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Chemical profiling of Wu-tou decoction by UPLC–Q-TOF-MS

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ABSTRACT

Wu-tou decoction (WTD), a traditional Chinese medicine (TCM) formula, is composed of Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata, and it has been used for more than a thousand years to treat rheumatic arthritis, rheumatoid arthritis and pain of joints, while the active constitutions of WTD are unclear. In this research, an ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF-MS) method in both positive and negative ion mode was established to investigate the major constitutions in WTD. A Waters ACQUITY UPLC BEH C18 column was used to separate the aqueous extract of WTD. Acetonitrile and 0.1% aqueous formic acid (v/v) were used as the mobile phase. 74 components including alkaloids, monoterpene glycosides, triterpene saponins, flavones and flavone glycosides were identified or tentatively characterized in WTD based on the accurate mass within 15 ppm error and tandem MS behavior. All the constitutions were also detected in the corresponding individual herbs. These results will provide a basis for further study *in vivo* of WTD and the information of potential new drug structure for treating rheumatic arthritis and rheumatoid arthritis.

1. Introduction

Traditional Chinese medicine (TCM) is regarded naturally and harmlessly and accepted gradually in the world in recent years [1,2]. Formula, a combination of plant species, is designed on the basis of patients' symptoms, clinical experience and TCM theories. It is characterized by multi-component, multi-target and synergistic therapeutic efficacies, but the working mechanisms remain unknown [3]. Wu-tou decoction (WTD), a TCM formula, has been used for more than a thousand years to treat rheumatic arthritis, rheumatoid arthritis and pain of joints. It was first recorded in "Jin Kui Yao Lve" written by Zhongjing Zhang, and it is composed of Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata with the mass ratio of 2:3:3:3:3. In recent years, researchers found that WTD could reduce the extent of footpad swelling and decline the concentration of NO in serum of adjuvant-induced arthritis rats [4]. Integrating network analysis showed that the predicted effector molecules of WTD were significantly associated with neuroactive ligand–receptor interaction and calcium signaling pathway [5].

Many constitutions of herbs in WTD have excellent anti-inflammatory and antioxidant activity. Total alkaloids of Aconiti Radix, total glycosides or polysaccharides of Paeoniae Radix Alba and polysaccharides of Astragali Radix could reduce multiple indices of arthritis in rat model of rheumatoid arthritis [6,7]. Ephedrine hydrochloride could contribute to the immune homeostasis of lipopolysaccharide induced endotoxic shock mouse model by inducing interleukin-10 secretion and inhibiting tumor necrosis factor-α through balancing the production of proinflammatory cytokines and anti-inflammatory cytokine in TLR4 signaling [8]. Glycyrrhiza uralensis extracts could strongly inhibit NF-κB-mediated inflammatory and Nrf2-ARE-anti-oxidative stress signaling pathways [9]. And active constitutions extracted by organic solvent, such as ethanol and n-butanol, in each herb of WTD have been studied. However, to our knowledge, the vast majority of TCM formulas need to be decocted in order to absorb better and produce the best therapeutic effects. Thus, it is necessary to get the chemical profiling of the aqueous extract of WTD for understanding its biological activity or pharmacological activity.

Recently, ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF-MS) has been widely used to characterize chemical profiling of plants [10–12]. UPLC columns packed with sub-2 μm particles could improve the separation efficiency, heighten the resolution and shorten the LC run time compared with traditional HPLC

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columns [13,14]. And high resolution mass spectrometry such as Q-TOF-MS could obtain a more specific and accurate mass when trying to identify constitutions of herbs. In order to obtain the chemical profiling of WTD, an UPLC–ESI–Q-TOF method has been established in both positive and negative ion mode. The constitutions in WTD were identified or tentatively characterized based on the accurate mass and tandem MS behavior, and the sources of these components were confirmed by comparing the base peak chromatograms of WTD and individual herbal extracts.

