

## Research Article

# Identification and Analysis of Chemical Constituents and Rat Serum Metabolites in Gushuling Using UPLC-Q-TOF/MS Coupled with Novel Informatics UNIFI Platform

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Gushuling (GSL), a well-known hospital preparation composed of traditional Chinese medicine (TCM), has been widely used in the clinical treatment of osteoporosis (OP) for decades due to its remarkable therapeutic effect. However, the chemical constituents of GSL are still unclear so far, which limits the in-depth study of its pharmacodynamic material basis and further restricts its clinical application. In this study, we developed a strategy for qualitative analysis of the chemical constituents of GSL *in vitro* and *in vivo*. Based on the results of ultra-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS) and the UNIFI informatics platform, the chemical constituents of GSL can be determined quickly and effectively. By comparing the retention time, accurate mass, and fragmentation spectrum of the compounds in GSL, a total of 93 compounds were identified or preliminarily identified, including flavonoids, terpenoids, phenylpropanoids, steroids, etc. Among them, nine compounds have been confirmed by standard substances, namely epimedin A, epimedin B, epimedin C, icariin, ecdysterone, calycosin, calycosin-7-glucoside, ononin, and ginsenoside Ro. Fragment patterns and characteristic ions of representative compounds with different chemical structure types were analyzed. At the same time, 20 prototype compounds and 42 metabolites were detected in rat serum. Oxidation, hydration, reduction, dehydration, glutathione S-conjugation, and acetylcysteine conjugation were the main transformation reactions of GSL in rat serum. In this research, the rapid method to characterize the *in vitro* and *in vivo* chemical constituents of GSL can not only be used for the standardization and quality control of GSL but also be helpful for further research on its pharmacodynamic material basis.

## 1. Introduction

Osteoporosis (OP) is a complex bone disease characterized by low bone density and impaired microstructure of bone tissue, leading to increased bone fragility and being prone to fractures [1]. At present, the treatment drugs for OP mainly include ingredients that inhibit bone resorption, such as bisphosphonates, calcitonin, and estrogen, and ingredients that promote bone formation, such as parathyroid hormone

[2]. However, taking bisphosphonates for a long time will increase the incidence of complications such as osteonecrosis of the jaw [3,4]. Long-term use of estrogen will also increase the incidence of breast cancer, endometrial cancer, and other diseases [5,6]. Nowadays, the importance of traditional Chinese medicine (TCM) and Chinese herbal compound prescription that have a therapeutic effect on OP were gradually been recognized because of their safety and effectiveness [7–9].

Gushuling (GSL) is a TCM preparation in the hospital of the First Affiliated Hospital of Anhui University of Chinese Medicine, consisting of *Herba Epimedii* (Yinyanghuo), *Radix Achyranthis Bidentatae* (Niuxi), *Radix Astragali* (Huangqi), and *Concha Ostreae* (Muli) as recorded in the Chinese Pharmacopoeia (Table S1). GSL has a good effect on the management of various types of OP in clinical treatment, such as senile osteoporosis [10,11], postmenopausal osteoporosis [12], and diabetes secondary osteoporosis [13]. However, the chemical constituents of TCM and related preparations are complex and the active ingredients are not fully understood [14,15]. Although the chemical constituents of GSL have been previously reported, only two or three bioactive ingredients have been found in GSL [16,17]. These efforts failed to reflect the overall chemical constituents of GSL, which made it difficult to comprehensively evaluate the quality of GSL. Therefore, there is an urgent need for a reliable and efficient analytical method to determine the chemical constituents of GSL and the effective ingredients that can enter the body to achieve the purpose of quality control. Fortunately, the combination of ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS) and the UNIFI analysis platform can solve this problem to some extent.

In recent years, UPLC-Q-TOF-MS has been widely used in various fields such as qualitative analysis of TCM ingredients, serum pharmacology, metabolomics, and new drug development due to its high resolution and high sensitivity [18–21]. As a powerful information processing platform, UNIFI can automatically extract the mass spectrometry data of the sample, determine the molecular formula, compare it with the compound in the database, which gives the possible cracking method of the compound according to the fragment information of the compound under high energy, and display the detailed information of the identified compound under the preset filter conditions [22,23]. The purpose of this study was to use the UPLC-Q-TOF-MS technology in conjunction with UNIFI analysis software to comprehensively analyze and identify the chemical constituents of hospital preparation GSL and to analyze the prototype compounds and metabolites in rat serum, which provides a basis for the in-depth research and quality control of its pharmacodynamic material basis.

## 2. Materials and Methods

**2.1. Materials and Reagents.** *Herba Epimedii*, *Radix Achyranthis Bidentatae*, *Radix Astragali*, and *Concha Ostreae* were purchased from Anhui Xiehecheng Co., Ltd. (Bozhou, China) and evaluated by Doctor Rongchun Han (College of Pharmacy, Anhui University of Chinese Medicine, Hefei, China). The standard substances of calycosin (Lot number: MUST-18120901), calycosin-7-glucoside (Lot number: MUST-19031920), ononin (Lot number: MUST-19041101), epimedin C (Lot number: MUST-19081310), icariin (Lot number: MUST-18091010), and ecdysterone (Lot number: MUST-18120209) were purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). Epimedin A (Lot

number: JOT-10354), epimedin B (Lot number: JOT-10353), and ginsenoside Ro (Lot number: JOT-10448) were purchased from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China). The purity of all standards was over 98.0%. Acetonitrile (HPLC grade; Lot number: SHBM1775) and methanol were purchased from Sigma Aldrich Trading Co., Ltd. (Shanghai, China). Formic acid (HPLC grade; Lot number: J2027129) was obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Ultrapure water was prepared by a Milli Q-Plus system (Millipore, Bedford, USA).

**2.2. Standards and Sample Preparation.** First, *Herba Epimedii* and *Radix Achyranthis Bidentatae* were mixed at the ratio of 5:4 and refluxed with 70% ethanol at 8-fold volume (11-fold volume for the first time) for 3 times for 2 h, respectively. We used a rotary evaporator to volatilize the ethanol in the extract to obtain a concentrated solution for use. Second, *Radix Astragali* and *Concha Ostreae* were mixed at the ratio of 5:2 and boiled with 9.5-fold and 8-fold volume ultrapure water for 1 h, respectively. Finally, the ethanol extract obtained in the first step was mixed with the water extract obtained in the second step and concentrated to obtain the GSL sample solution. *Herba Epimedii* refluxed with 70% ethanol at 8-fold volume (11-fold volume for the first time) for 3 times for 2 h, respectively. We used a rotary evaporator to volatilize the ethanol and water in the extract to obtain a sample solution of *Herba Epimedii*. The preparation method of *Radix Achyranthis Bidentatae* sample solution is the same as that of *Herba Epimedii* sample solution. *Radix Astragali* boiled with 9.5-fold and 8-fold volume ultrapure water for 1 h, respectively, and concentrated to obtain the *Radix Astragali* sample solution. An appropriate amount of sample solution was diluted with methanol, and the supernatant was taken and stored at 4°C. All the solutions were filtered through a 0.22 μm filter membrane before analysis.

Nine standard substances were completely dissolved in methanol. Before qualitative analysis, they were mixed to prepare a mixed standard solution with appropriate concentration and passed through a 0.22 μm filter membrane. All solutions were stored in a refrigerator at 4°C.

**2.3. Animals Handling and Serum Samples Preparation.** Sprague Dawley rats (200 ± 20 g) of specific pathogen-free grade were purchased from Animal Experiment Center, Bengbu Medical College. Approved by the Experimental Animal Management and Ethics Committee of Bengbu Medical College, animal experimental research meets relevant ethical requirements. They were randomly divided into blank groups and GSL extract groups, with 6 rats in each group. The rats were kept in an animal room with a suitable environment for 7 days before the experiment. Rats in the blank group and GSL extract group were intragastrically administered distilled water and 51.993 g·kg<sup>-1</sup>·d<sup>-1</sup> GSL sample solution for consecutive 3 days, respectively. The preparation process of the GSL sample solution is the same as that in the Standards and Sample Preparation section, and

there is no need for subsequent operations such as methanol treatment. Before the last oral administration, the rats fasted for 12 h with free drinking water. Blood samples (500  $\mu\text{L}$ ) were collected from the fundus venous plexus 15 min after oral administration on the 3rd day and centrifuged for 10 min at 3500 rpm·min<sup>-1</sup> at 4°C, and the supernatant was taken. Then, 1200  $\mu\text{L}$  methanol was added to the 300  $\mu\text{L}$  serum samples, vortexed, and centrifuged at 13000 rpm for 10 min. The supernatant was put into another tube and dried with nitrogen gas. The remaining was stored in acetonitrile (200  $\mu\text{L}$ ) and frozen at -80°C until analysis.

#### 2.4. Chromatography and Mass Spectrometry Conditions.

Chromatographic analysis was performed using a Waters Acquity™ UPLC system (Waters Corporation, Milford, USA). Chromatographic separation was carried on Waters ACQUITY UPLC® BEH C18 (2.1 × 100 mm, 1.7  $\mu\text{m}$ ) column by gradient elution with the optimal mobile phase of 0.1% formic acid aqueous solution (solvent A) and acetonitrile (solvent B), the column temperature was maintained at 35°C, and the temperature of the sample chamber was set to 8°C. The gradient elution was set as follows: 0–5 min, 5%–19% B; 5–10 min, 19% B; 10–11 min, 19%–25% B; 11–16 min, 25% B; 16–17 min, 25%–31% B; 17–22 min, 31%–51% B; 22–50 min, 51%–100% B; 50–55 min, 100% B; 55–60 min, 100%–5% B; 60–65 min, 5% B. The flow rate was 0.15 mL·min<sup>-1</sup>, and the injection volume was 2  $\mu\text{L}$ .

A Waters Xevo G2 Q-TOF mass spectrometer (Waters Corporation, Milford, USA) equipped with an electrospray ionization (ESI) source operating in both positive and negative ion modes was connected to the UPLC. The full scan data were collected from m/z 50 to m/z 1200. For positive and negative ion modes, the capillary and cone voltage were set to 3.0 kV, 40 V and 2.5 kV, 40 V, respectively. The temperature of the conservation gas was set to 350°C, and the flow rate was set to 600 L·h<sup>-1</sup>. The two ion source temperatures in positive ion and negative ion modes were set to 120°C and 110°C, respectively. The cone gas flow rate was set to 50 L·h<sup>-1</sup>, and leucine and enkephalin were used as calibration fluids to ensure accuracy and repeatability.

2.5. UNIFI Data Processing Method. The chemical constituents analysis strategy was mainly divided into the following three steps:

- (1) The establishment of the chemical constituents library of GSL: The complete information on the compounds of three herbal medicines (*Herba Epimedii*, *Radix Achyranthis Bidentatae*, and *Radix Astragali*) in GSL was collected and obtained by searching China National Knowledge Infrastructure (CNKI), PubMed, PubChem, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP), ChemSpider, and other databases. The self-built compound library was established, including compound name, molecular formula, chemical structure (saved in “mol” format),

accurate molecular mass, and other information. This information was imported into UNIFI. Among them, a total of 354 compounds were listed. When analyzing rat serum samples, the self-built database imported into UNIFI was the GSL *in vitro* compound information database that has been analyzed and confirmed.

- (2) Preliminary analysis of the results: We imported the original files of GSL sample solution, blank sample solution, rat administration serum, and blank serum analyzed by UPLC-Q-TOF-MS into the UNIFI software for samples comparison. Based on the automatic matching function of UNIFI software, compounds can be quickly identified. The parameter settings were as follows: analysis time range, 1–65 min; quality allowable error range,  $\pm 10$  ppm; quality testing range, 50 Da to 1500 Da; positive adducts including H<sup>+</sup>, Na<sup>+</sup>, and K<sup>+</sup>; and negative adducts containing H<sup>-</sup>, HCOO<sup>-</sup>, and Cl<sup>-</sup>.
- (3) Manual review: By the MassLynx workstation, the above identification results were reviewed in combination with the precise mass of excimer ions, retention time, fragment ion information, and literature.

