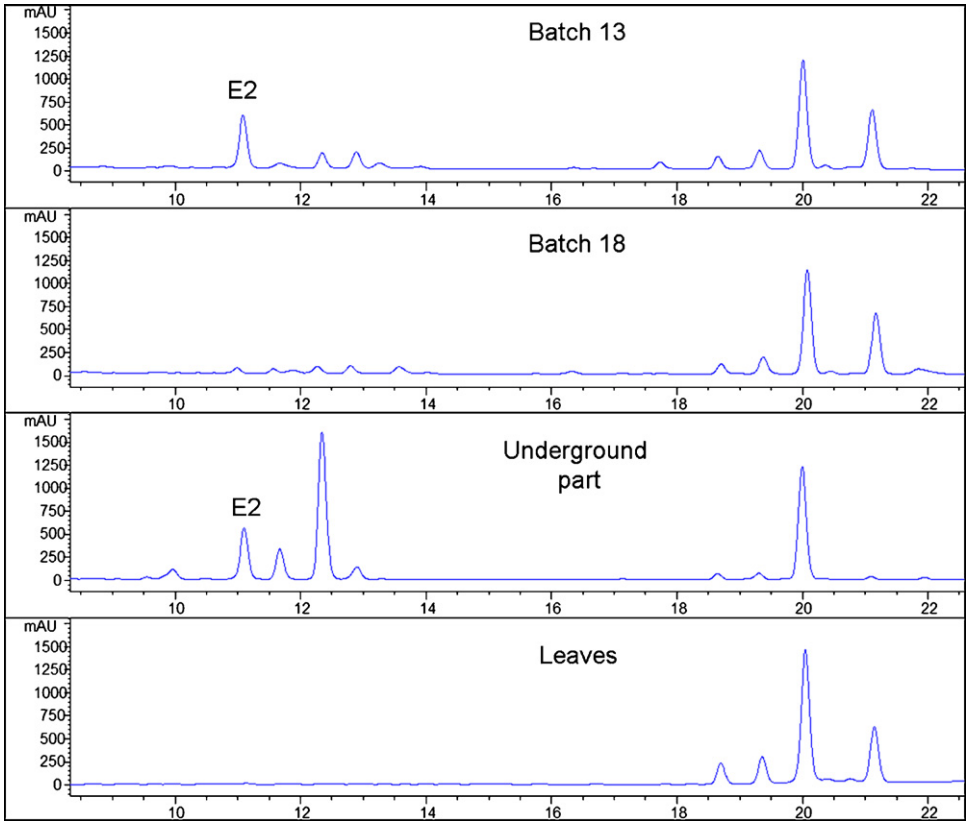

Fig. 9. HPLC chromatograms of XLGB (batch 13 and batch 18) and the underground parts and leaves of *E. wushanense*.

E. wushanense contains remarkably different constituents from other official *Epimedium* species, and it was listed as a new monograph as "Epimedii Wushanense Herba" in the recently published 2010 Edition of *Pharmacopoeia of People's Republic of China* [27]. Other *Epimedium* species, especially those indigenous to Guizhou province, could also be used for the manufacturing of XLGB. For instance, *E. myrianthum*, an unofficial species, might be used in batch 9 where hexandraside F (E8) was observed in moderate abundance (Fig. 8). E8 is a characteristic constituent of *E. myrianthum* [11,13].

As shown in Table 5, the contents of ten major compounds in XLGB among 18 batches were relatively consistent. The RSD values were basically lower than 25%. The only exception was epimedoside E (E2), for which the RSD was 110.04%. This variation may be caused by the adulterating of underground parts of *Epimedium* species. Although only dry leaves are officially used, the underground parts of *Epimedium* species are also used in local areas including Guizhou province [24]. E2 was present in the underground parts of *Epimedium* species in considerably large amount [13], and it was abundant in batches 12, 13, and 14 of XLGB (Fig. 9). A reasonable explanation is that the underground parts were used for the manufacturing of these batches.

4. Conclusion

This study provided a systematic approach for the quality control of XLGB for the first time, including qualitative and quantitative analyses. In HPLC-DAD–MS^n qualitative analysis, a total of 47 compounds were identified or tentatively characterized. These compounds included 27 flavonols, 4 coumarins, 3 flavonones, 1 chalcone, 3 isoflavones, 1 coumestrol, 3 triterpenoid saponins, 1 iridoid, 3 steroidal saponins, and 1 phenolic acid. In HPLC quantitative analysis, the contents of 10 major constituents of XLGB were determined simultaneously. The results were valuable for the quality control of XLGB.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.03.021.

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