2. Materials and methods

2.1. Materials

Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata were purchased from Beijing Huamiao Chinese Medicine Engineering Development Center (Beijing, China). All herb medicines were identified by Prof. Shumin Wang (Changchun University of Chinese Medicine). Hypaconitine, ephedrine, paeoniflorin, liquiritin, glycyrrhizic acid, calycosin, formononetin and astragaloside IV were obtained from the Chinese Authenticating Institute of Material and Biological Products (Beijing, China). Benzoylmesaconine, benzoylhypacoitine and benzoylaconine were purchased from LanYuan Biological technology Co., Ltd. (Shanghai, China). Leucine enkephalin and sodium formate was purchased from Waters (Milford, USA). HPLC-grade acetonitrile and formic acid were obtained from Fisher Scientific (Loughborough, UK). Ultrapure water was prepared by the Milli-Q plus (Milford, MA, USA) water purification system.

2.2. Extraction

Powder of Aconiti Radix Cocta 1 g, Ephedrae Herba 1.5 g, Paeoniae Radix Alba 1.5 g, Astragali Radix 1.5 g and Glycyrrhizae Radix Preparata 1.5 g were immersed in 70 mL deionized water for 1 h, and then heated to refluxing for 1.5 h. Water as 8 times of the above total weight was added for another 1.5 h refluxing after filtering. The filtered extraction solutions were combined and diluted to attain a solution of 35 mg crude drug per milliliter. Samples of individual medicines were extracted as the same way adopted above and all samples were filtered through a 0.22 μm filter membrane before UPLC–MS analysis.

2.3. UPLC–MS and UPLC–MS^2 analysis

A Waters Acquity UPLC system coupled with a Q-TOF SYNAPT G2 High Definition Mass Spectrometer (Waters, USA) was used to analyze the chemical profiling of WTD. Chromatographic separation was performed on a Waters ACQUITY UPLC BEH C18 Column (1.7 μm, 2.1 × 50 mm) keeping at 35 °C. 0.1% aqueous formic acid (v/v) (A) and acetonitrile (B) were used as the mobile phase. The gradient elution with the flow rate of 0.3 mL/min was performed as follows: 10% B at 0–2 min, 10–15% B at 2–7 min, 15–30% B at 7–15 min, 30–39% B at 15–21 min, 39% up to 100% at 21–25 min. The sample inject volume was 5 μL. The MS analysis was carried out by the ESI source in both positive and negative ion mode, and full-scan mass range was 100–1200 Da. The source temperature was 110 °C, and the desolvation gas temperature was 300 °C. The flow rates of cone and desolvation gas were set at 30 L/h and 600 L/h, respectively. The voltages of capillary, cone and extraction cone in positive ion mode were set at 2.5 kV, 35 V and 5.0 V, respectively, and in negative ion mode, they were set at 2.0 kV, 35 V and 5.0 V, respectively. Leucine enkephalin (m/z 556.2771 in positive ion mode and m/z 554.2615 in negative ion mode) was used as a reference mass. Sodium formate was used to set up mass spectrometer calibration in both positive and negative ion mode. MS^E was applied for the MS/MS analysis with the low collision energy of 5 eV and the high collision energy of 25–35 eV.

3. Results and discussion

3.1. Optimization of LC and MS conditions

As the components in TCM are complex, it is necessary to chose sensible mobile phase to improve reproducibility, selectivity, or peak shape. Mobile phase systems, such as acetonitrile-aqueous, methanol-aqueous, acetonitrile-aqueous with 0.1% formic acid and acetonitrile-aqueous with 5 mM ammonium bicarbonate adjusted to pH 10.5 with ammonia were selected to optimize the LC conditions. Both acetonitrile-aqueous with 0.1% formic acid and acetonitrile-aqueous with 5 mM ammonium bicarbonate adjusted to pH 10.5 with ammonia could reduce the peak tailing, but in negative ion mode it gave a weak signal response when alkaline mobile phase mentioned above was used. Thus, acetonitrile-aqueous with 0.1% formic acid on the optimized gradient were selected as the mobile phase. The MS parameters were optimized on the basis of improving the ion intensity, and the optimized parameters were set as Section 2.3. Base peak chromatograms of WTD and its five individual herbs in positive and negative ion mode are shown in Figs. 1 and 2.