### 3. Results and Discussion

3.1. Chemical Constituents Analysis of Three Herbal Medicines in GSL. The separate extraction method of the three herbal medicines in GSL (*Herba Epimedii*, *Radix Achyranthis Bidentatae*, and *Radix Astragali*) was the same as the sample preparation process. Then, the TIC of the three herbal medicines in positive and negative ion modes were qualitatively analyzed according to the above-mentioned method, and the results are shown in Figure S1.

3.2. Identification and Analysis of Chemical Constituents in GSL. High-resolution mass spectrometry data of GSL were rapidly acquired by the UPLC-Q-TOF-MS method. The TIC of GSL in positive and negative ion modes is portrayed in Figure 1. The UNIFI screening platform was used to process and analyze its mass spectrometry data, and then the data were spontaneously matched with the fragment information. After further manual verification, it was found that there were 93 *in vitro* chemical constituents in the GSL recipe, including 51 kinds of flavonoids, 18 kinds of terpenoids, 6 kinds of phenylpropanoids, 5 kinds of steroids, and 13 others. Among them, 55 compounds were from *Herba Epimedii*, 23 compounds from *Radix Achyranthis Bidentatae*, and 21 compounds from *Radix Astragali*. In addition, *Herba Epimedii* and *Radix Achyranthis Bidentatae* contain four common compounds, and *Herba Epimedii*, *Radix Achyranthis Bidentatae*, and *Radix Astragali* contain one identical compound (rutin). Of the identified components, 9 compounds were identified by comparison with the standard substance. The detailed information on these components is gathered in Table 1 and Figure 2.

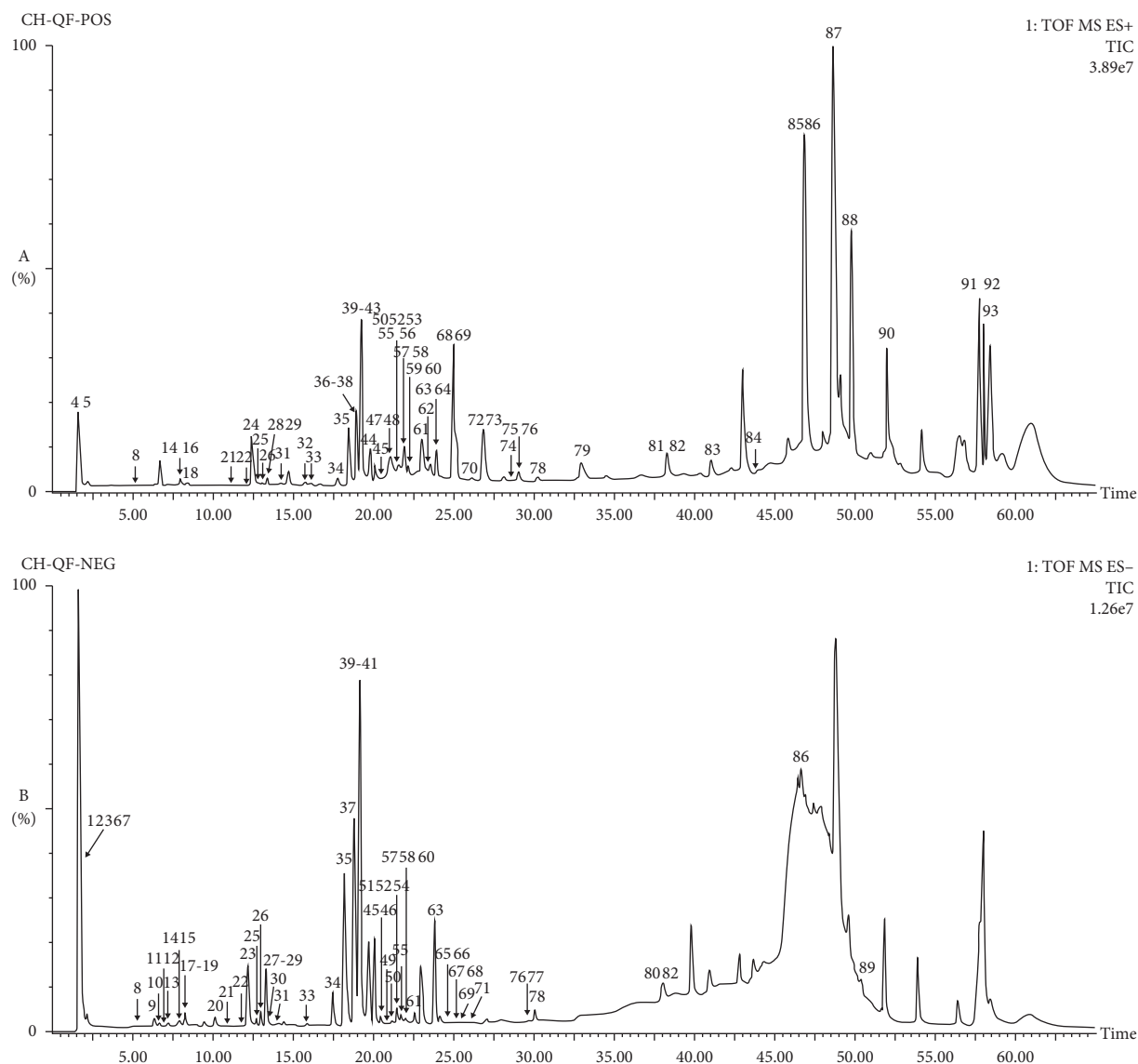


FIGURE 1: The total ion chromatography (TIC) of GSL from UPLC-Q-TOF-MS analysis. (a) Positive ion mode. (b) Negative ion mode.

TABLE 1: Identification of chemical constituents of GSL by UPLC-Q-TOF-MS.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
1	1.63	C <sub>6</sub> H <sub>12</sub> O <sub>5</sub>	209.0670	1.3	[M+HCOO] <sup>-</sup>	89.0255	RAM	Saccharide	HQ
2	1.64	C <sub>5</sub> H <sub>10</sub> O <sub>5</sub>	195.0510	-0.2	[M+HCOO] <sup>-</sup>	89.0255	XLS	Saccharide	HQ
3	1.66	C <sub>23</sub> H <sub>24</sub> O <sub>11</sub>	475.1287	8.6	[M-H] <sup>-</sup>	89.0255, 179.0563, 290.0864, 341.1084	7,2'-Dihydroxy-3',4'-dimethoxyisoflavone-7-O-β-D-glucoside	Flavonoids	HQ
4	1.67	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	203.0522	-2.2	[M+Na] <sup>+</sup> , [M+K] <sup>+</sup>	127.0388	Inositol	Others	YYH
5	1.68	C <sub>10</sub> H <sub>14</sub> O <sub>3</sub>	205.0836	0.4	[M+Na] <sup>+</sup>	127.0388, 89.0255, 179.0563,	3,4,5-Trimethoxytoluene	Others	YYH
6	1.69	C <sub>27</sub> H <sub>32</sub> O <sub>12</sub>	593.1879	0.6	[M+HCOO] <sup>-</sup>	290.0864, 341.1084, 377.0850	Maohuoside A	Flavonoids	YYH
7	1.76	C <sub>21</sub> H <sub>22</sub> O <sub>7</sub>	431.1381	7.8	[M+HCOO] <sup>-</sup>	128.0362	Wanepimidoside_qt	Flavonoids	YYH

TABLE 1: Continued.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
8	5.25	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	353.0867	-3.1	[M-H] <sup>-</sup>	191.0556	Chlorogenic acid	Phenylpropanoids (phenylpropionic acid)	YYH
		C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	355.1041	4.9	[M+H] <sup>+</sup> , [M+Na] <sup>+</sup>	163.0387	Chlorogenic acid	Phenylpropanoids (phenylpropionic acid)	YYH
9	6.44	C <sub>19</sub> H <sub>30</sub> O <sub>8</sub>	431.189	-6.6	[M+HCOO] <sup>-</sup>	163.0409, 173.0463, 219.0629	Icariside B1	Terpenoids (monocyclic monoterpenoids)	YYH
10	6.49	C <sub>19</sub> H <sub>28</sub> O <sub>10</sub>	461.1704	8.6	[M+HCOO] <sup>-</sup>	219.0629	Icariside D1	Terpenoids (monocyclic monoterpenoids)	YYH
11	6.87	C <sub>26</sub> H <sub>36</sub> O <sub>12</sub>	539.2149	2.7	[M-H] <sup>-</sup>	317.0238, 479.0789, 491.1857	Icariside E1	Phenylpropanoids (phenylpropanols)	YYH
12	6.92	C <sub>21</sub> H <sub>20</sub> O <sub>13</sub>	479.0807	-5.1	[M-H] <sup>-</sup>	316.0208	Isomyricitrin	Flavonoids	YYH
13	7.22	C <sub>32</sub> H <sub>38</sub> O <sub>15</sub>	697.1844	-8.8	[M+Cl] <sup>-</sup>	191.0563, 219.0647, 593.1515	Ikariside B	Flavonoids	YYH
14	7.86	C <sub>22</sub> H <sub>22</sub> O <sub>10</sub>	491.1183	-2.5	[M+HCOO] <sup>-</sup>	283.0601	Calycosin-7-glucoside*	Flavonoids	HQ
		C <sub>22</sub> H <sub>22</sub> O <sub>10</sub>	447.1279	-1.4	[M+H] <sup>+</sup>	285.0749	Calycosin-7-glucoside*	Flavonoids	HQ
15	7.92	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>	463.0849	-7.1	[M-H] <sup>-</sup>	285.0396, 431.0962	Hyperoside	Flavonoids	YYH NX
16	8.00	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	303.0520	6.8	[M+H] <sup>+</sup>	287.0543	Robinetin	Flavonoids	YYH
17	8.17	C <sub>28</sub> H <sub>34</sub> O <sub>12</sub>	597.1798	9.1	[M+Cl] <sup>-</sup>	193.0869, 219.0659, 237.0750, 399.1276	Caohuoside D	Flavonoids	YYH
18	8.39	C <sub>27</sub> H <sub>44</sub> O <sub>7</sub>	525.3065	-0.8	[M+HCOO] <sup>-</sup> , [M+Cl] <sup>-</sup>	159.1057, 193.0869, 219.0659	Ecdysterone*	Steroids	NX
		C <sub>27</sub> H <sub>44</sub> O <sub>7</sub>	481.3134	-5.5	[M+K] <sup>+</sup> , [M+Na] <sup>+</sup>	371.2244, 445.2923	Ecdysterone*	Steroids	NX
19	8.42	C <sub>22</sub> H <sub>32</sub> O <sub>11</sub>	517.1889	-7.2	[M+HCOO] <sup>-</sup> , [M-H] <sup>-</sup> , [M+Cl] <sup>-</sup>	177.0907, 471.1833	Eugenol rutinoside	Phenylpropanoids (Styrene)	YYH
20	10.13	C <sub>29</sub> H <sub>38</sub> O <sub>15</sub>	625.2131	-1.1	[M-H] <sup>-</sup>	261.0762, 279.0868, 291.0852	Isomucronulatol-7,2'-di-O-glucosiole	Flavonoids	HQ
21	11.14	C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>	283.0599	-4.7	[M-H] <sup>-</sup>	268.0381	Wogonin	Flavonoids	NX
		C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>	285.0769	4.0	[M+H] <sup>+</sup>	270.0515	Wogonin	Flavonoids	NX
22	12.07	C <sub>32</sub> H <sub>38</sub> O <sub>16</sub>	677.2074	-1.9	[M-H] <sup>-</sup>	353.0993, 514.1457, 515.1522, 557.1635	Hexandraside E	Flavonoids	YYH
		C <sub>32</sub> H <sub>38</sub> O <sub>16</sub>	679.2231	-0.3	[M+H] <sup>+</sup> , [M+Na] <sup>+</sup>	299.0524, 355.1186, 517.1787	Hexandraside E	Flavonoids	YYH
23	12.18	C <sub>43</sub> H <sub>68</sub> O <sub>14</sub>	853.4619	3.3	[M+HCOO] <sup>-</sup> , [M-H] <sup>-</sup>	96.9603, 451.3268	1-O-((3β,5ξ,9ξ)-3-[(6-Methyl-β-D-glucopyranuronosyl)oxy]-28-oxoolean-12-en-28-yl)-β-D-glucopyranose	Triterpenoids	NX
24	12.41	C <sub>29</sub> H <sub>50</sub> O	453.3471	-4.8	[M+K] <sup>+</sup>	343.3055	Sitosterol	Steroids	YYH NX



TABLE 1: Continued.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
25	12.81	$C_{38}H_{48}O_{20}$	825.2805	-0.8	$[M+H]^+$	299.0541, 355.1165, 517.1669, 663.2131 146.9657,	Diphyllaside A	Flavonoids	YYH
		$C_{38}H_{48}O_{20}$	869.2693	-3.2	$[M+HCOO]^-$ , $[M-H]^-$ , $[M+Cl]^-$	353.1024, 514.1468, 661.2136, 807.2701	Diphyllaside A	Flavonoids	YYH
26	13.06	$C_{37}H_{46}O_{19}$	793.2564	0.4	$[M-H]^-$ , $[M+Cl]^-$ , $[M+HCOO]^-$	285.0389, 431.0974, 631.2028	Epimedeside D	Flavonoids	YYH
		$C_{37}H_{46}O_{19}$	795.2681	-3.2	$[M+H]^+$	287.0541, 299.0538, 355.1168, 517.1700 146.9657,	Epimedeside D	Flavonoids	YYH
27	13.29	$C_{37}H_{44}O_{17}$	805.2492	-8.5	$[M+HCOO]^-$	353.1024, 514.1468, 661.2136 146.9657,	Epimedeside	Flavonoids	YYH
28	13.32	$C_{38}H_{48}O_{19}$	807.2703	-1.8	$[M-H]^-$	353.1024, 514.1468, 661.2136 121.0269,	Diphyllaside B	Flavonoids	YYH
		$C_{38}H_{48}O_{19}$	831.2681	-0.2	$[M+Na]^+$	299.0541, 355.1166, 547.1760 146.9657,	Diphyllaside B	Flavonoids	YYH
29	13.39	$C_{32}H_{38}O_{15}$	707.2195	0.3	$[M+HCOO]^-$ , $[M-H]^-$ , $[M+Cl]^-$	146.9657, 353.1024, 514.1468 121.0269,	Epimedeside A	Flavonoids	YYH
		$C_{32}H_{38}O_{15}$	663.2275	-1.2	$[M+H]^+$ , $[M+Na]^+$	299.0541, 355.1166, 547.1760	Epimedeside A	Flavonoids	YYH
30	13.41	$C_{26}H_{30}O_{11}$	517.1680	-6.9	$[M-H]^-$	219.0671, 327.1270, 383.1110	(2S,3S)-3,5-dihydroxy-2-(4-hydroxyphenyl)-8-(3-methyl-2-buten-1-yl)-4-oxo-3,4-dihydro-2H-chromen-7-yl $\beta$ -D-glucopyranoside	Flavonoids	YYH
31	14.13	$C_{22}H_{22}O_9$	475.1212	-7.0	$[M+HCOO]^-$	267.0642, 353.0994	Ononin*	Flavonoids	HQ
		$C_{22}H_{22}O_9$	431.1325	-2.8	$[M+H]^+$	269.0797	Ononin*	Flavonoids	HQ
32	15.81	$C_{12}H_{22}O_6$	301.1069	6.9	$[M+K]^+$	167.0713	3-Hexenyl- $\beta$ -glucopyranoside	Saccharide	YYH
33	16.05	$C_{16}H_{12}O_5$	283.0604	-2.9	$[M-H]^-$	148.0159, 268.0364	Calycosin*	Flavonoids	HQ
		$C_{16}H_{12}O_5$	285.0751	-2.3	$[M+K]^+$ , $[M+Na]^+$	270.0508	Calycosin*	Flavonoids	HQ
34	17.76	$C_{39}H_{50}O_{19}$	883.2882	0.6	$[M+HCOO]^-$ , $[M-H]^-$ , $[M+Cl]^-$	146.9654, 251.0307, 529.1697, 675.2292 313.0700,	Epimedin A*	Flavonoids	YYH
		$C_{39}H_{50}O_{19}$	839.2961	-0.9	$[M+K]^+$ , $[M+Na]^+$	369.1326, 531.1852	Epimedin A*	Flavonoids	YYH

TABLE 1: Continued.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
35	18.46	C <sub>38</sub> H <sub>48</sub> O <sub>18</sub>	853.2774	0.2	[M+HCOO] <sup>-</sup> , [M-H] <sup>-</sup> , [M+Cl] <sup>-</sup>	146.9656, 366.1101, 551.1510, 645.2186 313.0698,	Epimedin B*	Flavonoids	YYH
		C <sub>38</sub> H <sub>48</sub> O <sub>18</sub>	809.2855	-0.9	[M+H] <sup>+</sup> , [M+K] <sup>+</sup>	369.1322, 531.1849, 677.2419	Epimedin B*	Flavonoids	YYH
36	18.88	C <sub>21</sub> H <sub>20</sub> O <sub>6</sub>	369.1322	-2.9	[M+H] <sup>+</sup>	243.0640, 313.0699	Icaritin	Flavonoids	YYH
37	18.93	C <sub>39</sub> H <sub>50</sub> O <sub>19</sub>	867.2924	-0.5	[M+HCOO] <sup>-</sup> , [M-H] <sup>-</sup> , [M+Cl] <sup>-</sup>	146.9660, 366.1099, 551.1497, 659.2339 129.0545, 313.0699,	Epimedin C*	Flavonoids	YYH
		C <sub>39</sub> H <sub>50</sub> O <sub>19</sub>	823.3014	-0.6	[M+H] <sup>+</sup> , [M+K] <sup>+</sup>	369.1322, 515.1931, 531.1852	Epimedin C*	Flavonoids	YYH
38	18.99	C <sub>22</sub> H <sub>24</sub> O <sub>7</sub>	439.1143	-2.5	[M+K] <sup>+</sup> , [M+Na] <sup>+</sup>	239.0905, 301.0677	3,5,7-Trihydroxy-8-(3-methoxy-3-methylbutyl)-2-(4-methoxyphenyl)-4H-chromen-4-one	Flavonoids	YYH
39	19.17	C <sub>41</sub> H <sub>52</sub> O <sub>21</sub>	881.3114	4.6	[M+H] <sup>+</sup>	85.0294, 135.0437, 243.0645, 313.0698, 369.1321 267.0300, 367.0621,	Hexandraside B	Flavonoids	YYH
		C <sub>41</sub> H <sub>52</sub> O <sub>21</sub>	879.2921	-0.8	[M-H] <sup>-</sup>	451.0672, 513.1100, 613.1210 175.0025,	Hexandraside B	Flavonoids	YYH
40	19.21	C <sub>27</sub> H <sub>30</sub> O <sub>11</sub>	575.1754	-2.9	[M+HCOO] <sup>-</sup> , [M-H] <sup>-</sup>	351.0861, 367.1169, 513.1748 85.0294, 135.0437,	Icariside I	Flavonoids	YYH
		C <sub>27</sub> H <sub>30</sub> O <sub>11</sub>	531.1850	-2.1	[M+H] <sup>+</sup> , [M+Na] <sup>+</sup>	243.0645, 313.0698, 369.1321 175.0025, 281.0437,	Icariside I	Flavonoids	YYH
41	19.22	C <sub>33</sub> H <sub>40</sub> O <sub>15</sub>	721.2337	-1.7	[M+HCOO] <sup>-</sup> , [M+Cl] <sup>-</sup>	367.1169, 451.0672, 513.1748 135.0437,	Icariin*	Flavonoids	YYH
		C <sub>33</sub> H <sub>40</sub> O <sub>15</sub>	677.2433	-1.1	[M+H] <sup>+</sup> , [M+K] <sup>+</sup>	313.0698, 369.1321, 531.1851	Icariin*	Flavonoids	YYH
42	19.23	C <sub>20</sub> H <sub>24</sub> O <sub>6</sub>	399.1215	2.7	[M+K] <sup>+</sup>	135.0437	Lariciresinol	Phenylpropanoids (lignans)	HQ

TABLE 1: Continued.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
43	19.26	C <sub>28</sub> H <sub>24</sub> O <sub>13</sub>	569.1236	-9.4	[M+H] <sup>+</sup>	85.0294, 135.0437, 313.0698, 369.1321	8-Hydroxy-6-methyl-9,10-dioxo-9,10-dihydro-1-anthracenyl 6-O-(3,4,5-trihydroxybenzoyl)-β-D-glucopyranoside	Anthraquinones	NX
44	19.63	C <sub>25</sub> H <sub>30</sub> O <sub>8</sub>	481.1785	-9.9	[M+Na] <sup>+</sup>	387.1427	Rubschisantherin	Phenylpropanoids (lignans)	NX
45	20.82	C <sub>36</sub> H <sub>42</sub> O <sub>17</sub>	747.2483	-1.5	[M+H] <sup>+</sup>	299.0532, 355.1114 121.0304, 352.0934,	Korepimedeside A	Flavonoids	YYH
		C <sub>36</sub> H <sub>42</sub> O <sub>17</sub>	745.2332	-2.3	[M-H] <sup>-</sup>	367.1168, 499.1620, 583.1812 367.1156,	Korepimedeside A	Flavonoids	YYH
46	20.94	C <sub>41</sub> H <sub>52</sub> O <sub>21</sub>	879.2892	-4.1	[M-H] <sup>-</sup>	381.0964, 571.1715, 673.2076 369.1320,	Korepimedeside C	Flavonoids	YYH
47	21.01	C <sub>56</sub> H <sub>88</sub> O <sub>25</sub>	1199.5264	1.5	[M+K] <sup>+</sup>	383.1110, 882.4549, 1059.5290 295.0576,	Achyranthoside D trimethyl ester	Triterpenoids	NX
48	21.08	C <sub>52</sub> H <sub>84</sub> O <sub>19</sub>	1013.5723	4.3	[M+H] <sup>+</sup>	311.0891, 385.1281, 882.4549 312.0607, 383.1097,	Chikusetsusaponin V butyl ester	Triterpenoids	NX
49	21.09	C <sub>53</sub> H <sub>82</sub> O <sub>25</sub>	1117.5026	-4.1	[M-H] <sup>-</sup>	529.1652, 631.2021, 997.4956 295.0576,	Achyranthoside D	Triterpenoids	NX
50	21.11	C <sub>47</sub> H <sub>74</sub> O <sub>18</sub>	949.4763	-0.5	[M+Na] <sup>+</sup>	311.0891, 882.4549 139.1120, 171.1024,	Chikusetsusaponin IV	Triterpenoids	NX
		C <sub>47</sub> H <sub>74</sub> O <sub>18</sub>	925.4757	-4.9	[M-H] <sup>-</sup>	211.1335, 229.1434, 367.1184 352.0934,	Chikusetsusaponin IV	Triterpenoids	NX
51	21.19	C <sub>48</sub> H <sub>76</sub> O <sub>19</sub>	955.4884	-2.5	[M-H] <sup>-</sup> , [M+HCOO] <sup>+</sup>	367.1168, 631.2021, 793.4236 121.0304, 352.0934,	Ginsenoside Ro*	Triterpenoids	NX
52	21.24	C <sub>31</sub> H <sub>36</sub> O <sub>14</sub>	631.2021	-1.8	[M-H] <sup>-</sup>	367.1168, 583.1812 299.0526,	Ikariside F	Flavonoids	YYH
		C <sub>31</sub> H <sub>36</sub> O <sub>14</sub>	633.2146	-5.0	[M+H] <sup>+</sup>	369.1327, 385.1281 299.0526,	Ikariside F	Flavonoids	YYH
53	21.28	C <sub>45</sub> H <sub>60</sub> O <sub>24</sub>	1023.3186	7.8	[M+K] <sup>+</sup>	369.1327, 531.1847, 915.3315	Acuminatoside	Flavonoids	YYH