3.2. Identification of main constituents in WTD

74 components including alkaloids, monoterpene glycosides, triterpene saponins, flavones and flavone glycosides in WTD were identified (Tables 1 and 2). And their sources were confirmed by comparing the base peak chromatograms of WTD and its five individual herbal extracts. For the standard available compound, it was identified by comparing retention time and accurate mass. For the standard unavailable compound, the structure was presumed mainly based on accurate mass and tandem mass spectra. In this study, the molecular formula was established by high-accuracy quasi-molecular ion such as $[M+H]^+$, $[M+Na]^+$, $[M-H]^-$ and $[M+HCOO]^-$ within mass error of 15 ppm and fractional isotope abundance. Then the most rational molecular formula was searched in chemical databases, such as Chemspider (www.chemspider.com) and Massbank (http://www.massbank.jp). When several isomers were matched, the structure that had been reported previously from the five individual herbs of WTD would have higher possibility than the other isomers. Finally, fragment ions were used to further confirm the chemical structure. The structures of main active constitutions in WTD are showed in Fig. 3.

3.2.1. Alkaloids

43 alkaloids in WTD were indentified in positive ion mode. Four of them were the major constituents in Ephedrae Herba and the others were diterpenoid alkaloids in Aconiti Radix Cocta. Compound 8 was determined as ephedrine by comparing with the reference compound. To indentify other alkaloids in WTD from Ephedrae Herba, the feature fragment ions of ephedrine were investigated. The possible fragmentation mechanism is depicted in Fig. 4A. It is shown that the neutral losses like H_2O, CH_4 and CH_3NH_2 are the main fragment patterns of ephedrine. Compounds 5 and 6 showed $[M+H]^+$ ion at m/z 152, 14 Da less than that of ephedrine. They may be the previously reported norephedrine or norpseudoephedrine. Because the information obtained by MS was not enough, these optical isomers could not be distinguished. Compound 11 gave $[M+H]^+$ ion at m/z 180 and similar fragment patterns with ephedrine, and it could be identified as methylephedrine.