TABLE 1: Continued.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
54	21.31	C <sub>27</sub> H <sub>30</sub> O <sub>11</sub>	529.1669	-8.8	[M-H] <sup>-</sup>	121.0304, 219.0656, 383.1102, 513.1729 367.1184,	Wushanicariin	Flavonoids	YYH
55	21.56	C <sub>35</sub> H <sub>42</sub> O <sub>16</sub>	763.2415	-5.2	[M+HCOO] <sup>-</sup>	381.0975, 555.1833, 645.2117 313.0706, 369.1324,	Sagittatoside C	Flavonoids	YYH
		C <sub>35</sub> H <sub>42</sub> O <sub>16</sub>	719.2591	6.3	[M+H] <sup>+</sup>	383.1134, 385.1288, 531.1848	Sagittatoside C	Flavonoids	YYH
56	21.61	C <sub>46</sub> H <sub>74</sub> O <sub>14</sub>	873.4927	-5.0	[M+Na] <sup>+</sup>	729.3908	Taibaienoside IV	Triterpenoids	NX
57	21.78	C <sub>20</sub> H <sub>18</sub> O <sub>6</sub>	353.1015	-4.3	[M-H] <sup>-</sup>	252.0419 253.0480,	8-Prenylkaempferol	Flavonoids	YYH
		C <sub>20</sub> H <sub>18</sub> O <sub>6</sub>	355.1173	-0.9	[M+H] <sup>+</sup>	269.0803, 299.0550	8-Prenylkaempferol	Flavonoids	YYH
58	21.79	C <sub>16</sub> H <sub>12</sub> O <sub>4</sub>	267.0656	-2.4	[M-H] <sup>-</sup>	252.0419	Formononetin	Flavonoids	HQ
		C <sub>16</sub> H <sub>12</sub> O <sub>4</sub>	269.0803	-2.0	[M+H] <sup>+</sup> , [M+Na] <sup>+</sup>	118.0413, 253.0480	Formononetin	Flavonoids	HQ
59	22.23	C <sub>17</sub> H <sub>16</sub> O <sub>5</sub>	301.1080	3.3	[M+H] <sup>+</sup>	167.0694	(6aR,11aR)-9,10-dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol	Flavonoids	HQ
60	22.33	C <sub>47</sub> H <sub>72</sub> O <sub>20</sub>	955.4528	-1.7	[M-H] <sup>-</sup>	382.1079, 645.2166, 835.4451, 925.4768 272.2571,	Achyranthoside C	Triterpenoids	NX
		C <sub>47</sub> H <sub>72</sub> O <sub>20</sub>	995.4167	-8.2	[M+K] <sup>+</sup>	299.0537, 383.1132	Achyranthoside C	Triterpenoids	NX
61	23.03	C <sub>32</sub> H <sub>38</sub> O <sub>14</sub>	645.2190	0.2	[M-H] <sup>-</sup> , [M+Cl] <sup>-</sup> [M+HCOO] <sup>-</sup>	223.0282, 366.1100	Sagittatoside B	Flavonoids	YYH
		C <sub>32</sub> H <sub>38</sub> O <sub>14</sub>	647.2329	-0.7	[M+H] <sup>+</sup> , [M+Na] <sup>+</sup>	191.0005, 207.0315, 313.0700, 369.1321	Sagittatoside B	Flavonoids	YYH
62	23.54	C <sub>17</sub> H <sub>20</sub> O <sub>6</sub>	343.1163	3.2	[M+Na] <sup>+</sup>	131.0491	1,2-Bis(4-hydroxy-3-methoxyphenyl)-1,3-propanediol	Phenylpropanoids (phenylpropionic acid)	YYH
63	23.89	C <sub>27</sub> H <sub>30</sub> O <sub>10</sub>	513.1762	-0.7	[M-H] <sup>-</sup>	146.9659, 217.0478, 351.0867, 366.1100 135.0434,	Icariside II	Flavonoids	YYH
		C <sub>27</sub> H <sub>30</sub> O <sub>10</sub>	515.1900	-2.2	[M+H] <sup>+</sup> , [M+K] <sup>+</sup> , [M+Na] <sup>+</sup>	243.0638, 313.0698, 369.1322 135.0434,	Icariside II	Flavonoids	YYH
64	23.90	C <sub>21</sub> H <sub>20</sub> O <sub>6</sub>	369.1321	-3.2	[M+H] <sup>+</sup>	243.0638, 313.0698	Anhydroicaritin	Flavonoids	YYH
65	24.53	C <sub>42</sub> H <sub>66</sub> O <sub>14</sub>	793.4358	-2.7	[M-H] <sup>-</sup> , [M+HCOO] <sup>-</sup>	209.0450, 367.1171	Zingibroside R1	Triterpenoids	NX

TABLE 1: Continued.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
66	24.58	C <sub>19</sub> H <sub>30</sub> O <sub>9</sub>	437.1603	4.3	[M+Cl] <sup>-</sup>	209.0450	Icariside B3	Terpenoids (Monocyclic monoterpenoids)	YYH
67	25.14	C <sub>41</sub> H <sub>64</sub> O <sub>12</sub>	793.4391	1.5	[M+HCOO] <sup>-</sup>	469.3326, 487.3421	Acetylastragaloside I <sub>qt</sub>	Triterpenoids	HQ
68	25.55	C <sub>20</sub> H <sub>18</sub> O <sub>5</sub>	337.1068	-4.0	[M-H] <sup>-</sup>	282.0493	Yinyanghuo D	Flavonoids	YYH
69	25.84	C <sub>20</sub> H <sub>18</sub> O <sub>5</sub>	339.1227	0.0	[M+H] <sup>+</sup>	283.0583	Yinyanghuo D	Flavonoids	YYH
		C <sub>25</sub> H <sub>26</sub> O <sub>6</sub>	421.1666	2.2	[M-H] <sup>-</sup>	351.1189	Yinyanghuo B	Flavonoids	YYH
70	26.15	C <sub>18</sub> H <sub>32</sub> O <sub>3</sub>	423.1829	6.5	[M+H] <sup>+</sup>	349.1055	Yinyanghuo B	Flavonoids	YYH
			297.2423	-0.3	[M+H] <sup>+</sup>	119.0855	13-Hydroxy-9,11-octadecadienoic acid	Organic acids	HQ
71	26.23	C <sub>41</sub> H <sub>62</sub> O <sub>15</sub>	793.4010	-0.8	[M-H] <sup>-</sup>	631.3795, 673.3972	Achyranthoside C <sub>qt</sub>	Triterpenoids	NX
72	26.71	C <sub>45</sub> H <sub>56</sub> O <sub>23</sub>	1003.2895	5.1	[M+K] <sup>+</sup>	313.0709	Korepimodoside B	Flavonoids	YYH
73	26.94	C <sub>27</sub> H <sub>44</sub> O <sub>7</sub>	481.3162	0.4	[M+H] <sup>+</sup>	251.1605	Inokosterone	Steroids	NX
74	28.62	C <sub>16</sub> H <sub>30</sub> O <sub>2</sub>	277.2149	4.1	[M+Na] <sup>+</sup>	223.1695	2,15-Hexadecanedione	Others	YYH
75	29.06	C <sub>25</sub> H <sub>24</sub> O <sub>6</sub>	421.1631	-3.6	[M+H] <sup>+</sup>	367.1135	Yinyanghuo A	Flavonoids	YYH
76	29.63	C <sub>21</sub> H <sub>20</sub> O <sub>5</sub>	351.1228	-2.9	[M-H] <sup>-</sup>	293.0440	Artonin U	Flavonoids	YYH
		C <sub>21</sub> H <sub>20</sub> O <sub>5</sub>	353.1355	-8.2	[M+H] <sup>+</sup>	297.0770	Artonin U	Flavonoids	YYH
77	29.76	C <sub>37</sub> H <sub>60</sub> O <sub>10</sub>	699.3821	-8.5	[M+Cl] <sup>-</sup>	277.2148, 353.2030	AstragalosideII <sub>qt</sub>	Triterpenoids	HQ
78	30.20	C <sub>25</sub> H <sub>26</sub> O <sub>5</sub>	405.1700	-1.8	[M-H] <sup>-</sup>	295.0594	8,3'-diprenylapigenin	Flavonoids	YYH
		C <sub>25</sub> H <sub>26</sub> O <sub>5</sub>	407.1848	-1.2	[M+H] <sup>+</sup>	295.0598, 351.1224	8,3'-diprenylapigenin	Flavonoids	YYH
79	32.96	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	301.1402	-2.7	[M+Na] <sup>+</sup> , [M+H] <sup>+</sup> , [M+K] <sup>+</sup>	149.0231	DBP	Others	NX
80	37.98	C <sub>30</sub> H <sub>48</sub> O <sub>3</sub>	455.3506	-5.3	[M-H] <sup>-</sup> , [M+HCOO] <sup>-</sup>	277.2164	Oleanolic acid	Triterpenoids	YYH NX
81	38.19	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	279.2309	-3.4	[M+H] <sup>+</sup>	95.0854	Linolenic acid	Organic acids	HQ
82	38.31	C <sub>22</sub> H <sub>22</sub> O <sub>11</sub>	507.1170	5.2	[M+HCOO] <sup>-</sup> , [M+Cl] <sup>-</sup>	223.0281	Rhamnocitrin-3-O-glucoside	Flavonoids	HQ
		C <sub>22</sub> H <sub>22</sub> O <sub>11</sub>	501.0842	9.7	[M+K] <sup>+</sup>	191.0004, 207.0315, 281.0502	Rhamnocitrin-3-O-glucoside	Flavonoids	HQ
83	41.16	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	281.2470	-1.9	[M+H] <sup>+</sup>	95.0852	EIC	Organic acids	HQ
84	44.22	C <sub>21</sub> H <sub>18</sub> O <sub>11</sub>	447.0959	8.4	[M+H] <sup>+</sup>	191.0001	Baicalin	Flavonoids	NX
						115.0034,			
85	46.85	C <sub>47</sub> H <sub>78</sub> O <sub>18</sub>	931.5353	9.8	[M+H] <sup>+</sup>	147.0651, 553.3685	Asernestioside A	Triterpenoids	HQ
86	46.86	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	655.1574	8.8	[M+HCOO] <sup>-</sup>	91.0223, 223.0270	Rutin	Flavonoids	YYH NX HQ
		C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	633.1487	9.6	[M+Na] <sup>+</sup> , [M+H] <sup>+</sup> , [M+K] <sup>+</sup>	115.0034, 147.0651, 190.9999, 207.0309, 355.068	Rutin	Flavonoids	YYH NX HQ
87	48.41	C <sub>49</sub> H <sub>80</sub> O <sub>19</sub>	1011.4944	1.8	[M+K] <sup>+</sup>	147.0656, 184.0726, 621.3171	Asernestioside B	Triterpenoids	HQ
88	49.83	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>	315.0886	7.2	[M+H] <sup>+</sup>	207.0301, 281.0486	Jaranol	Flavonoids	HQ
89	50.62	C <sub>35</sub> H <sub>60</sub> O <sub>6</sub>	621.4377	0.8	[M+HCOO] <sup>-</sup> , [M+Cl] <sup>-</sup>	277.2198	Daucosterol	Steroids	YYH NX
90	52.31	C <sub>33</sub> H <sub>42</sub> O <sub>15</sub>	717.2087	-9.5	[M+K] <sup>+</sup>	221.0835, 429.0876	Wanepimodoside A	Flavonoids	YYH

TABLE 1: Continued.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
91	57.66	C <sub>20</sub> H <sub>36</sub> O <sub>2</sub>	309.2763	-8.2	[M+H] <sup>+</sup>	89.0612	Linoelaidyl acetate	Others	YYH
92	57.75	C <sub>29</sub> H <sub>38</sub> O <sub>16</sub>	665.2115	9.5	[M+Na] <sup>+</sup>	133.0854	5'-Hydroxyisomuronulatol-2',5'-di-O-glucoside	Saccharide	HQ
93	57.90	C <sub>29</sub> H <sub>48</sub> O	413.3796	4.3	[M+H] <sup>+</sup>	95.0869	Stigmasterol	Steroids	NX

YYH: *Herba Epimedii*; NX: *Radix Achyranthis Bidentatae*; HQ: *Radix Astragali*; \*Compared with a reference standard.

**3.3. Identification and Analysis of Chemical Constituents in Rat Serum.** When analyzing the rat serum samples of GSL, the selected database was a self-built database constructed from analyzed and confirmed GSL *in vitro* compound information. The analysis steps of GSL *in vivo* components were the same as *in vitro* processing. The results showed that there were 20 chemical constituents in rat serum, including 10 kinds of flavonoids, 4 kinds of triterpenoids, 2 kinds of phenylpropanoids, 1 kind of steroid, 1 kind of organic acid, and 2 others. Among them, 12 compounds were from *Herba Epimedii*, 8 compounds from *Radix Achyranthis Bidentatae*, and 2 compounds from *Radix Astragali*. At the same time, rutin is a common compound of three Chinese herbal medicines. Five compounds were identified by the standard substance. The specific information is shown in Table 2 and Figure S2.

**3.4. Identification and Analysis of Metabolites in Rat Serum.** After the chemical constituents from the GSL get into the body, some components exist in the form of a prototype, but most of them are structurally modified based on the original components, such as oxidation, reduction, and hydration. With the unique data postprocessing function of UNIFI, we analyzed the possible metabolites based on the screened prototype compounds *in vivo* and finally obtained 42 metabolites in rat serum through artificial screening. After analysis, these 42 metabolites mainly undergo oxidation, hydration, reduction, dehydration, glutathione S-conjugation, and acetylcysteine conjugation reactions based on the prototype compounds. The detailed information of the prototype, metabolite name, molecular weight, and molecular formula is given in Table 3.

### 3.5. Analysis of GSL by UPLC-Q-TOF-MS

**3.5.1. Flavonoids.** Flavonoids and their glycosides are the main ingredients and the major bioactive components of GSL. In this study, 3 flavones, 41 flavonols, 5 isoflavones, 1 isoflavanone, and 1 flavanol were determined by the matching of the mass spectrometry data with the UNIFI analysis platform.

For flavonoid glycosides, the glycosidic bonds connected by oxygen atoms could be cleaved in both positive and negative ion modes, and most of them were characterized by neutral losses such as 162 Da (Glc) and 146 Da (Rha) [24]. It was difficult to directly remove the glycosyl groups

connected by carbon-glycosidic bonds and often produce [M+H-120]<sup>+</sup> fragment [25]. As we all know, the main cleavage behavior of flavonoid aglycones was the retro Diels-Alder reaction (RDA) cleavage pathway and the loss of free radicals and/or small molecules (such as CH<sub>3</sub>, CO, and CO<sub>2</sub>) [26]. By comparing the retention time and fragmentation patterns with standard substance, peaks 14, 31, 33, 34, 35, 37, and 41 in Figure 1 were exactly identified as calycosin-7-glucoside, ononin, calycosin, epimedin A, epimedin B, epimedin C, and icariin, respectively. Here, we took epimedin C and icariin as examples to depict the fragmentation mode of these ingredients.

Epimedin C showed quasi-molecular ion [M+H]<sup>+</sup> at m/z 823.3014 in positive ion mode and yielded fragment ions at m/z 531.1852 and 515.1931 by losses of 2 molecules of rhamnose and a molecule of ORha-Rha group, respectively. Then, the ion at m/z 515.1931 loses a molecule of Glc to generate an ion at m/z 369.1322, and the ion at m/z 369.1322 further loses a functional group of C<sub>4</sub>H<sub>8</sub> to produce ion at m/z 313.0699. The mass spectrogram and possible fragmentation pathways of epimedin C in positive ion mode are shown in Figure 3.

In the positive ion mode, the mass-to-charge ratio of the quasi-molecular ion peak of compound 41 was 677.2433 [M+H]<sup>+</sup> as shown in Table 1. The mass-to-charge ratio of the fragment ions produced by the precursor ions were 531.1851 [M+H-Rha]<sup>+</sup>, 369.1321 [M+H-Glc]<sup>+</sup>, and 313.0698 [M+H-C<sub>4</sub>H<sub>8</sub>]<sup>+</sup>, which are consistent with those of icariin. The mass spectrogram and potential fragmentation pathways of icariin in positive ion mode are shown in Figure S3.

**3.5.2. Terpenoids.** The terpenoids in GSL primarily included monocyclic monoterpenoids, cycloartane-type tetracyclic triterpenoids, and oleanane-type pentacyclic triterpenoids. Among them, the number of the above categories in GSL was 3, 4, and 11, respectively.

Triterpene saponins in GSL mainly exist in the form of aglycones binding with sugars, such as glucose, rhamnose, and xylose. In mass spectrometric analysis, triterpenoid saponins are mostly in the form of de-sugar or continuous de-sugar fragment ions [27]. Such compounds also had the loss of CO<sub>2</sub>, COOH, CH<sub>2</sub>OH, and other complex groups. Compound 49 gave a deprotonated molecule [M-H]<sup>-</sup> at m/z 1117.5026 and produced predominant fragment ions at m/z 997.4956 [M-H-COOH-CH<sub>2</sub>OH-CO<sub>2</sub>]<sup>-</sup> and m/z 631.2021 [M-H-C<sub>2</sub>H<sub>2</sub>O-2Glc]<sup>-</sup> in negative ion mode (Figure 4). It

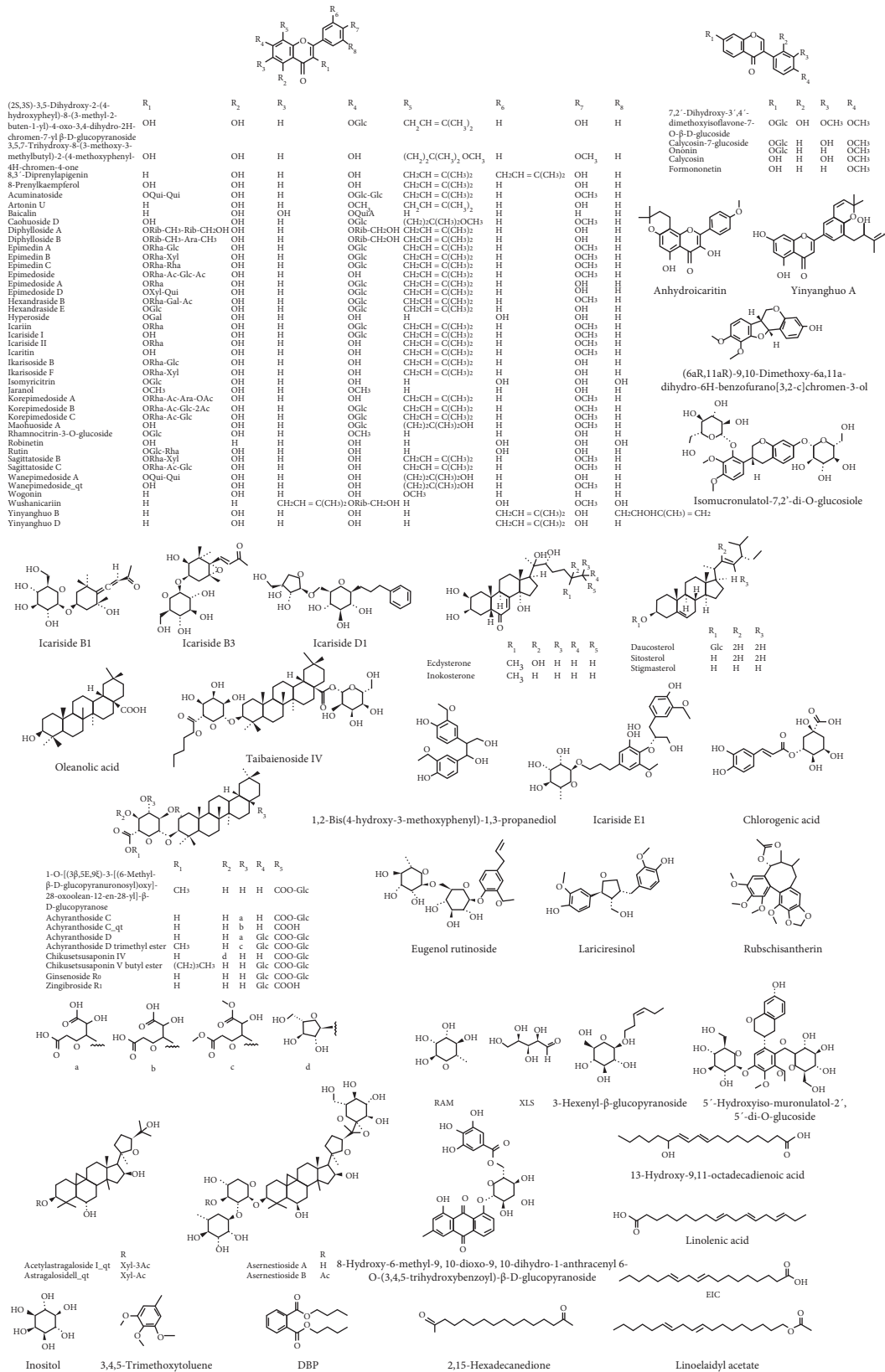


FIGURE 2: Chemical structures of compounds identified in GSL.

TABLE 2: The chemical constituents of the GSL blood prototype identified by UPLC-Q-TOF-MS.

NO.	Observed RT (min)	Formula	Observed m/z	Mass error (ppm)	Adducts	Fragment (+/-)	Identification	Structure class	Origin
1	5.26	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	353.0846	-9.2	[M-H] <sup>-</sup>	93.0350	Chlorogenic acid	Phenylpropanoids (phenylpropionic acid)	YYH
2	8.40	C <sub>27</sub> H <sub>44</sub> O <sub>7</sub>	481.3201	8.5	[M+H] <sup>+</sup>	175.1189, 288.2025	Ecdysterone*	Steroids	NX
3	12.30	C <sub>43</sub> H <sub>68</sub> O <sub>14</sub>	853.4568	-2.8	[M+HCOO] <sup>-</sup>	137.0231, 312.1296	1-O-((3β,5ξ,9ξ)-3-[(6-Methyl-β-D-glucopyranuronosyl)oxy]-28-oxoolean-12-en-28-yl)-β-D-glucopyranose	Triterpenoids	NX
4	12.72	C <sub>38</sub> H <sub>48</sub> O <sub>20</sub>	823.2644	-2.7	[M-H] <sup>-</sup>	214.9992, 312.1294	Diphyllaside A	Flavonoids	YYH
5	13.01	C <sub>37</sub> H <sub>46</sub> O <sub>19</sub>	793.2537	-3.0	[M-H] <sup>-</sup>	631.2019	Epimedeside D	Flavonoids	YYH
6	13.34	C <sub>32</sub> H <sub>38</sub> O <sub>15</sub>	661.2083	-8.3	[M-H] <sup>-</sup> , [M+HCOO] <sup>-</sup>	353.0997	Epimedeside A	Flavonoids	YYH
7	17.58	C <sub>39</sub> H <sub>50</sub> O <sub>19</sub>	883.2807	-8.0	[M+HCOO] <sup>-</sup>	675.2247	Epimedin A*	Flavonoids	YYH
8	18.44	C <sub>38</sub> H <sub>48</sub> O <sub>18</sub>	853.2745	-3.2	[M+HCOO] <sup>-</sup> , [M+Cl] <sup>-</sup>	252.0406, 366.1081, 645.2177	Epimedin B*	Flavonoids	YYH
		C <sub>38</sub> H <sub>48</sub> O <sub>18</sub>	809.2861	-0.2	[M+H] <sup>+</sup>	369.1333, 531.1842	Epimedin B*	Flavonoids	YYH
9	18.92	C <sub>39</sub> H <sub>50</sub> O <sub>19</sub>	867.2915	-1.6	[M+HCOO] <sup>-</sup>	366.1059, 659.2331	Epimedin C*	Flavonoids	YYH
		C <sub>39</sub> H <sub>50</sub> O <sub>19</sub>	823.3031	1.5	[M+H] <sup>+</sup>	136.0765, 531.1840	Epimedin C*	Flavonoids	YYH
10	19.20	C <sub>33</sub> H <sub>40</sub> O <sub>15</sub>	721.2341	-1.2	[M+HCOO] <sup>-</sup> , [M+Cl] <sup>-</sup>	367.1163	Icariin*	Flavonoids	YYH
		C <sub>33</sub> H <sub>40</sub> O <sub>15</sub>	677.2413	-4.0	[M+H] <sup>+</sup>	313.0683, 369.1314, 531.1844	Icariin*	Flavonoids	YYH
11	19.45	C <sub>25</sub> H <sub>30</sub> O <sub>8</sub>	481.1812	-4.3	[M+Na] <sup>+</sup>	97.0644	Rubschisantherin	Phenylpropanoids (lignans)	NX
12	21.12	C <sub>53</sub> H <sub>82</sub> O <sub>25</sub>	1117.5037	-3.2	[M-H] <sup>-</sup>	217.0817, 365.2313, 413.1985, 585.2846, 785.4175	Achyranthoside D	Triterpenoids	NX
13	21.21	C <sub>48</sub> H <sub>76</sub> O <sub>19</sub>	955.4937	3.0	[M-H] <sup>-</sup>	413.1985, 585.2846, 785.4175	Ginsenoside Ro*	Triterpenoids	NX
14	21.92	C <sub>47</sub> H <sub>72</sub> O <sub>20</sub>	955.4622	8.2	[M-H] <sup>-</sup>	353.2213, 405.2627	Achyranthoside C	Triterpenoids	NX
15	22.99	C <sub>32</sub> H <sub>38</sub> O <sub>14</sub>	645.2157	-4.9	[M-H] <sup>-</sup>	223.0270	Sagittatoside B	Flavonoids	YYH
16	23.89	C <sub>27</sub> H <sub>30</sub> O <sub>10</sub>	513.1739	-5.3	[M-H] <sup>-</sup>	366.1082	Icariside II	Flavonoids	YYH
		C <sub>27</sub> H <sub>30</sub> O <sub>10</sub>	515.1908	-0.8	[M+H] <sup>+</sup>	313.0671	Icariside II	Flavonoids	YYH
17	28.61	C <sub>16</sub> H <sub>30</sub> O <sub>2</sub>	277.2140	0.6	[M+Na] <sup>+</sup>	223.1695	2,15-Hexadecanedione	Others	YYH
18	32.96	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	301.1382	-9.4	[M+Na] <sup>+</sup> , [M+H] <sup>+</sup> , [M+K] <sup>+</sup>	149.0216	DBP	Others	NX
19	38.18	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	279.2323	1.7	[M+H] <sup>+</sup>	95.0865	Linolenic acid	Organic acids	HQ YYH
20	46.60	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	645.1256	4.4	[M+Cl] <sup>-</sup>	168.0420	Rutin	Flavonoids	NX HQ YYH
		C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	633.1444	2.8	[M+Na] <sup>+</sup> , [M+K] <sup>+</sup>	207.0296	Rutin	Flavonoids	NX HQ

YYH: *Herba Epimedii*; NX: *Radix Achyranthis Bidentatae*; HQ: *Radix Astragali*; \*Compared with a reference standard.



TABLE 3: Identification of the metabolites from GSL *in vivo*.

NO.	Observed RT (min)	Formula	Observed $m/z$	Mass error (ppm)	Adducts	Identification
M1	4.8	C <sub>39</sub> H <sub>52</sub> O <sub>20</sub>	875.2792	5.2	[M+Cl] <sup>-</sup>	Epimedin C + H <sub>2</sub> + O
M2	7.4	C <sub>28</sub> H <sub>47</sub> N <sub>3</sub> O <sub>9</sub> S	646.2978	-5.8	[M+HCOO] <sup>-</sup>	Linolenic acid + H <sub>2</sub> O + C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S
M3	7.57	C <sub>32</sub> H <sub>55</sub> NO <sub>11</sub> S	696.3213	3.2	[M+Cl] <sup>-</sup>	Ecdysterone + H <sub>2</sub> +H <sub>2</sub> O + C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub> S
M4	7.72	C <sub>27</sub> H <sub>45</sub> O <sub>12</sub> P	591.2631	9.3	[M-H] <sup>-</sup>	Ecdysterone + 2x(+O) + HPO <sub>3</sub>
M5	7.73	C <sub>27</sub> H <sub>47</sub> O <sub>12</sub> P	629.2539	6.4	[M+Cl] <sup>-</sup>	Ecdysterone + O + H <sub>2</sub> O + HPO <sub>3</sub>
M6	7.89	C <sub>24</sub> H <sub>31</sub> O <sub>9</sub> P	493.1677	8.9	[M-H] <sup>-</sup>	Rubschisantherin - COO + HPO <sub>3</sub>
M7	8.24	C <sub>25</sub> H <sub>30</sub> O <sub>10</sub>	535.1793	-5.2	[M+HCOO] <sup>-</sup>	Rubschisantherin + 2x(+O)
M8	8.41	C <sub>37</sub> H <sub>59</sub> N <sub>3</sub> O <sub>15</sub> S	852.3433	8.4	[M+Cl] <sup>-</sup>	Ecdysterone + 2x(+O) + C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S
M9	14.8	C <sub>24</sub> H <sub>30</sub> O <sub>7</sub>	429.193	2.7	[M-H] <sup>-</sup>	Rubschisantherin + O - COO
M10	16.62	C <sub>33</sub> H <sub>36</sub> O <sub>21</sub>	803.1385	-7.2	[M+Cl] <sup>-</sup>	Rutin - H <sub>2</sub> O + C <sub>6</sub> H <sub>8</sub> O <sub>6</sub>
M11	19.74	C <sub>39</sub> H <sub>48</sub> O <sub>19</sub>	865.2694	-8.9	[M+HCOO] <sup>-</sup>	Epimedin A - H <sub>2</sub> O
M12	19.79	C <sub>38</sub> H <sub>46</sub> O <sub>20</sub>	821.2456	-6.6	[M-H] <sup>-</sup>	Sagittatoside B + C <sub>6</sub> H <sub>8</sub> O <sub>6</sub>
M13	20.03	C <sub>24</sub> H <sub>30</sub> O <sub>6</sub>	413.199	4.9	[M-H] <sup>-</sup>	Rubschisantherin - COO
M14	20.04	C <sub>38</sub> H <sub>46</sub> O <sub>18</sub>	835.2618	-5.8	[M + HCOO] <sup>-</sup>	Epimedin B - H <sub>2</sub> O
M15	22.34	C <sub>27</sub> H <sub>46</sub> O <sub>8</sub>	497.3131	2.2	[M-H] <sup>-</sup>	Ecdysterone + H <sub>2</sub> O
M16	23.09	C <sub>33</sub> H <sub>40</sub> O <sub>14</sub>	659.2317	-4.3	[M-H] <sup>-</sup>	Icariin + H <sub>2</sub> - H <sub>2</sub> O
M17	24.63	C <sub>32</sub> H <sub>51</sub> NO <sub>12</sub> S	672.312	9.1	[M-H] <sup>-</sup>	Ecdysterone + 2x(+O) + C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub> S
M18	26.05	C <sub>18</sub> H <sub>34</sub> O <sub>4</sub>	313.2376	-2.9	[M-H] <sup>-</sup>	Linolenic acid + 2x(+H <sub>2</sub> O)
M19	27.3	C <sub>18</sub> H <sub>31</sub> O <sub>6</sub> P	409.1519	-8.1	[M+Cl] <sup>-</sup>	Linolenic acid + O + HPO <sub>3</sub>
M20	27.48	C <sub>27</sub> H <sub>49</sub> O <sub>11</sub> P	579.2972	5.5	[M-H] <sup>-</sup>	Ecdysterone + H <sub>2</sub> + H <sub>2</sub> O + HPO <sub>3</sub>
M21	27.59	C <sub>28</sub> H <sub>49</sub> N <sub>3</sub> O <sub>8</sub> S	586.3145	-4.1	[M-H] <sup>-</sup>	Linolenic acid + 2x(+H <sub>2</sub> ) + C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S
M22	27.85	C <sub>28</sub> H <sub>47</sub> N <sub>3</sub> O <sub>8</sub> S	630.3034	-5.3	[M+HCOO] <sup>-</sup>	Linolenic acid + H <sub>2</sub> + C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S
M23	27.88	C <sub>18</sub> H <sub>30</sub> O <sub>4</sub>	309.2043	-9.1	[M-H] <sup>-</sup>	Linolenic acid + 2x(+O)
M24	28.66	C <sub>27</sub> H <sub>46</sub> O <sub>7</sub>	527.3212	-2.7	[M+HCOO] <sup>-</sup>	Ecdysterone + H <sub>2</sub>
M25	28.82	C <sub>27</sub> H <sub>44</sub> O <sub>9</sub>	557.2997	5.4	[M+HCOO] <sup>-</sup>	Ecdysterone + 2x(+O)
M26	28.83	C <sub>28</sub> H <sub>47</sub> N <sub>3</sub> O <sub>10</sub> S	616.2894	-2.7	[M-H] <sup>-</sup>	Linolenic acid + O + H <sub>2</sub> O + C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S
M27	28.86	C <sub>32</sub> H <sub>55</sub> NO <sub>10</sub> S	680.3184	-8.6	[M+Cl] <sup>-</sup>	Ecdysterone + 2x(+H <sub>2</sub> ) + C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub> S
M28	28.97	C <sub>32</sub> H <sub>51</sub> NO <sub>11</sub> S	656.3155	6.9	[M-H] <sup>-</sup>	Ecdysterone + O + C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub> S
M29	29.08	C <sub>27</sub> H <sub>44</sub> O <sub>8</sub>	531.2733	0.5	[M+Cl] <sup>-</sup>	Ecdysterone + O
M30	30.24	C <sub>18</sub> H <sub>32</sub> O <sub>3</sub>	295.2255	-8	[M-H] <sup>-</sup>	Linolenic acid + H <sub>2</sub> O
M31	31.03	C <sub>23</sub> H <sub>41</sub> NO <sub>7</sub> S	520.2627	7.8	[M+HCOO] <sup>-</sup>	Linolenic acid + 2x(+H <sub>2</sub> O) + C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub> S
M32	32.47	C <sub>32</sub> H <sub>47</sub> NO <sub>8</sub> S	640.2711	-0.7	[M+Cl] <sup>-</sup>	Ecdysterone + 2x(-H <sub>2</sub> O) + C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub> S
M33	34.78	C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>	347.1975	-5.8	[M+Cl] <sup>-</sup>	Linolenic acid + O + H <sub>2</sub> O
M34	34.9	C <sub>32</sub> H <sub>49</sub> NO <sub>10</sub> S	638.2981	-3.6	[M-H] <sup>-</sup>	Ecdysterone + O - H <sub>2</sub> O + C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub> S
M35	37.62	C <sub>23</sub> H <sub>30</sub> O <sub>4</sub>	369.2044	-7.4	[M-H] <sup>-</sup>	Rubschisantherin + 2x(-COO)
M36	38.5	C <sub>34</sub> H <sub>45</sub> N <sub>3</sub> O <sub>12</sub> S	754.2396	-3	[M+Cl] <sup>-</sup>	Rubschisantherin-COO + C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S
M37	40.72	C <sub>33</sub> H <sub>40</sub> O <sub>17</sub>	753.2191	-7.5	[M+HCOO] <sup>-</sup>	Icariin + 2x(+O)
M38	41.15	C <sub>33</sub> H <sub>52</sub> O <sub>15</sub>	733.3252	-5	[M+HCOO] <sup>-</sup>	Ecdysterone + 2x(+O) + C <sub>6</sub> H <sub>8</sub> O <sub>6</sub>
M39	44.28	C <sub>33</sub> H <sub>56</sub> O <sub>15</sub>	737.3607	0.7	[M+HCOO] <sup>-</sup>	Ecdysterone + 2x(+H <sub>2</sub> O) + C <sub>6</sub> H <sub>8</sub> O <sub>6</sub>
M40	51.66	C <sub>33</sub> H <sub>40</sub> O <sub>23</sub>	803.1956	8.5	[M-H] <sup>-</sup>	Rutin + H <sub>2</sub> O + C <sub>6</sub> H <sub>8</sub> O <sub>6</sub>
M41	53.66	C <sub>39</sub> H <sub>51</sub> O <sub>21</sub> P	921.2341	-1.5	[M+Cl] <sup>-</sup>	Epimedin C + H <sub>2</sub> -H <sub>2</sub> O + HPO <sub>3</sub>
M42	53.67	C <sub>37</sub> H <sub>47</sub> N <sub>3</sub> O <sub>22</sub> S	916.2292	-0.9	[M-H] <sup>-</sup>	Rutin + H <sub>2</sub> + C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S

+H<sub>2</sub>: reduction; +O: oxidation; +H<sub>2</sub>O: hydration; +HPO<sub>3</sub>: phosphorylation; +C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>: glucuronidation; +C<sub>5</sub>H<sub>7</sub>NO<sub>3</sub>S: acetyl cysteine conjugation; +C<sub>10</sub>H<sub>15</sub>N<sub>3</sub>O<sub>6</sub>S: glutathione S-conjugation; -H<sub>2</sub>O: dehydration; -COO: decarboxylation.

was consistent with previous literature [28] and was finally identified as achyranthoside D. Peak 51 was identified clearly as ginsenoside Ro with a standard substance, and its mass fragmentation pattern is demonstrated in detail (Figure S4). In the negative ion mode, ginsenoside Ro gave [M-H]<sup>-</sup> ion at  $m/z$  955.4884, along with two major fragment ions at  $m/z$  793.4236 [M-H-Glc]<sup>-</sup> and 631.2021[M-H-2Glc]<sup>-</sup> in mass spectrometry under high-energy conditions. From the cleavage pathway of these triterpenoid saponins, they tend to lose the sugar group at the C28 position first under the action of high energy of MS<sup>E</sup>. The possible reason for this phenomenon is that the ester bond at the C28 position is easier to break than the ether bond at the C3 position.

**3.5.3. Phenylpropanoids.** Six phenylpropanoids were recognized as the major active ingredients in GSL. Among them, a total of 2 phenylpropionic acids, 1 phenylpropanol, 1 styrene, and 2 lignans were identified. Simple phenylpropanoids belong to phenylpropane derivatives in structure and exist in plants in the form of glycosides or esters, which can be combined with sugars and polyols. In mass spectrometric analysis, phenylpropanoids are mainly manifested by the loss of sugar, neutral loss, and loss of other complex groups [29]. Compound 11 showed a deprotonated molecule [M-H]<sup>-</sup> at  $m/z$  539.2149 and produced predominant fragment ions at  $m/z$  491.1857 [M-H-CH<sub>2</sub>O-H<sub>2</sub>O]<sup>-</sup>,  $m/z$  479.0789 [M-H-C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>]<sup>-</sup>, and  $m/z$  317.0238



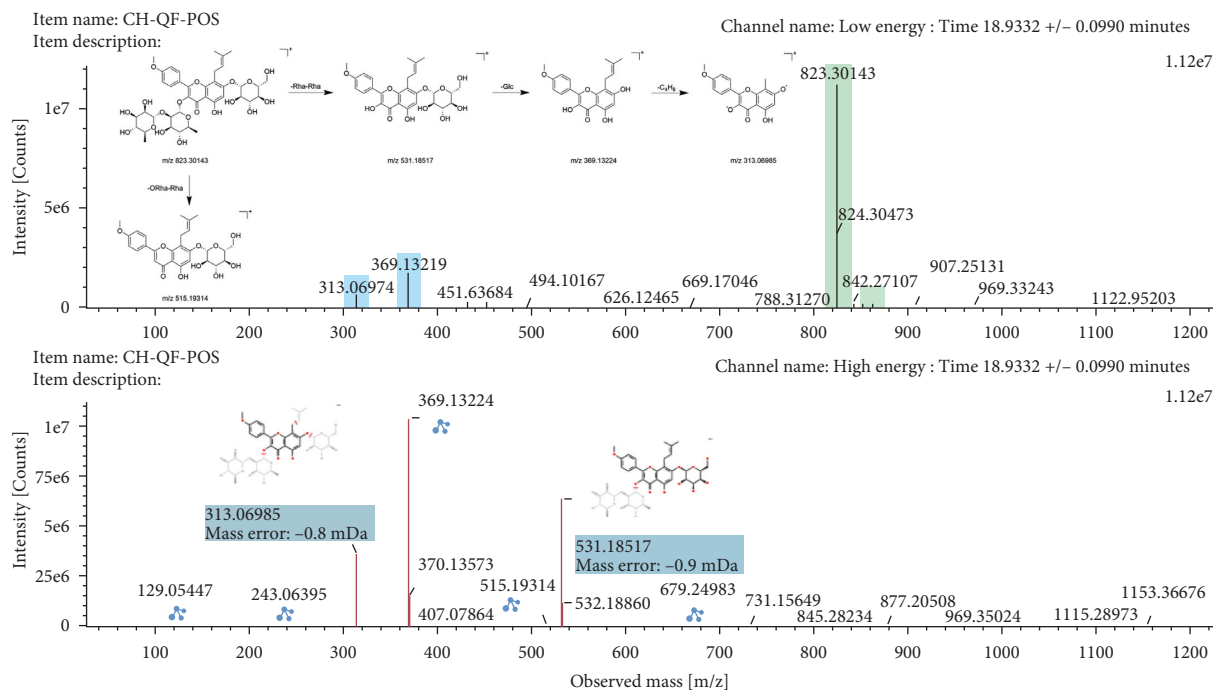


FIGURE 3: The mass spectrogram and fragmentation pathways of epimedinin C in positive ion mode.

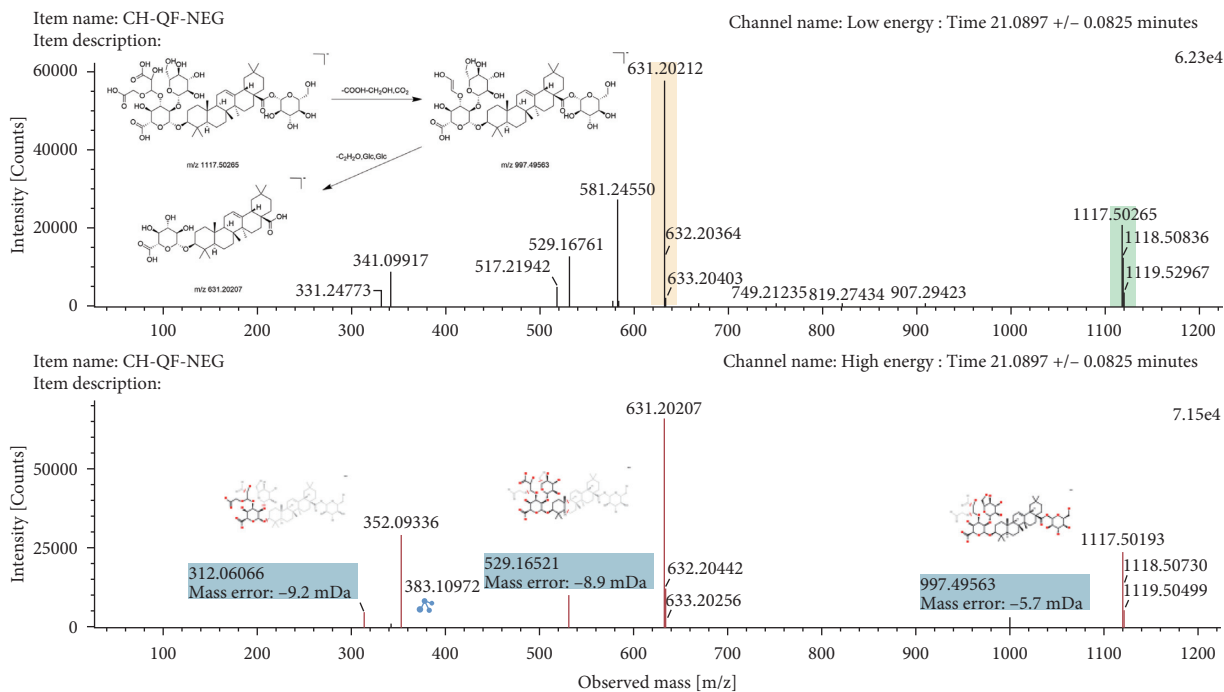


FIGURE 4: The mass spectrogram and fragmentation pathways of achyranthoside D in negative ion mode.

[M-H-C<sub>3</sub>H<sub>6</sub>O-Rha-OH]<sup>-</sup> in negative ion mode. By confirmation of fragment ions, we preliminarily identified compound 11 as icaraside E1. In the case of compound 44, it generated a base peak ion at m/z 481.1785 [M+Na]<sup>+</sup> in positive ion mode, along with a major fragment ion at m/z 387.1427 [M+Na-C<sub>4</sub>H<sub>8</sub>O]<sup>+</sup>, which was consistent with a previous study [30]. Finally, it was assigned to be

rubischisantherin. The detailed mass spectrogram and fragmentation pathways are shown in Figures 5 and S5.

3.5.4. Steroids. In the UNIFI results interface, 5 steroids were automatically matched. The cleavage of steroids and their aglycones is complicated. Besides RDA cleavage,

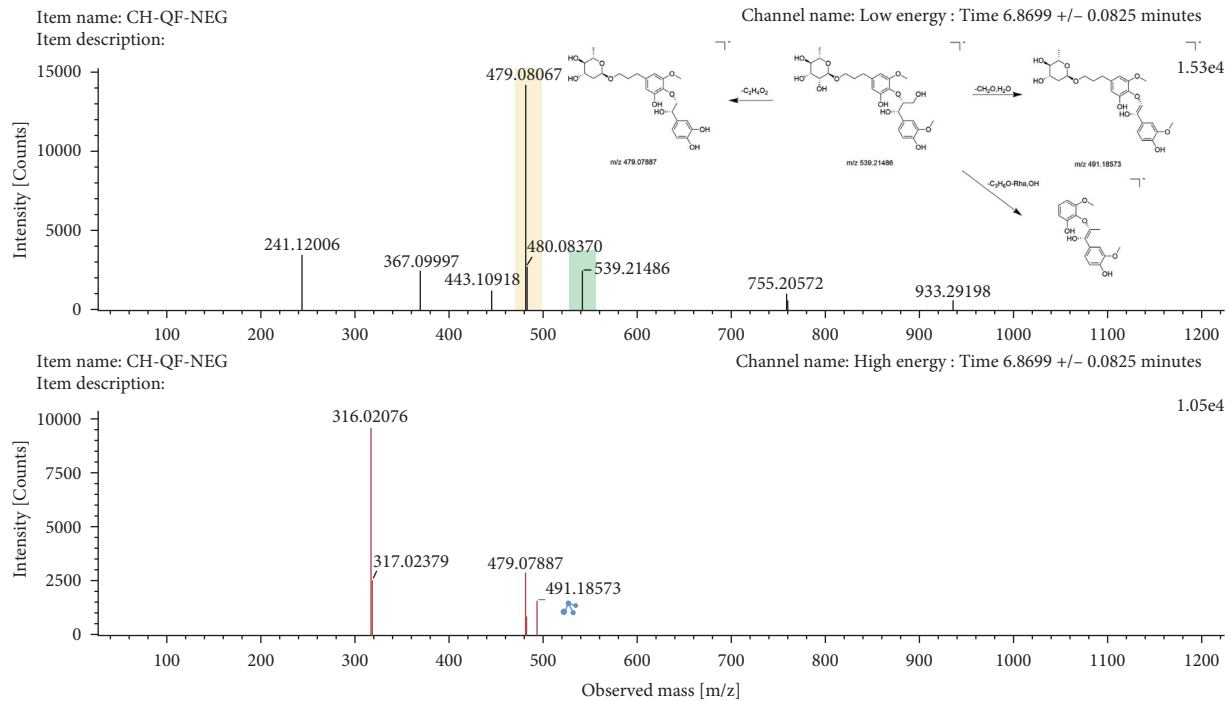


FIGURE 5: The mass spectrogram and fragmentation pathways of icariside E1 in negative ion mode.

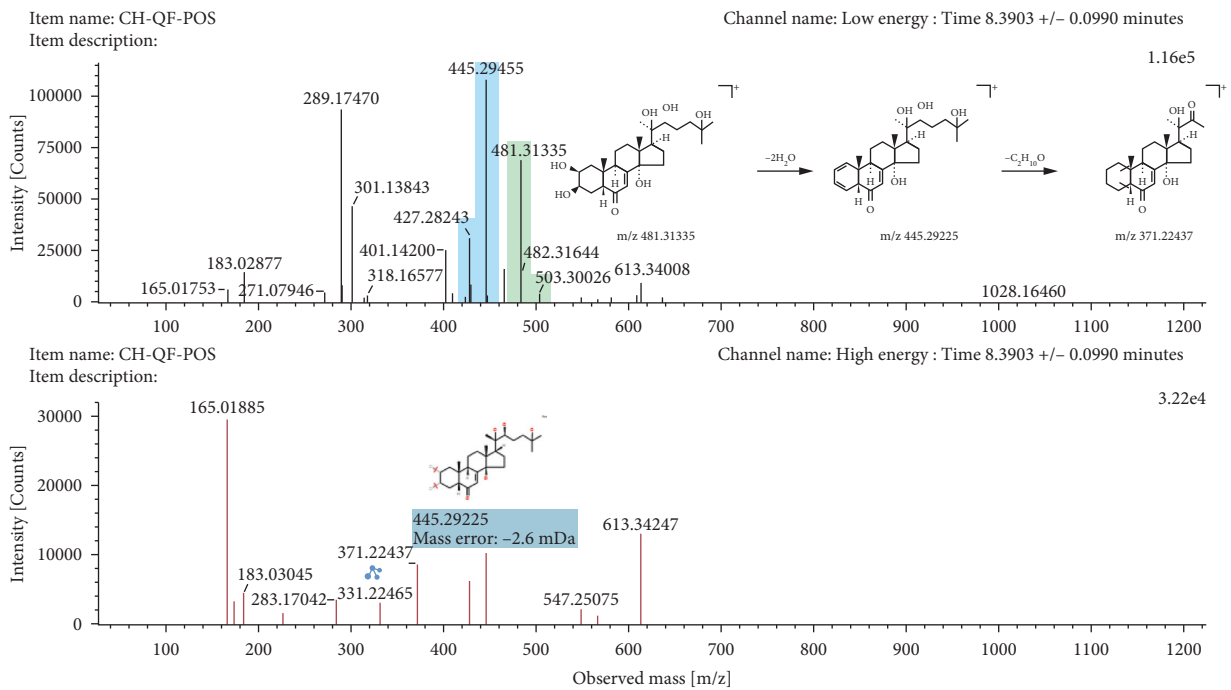


FIGURE 6: The mass spectrogram and fragmentation pathways of ecdysterone in positive ion mode.

dehydration, and demethylation of the hydroxyl group, the side chain at position 17 often falls off. Peak 18 was ascertained to be ecdysterone by contrast with reference

standards. As shown in Figure 6, ecdysterone displayed a hydrogenated ion at  $m/z$  481.3134  $[M+H]^+$  with a molecular formula  $C_{27}H_{44}O_7$  and lost  $H_2O$  to generate an ion at  $m/z$

445.2923 [M+H-2H<sub>2</sub>O]<sup>+</sup>. Further loss of the C<sub>4</sub>H<sub>10</sub>O group resulted in fragmentation with a m/z of 371.2244 [M+H-2H<sub>2</sub>O-C<sub>4</sub>H<sub>10</sub>O]<sup>+</sup>.

3.5.5. *Others.* Some compounds with fewer species and lower concentrations are assigned to this category. The mass spectra data extracted from MassLynx workstation were matched with UNIFI software, and the results were verified by literature analysis [31]. A total of 13 compounds were inferred, including anthraquinones, glycosides, organic acids, and others. Specific mass spectrometry data are listed in Table 1.

#### 4. Conclusions

In this experiment, UPLC-Q-TOF-MS technology combined with UNIFI software was used for the first time to comprehensively and systematically analyze the *in vitro* and *in vivo* chemical constituents of GSL. We summarized the cleavage law of flavonoids, triterpene saponins, phenylpropanoids, and steroids in the mass spectrum and initially explored the prototype compounds and metabolites of GSL in rat serum. These results provide a technical basis for the comprehensive and effective quality control and pharmacodynamic material basis of GSL. In addition, some chromatographic peaks with better response in GSL are unknown ingredients, which deserve further study.

#### Data Availability

The data used to support the findings of this study are included within the article.

#### Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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#### Supplementary Materials

See Table S1 and Figures S1-S5 in Supplementary Materials for comprehensive images and value analysis. (*Supplementary Materials*)

#### References

- [1] M. Rossini, S. Adami, F. Bertoldo et al., "Guidelines for the diagnosis, prevention and management of osteoporosis," *Reumatismo*, vol. 68, no. 1, pp. 1-39, 2016.
- [2] R. Nuti, M. L. Brandi, G. Checchia et al., "Guidelines for the management of osteoporosis and fragility fractures," *Internal and Emergency Medicine*, vol. 14, no. 1, pp. 85-102, 2019.
- [3] F. S. O'Ryan and J. C. Lo, "Bisphosphonate-related osteonecrosis of the jaw in patients with oral bisphosphonate exposure: clinical course and outcomes," *Journal of Oral and Maxillofacial Surgery*, vol. 70, no. 8, pp. 1844-1853, 2012.
- [4] T. D. Rachner, U. Platzbecker, D. Felsenberg, and L. C. Hofbauer, "Osteonecrosis of the jaw after osteoporosis therapy with denosumab following long-term bisphosphonate therapy," *Mayo Clinic Proceedings*, vol. 88, no. 4, pp. 418-419, 2013.
- [5] J. E. Rossouw, G. L. Anderson, R. L. Prentice et al., "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial," *Journal of the American Medical Association*, vol. 288, pp. 321-333, 2002.
- [6] M. R. Meyer and M. Barton, "Estrogens and coronary artery disease," *Advances in Pharmacology*, vol. 77, pp. 307-360, 2016.
- [7] T. Wang, Q. Liu, W. Tjhiue et al., "Therapeutic potential and outlook of alternative medicine for osteoporosis," *Current Drug Targets*, vol. 18, pp. 1051-1068, 2017.
- [8] N.-D. Zhang, T. Han, B.-K. Huang et al., "Traditional Chinese medicine formulas for the treatment of osteoporosis: implication for antiosteoporotic drug discovery," *Journal of Ethnopharmacology*, vol. 189, pp. 61-80, 2016.
- [9] L. Wang, Y. Li, Y. Guo et al., "Herba Epimedii: an ancient Chinese Herbal Medicine in the prevention and treatment of osteoporosis," *Current Pharmaceutical Design*, vol. 22, pp. 328-349, 2016.
- [10] C. H. Fang, J. Liu, X. P. Zhang, M. X. Han, Y. Z. Zhang, and H. M. Nie, "Clinical observation on 60 cases of senile osteoporosis treated by Gushuling," *Traditional Chinese Medicine and Clinical Pharmacology*, vol. 14, pp. 30-32, 1999.
- [11] D. Y. Wang and C. H. Fang, "Report of 60 cases of senile osteoporosis treated by Gushuling," *Traditional Chinese Medicine and Clinical Pharmacology*, vol. 20, pp. 55-56, 2008.
- [12] T. Yin and C. H. Fang, "Clinical observation of Gushuling in preventing and treating postmenopausal osteoporosis," *Anhui Medical and Pharmaceutical Journal*, vol. 12, pp. 863-864, 2008.
- [13] Y. Q. Shu, C. H. Fang, and T. T. Ba, "Clinical observation on treatment of diabetic osteoporosis by warming yang, nourishing qi and activating blood," *Journal of Emergency in Traditional Chinese Medicine*, vol. 19, pp. 49-50, 2010.
- [14] J. S. Rooney, A. McDowell, C. J. Strachan, and K. C. Gordon, "Evaluation of vibrational spectroscopic methods to identify and quantify multiple adulterants in herbal medicines," *Talanta*, vol. 138, pp. 77-85, 2015.
- [15] Y. Jiang, B. David, P. Tu, and Y. Barbin, "Recent analytical approaches in quality control of traditional Chinese medicines-a review," *Analytica Chimica Acta*, vol. 657, no. 1, pp. 9-18, 2010.
- [16] J. Yu, R. Zhu, Y. J. Dong, Y. L. Zhu, R. Sang, and W. D. Chen, "Effects of the compatibility of Gushuling granules on the icariin content," *Chinese Journal of Pharmaceuticals*, vol. 27, pp. 79-82, 2016.
- [17] R. Sang, X. X. Wang, Y. Y. Zhu, Y. L. Zhu, and W. D. Chen, "Serum pharmacochimistry analysis of Gushuling in rats," *Traditional Chinese Medicine in the Treatment of Patients*, vol. 21, pp. 78-81, 2015.
- [18] G. Zhou, M. Wang, Y. Li, R. Xu, and X. Li, "Comprehensive analysis of 61 characteristic constituents from *Siraitia fructus*

- using ultrahigh-pressure liquid chromatography with time-of-flight mass spectrometry,” *Journal of Pharmaceutical and Biomedical Analysis*, vol. 125, pp. 1–14, 2016.
- [19] M. Ganzera and S. Sturm, “Recent advances on HPLC/MS in medicinal plant analysis-An update covering 2011-2016,” *Journal of Pharmaceutical and Biomedical Analysis*, vol. 147, pp. 211–233, 2018.
- [20] H. Wu, Y. Chen, Q. Li et al., “Intervention effect of Qi-Yu-San-Long decoction on lewis lung carcinoma in C57BL/6 mice: insights from UPLC-QTOF/MS-based metabolic profiling,” *Journal of Chromatography B*, vol. 1102-1103, pp. 23–33, 2018.
- [21] B. Zhu, H. Cao, L. Sun et al., “Metabolomics-based mechanisms exploration of Huang-Lian Jie-Du decoction on cerebral ischemia via UPLC-Q-TOF/MS analysis on rat serum,” *Journal of Ethnopharmacology*, vol. 216, pp. 147–156, 2018.
- [22] F.-X. Zhang, M. Li, L.-R. Qiao et al., “Rapid characterization of Ziziphi Spinosae Semen by UPLC/Qtof MS with novel informatics platform and its application in evaluation of two seeds from Ziziphus species,” *Journal of Pharmaceutical and Biomedical Analysis*, vol. 122, pp. 59–80, 2016.
- [23] L. Deng, A.-M. Shi, H.-Z. Liu et al., “Identification of chemical ingredients of peanut stems and leaves extracts using UPLC-QTOF-MS coupled with novel informatics UNIFI platform,” *Journal of Mass Spectrometry*, vol. 51, no. 12, pp. 1157–1167, 2016.
- [24] L. Xu, Y. Liu, H. Wu, H. Wu, X. Liu, and A. Zhou, “Rapid identification of chemical profile in Gandou decoction by UPLC-Q-TOF-MSE coupled with novel informatics UNIFI platform,” *Journal of Pharmaceutical Analysis*, vol. 10, no. 1, pp. 35–48, 2020.
- [25] J. R. Gao, M. Q. Zhu, X. L. Wang, H. Wu, L. Shan, and Y. Wang, “Identification of chemical constituents in Huangdi Anxiao Capsules by UPLC-Q-TOF-MS<sup>E</sup> combined with UNIFI software,” *China Journal of Chinese Materia Medica*, vol. 45, pp. 2395–2405, 2020.
- [26] S. Wang, L. Chen, J. Leng, P. Chen, X. Fan, and Y. Cheng, “Fragment ion diagnostic strategies for the comprehensive identification of chemical profile of Gui-Zhi-Tang by integrating high-resolution MS, multiple-stage MS and UV information,” *Journal of Pharmaceutical and Biomedical Analysis*, vol. 98, pp. 22–35, 2014.
- [27] C.-J.-S. Lai, T. Tan, S.-L. Zeng et al., “An integrated high resolution mass spectrometric data acquisition method for rapid screening of saponins in Panax notoginseng (Sanqi),” *Journal of Pharmaceutical and Biomedical Analysis*, vol. 109, pp. 184–191, 2015.
- [28] J. Fu, H. Wu, and H. Wu, “Rapid characterization of triterpenoid saponins in Achyranthes bidentate Blume by UPLC-Q -TOF/MS in MS<sup>E</sup> mode combined with UNIFI screening platform,” *Natural Product Research and Development*, vol. 31, pp. 1054–1061+1090, 2019.
- [29] J. Xie, L. Zhang, J. X. Zeng et al., “Fast identification of constituents of Lagotis breviflora by using UPLC-Q-TOF-MS/MS method,” *China Journal of Chinese Materia Medica*, vol. 42, pp. 2123–2130, 2017.
- [30] X.-M. Gao, R.-R. Wang, D.-Y. Niu et al., “Bioactive dibenzocyclooctadiene lignans from the stems of Schisandra neglecta,” *Journal of Natural Products*, vol. 76, no. 6, pp. 1052–1057, 2013.
- [31] D. Y. Lee, B.-R. Choi, J. W. Lee et al., “Simultaneous determination of various platycosides in Four Platycodon grandiflorum cultivars by UPLC-QTOF/MS,” *Applied Biological Chemistry*, vol. 62, no. 1, p. 47, 2019.