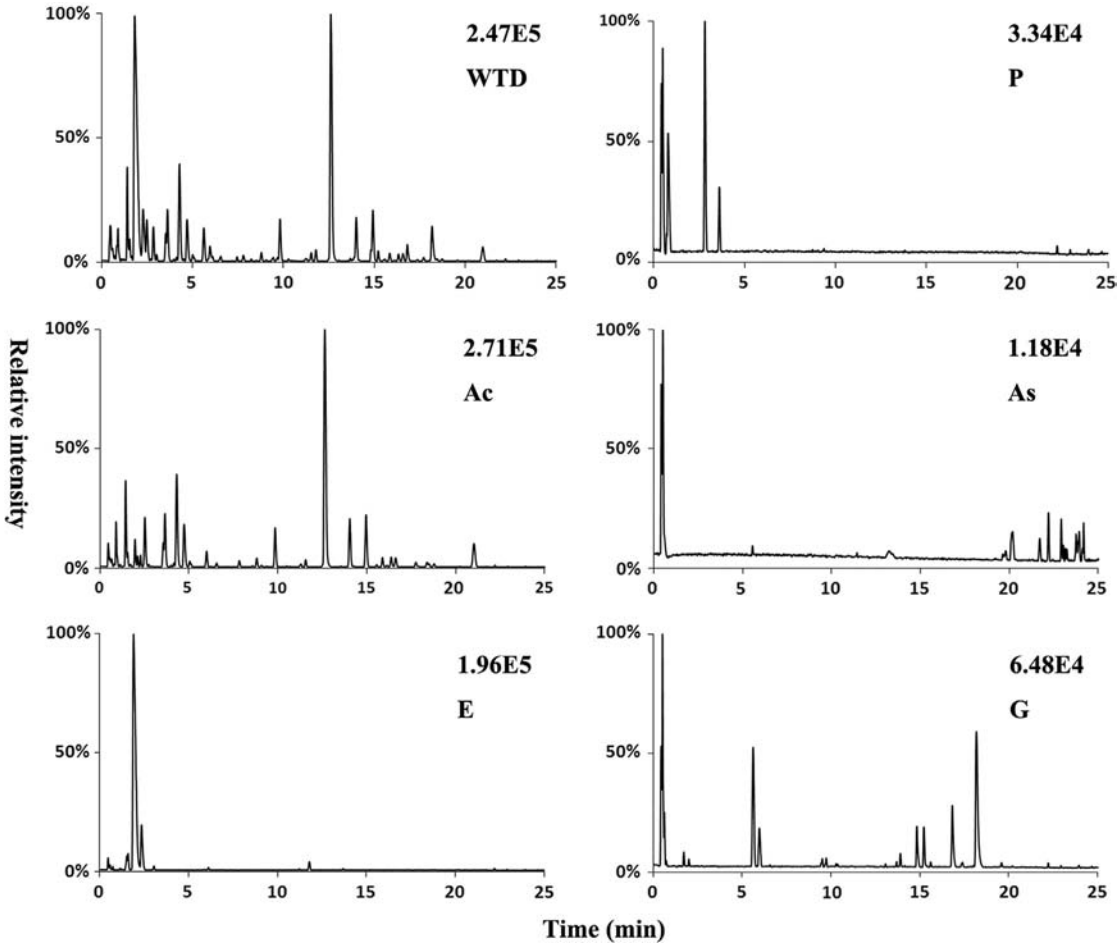


Fig. 1. Base peak chromatograms of WTD and the five individual herbs in positive ion mode. Ac=Aconiti Radix Cocta, E=Ephedrae Herba, P=Paeoniae Radix Alba, As=Astragali Radix, and G=Glycyrrhiza Radix Preparata.

The fragmentation pathways of aconitum alkaloids have been investigated previously [15–17]. The neutral losses of H_2O, MeOH, AcOH or BzOH were commonly observed in tandem mass spectrum of aconitum alkaloids. In addition, the neutral loss of 28 Da corresponding to eliminate one molecule of CO or C_2H_4 was the feature loss in diester-diterpenoid alkaloids (DDA). The order of eliminations of carboxyl, benzyl, ethyl or methyl and methoxy in DDAs was also investigated previously [18]. Hydroxyl at C1 position was the most active site for monoester-diterpenoid alkaloids (MDA), and moreover, hydroxyl at C15 position could not be eliminated even at a high fragmentor voltage in amine diterpenoid alkaloids (ADA) [17]. These findings could provide principles for understanding tandem mass spectrum of aconitum alkaloids. Take hypaconitine as an example (Fig. 4B), fragment ions at *m/z* 584, 556, 524, 496, 492, 464 and 338 were corresponding to $[M+H-CH_3OH]^+$, $[M+H-AcOH]^+$, $[M+H-AcOH-CH_3OH]^+$, $[M+H-AcOH-CH_3OH-CO]^+$, $[M+H-AcOH-2CH_3OH]^+$, $[M+H-AcOH-2CH_3OH-CO]^+$ and $[M+H-3CH_3OH-BzOH]^+$, respectively.

Hypaconitine, benzoylmesaconine, benzoylhypacoitine and benzoylaconine were identified by pure standards. Compounds 34, 38, 52 and 62 showed $[M+H]^+$ ion at *m/z* 606, 620, 648 and 662, 16 Da greater than that of benzoylmesaconine, benzoylaconine, mesaconine and aconine, respectively. As C10 position was commonly substituted by hydroxyl in aconitum alkaloids, they were presumed as 10-OH benzoylmesaconine, 10-OH benzoylaconine, 10-OH mesaconine and 10-OH aconine, respectively. Compounds 53 and 66 gave $[M+H]^+$ ion at *m/z* 630 and 588, 16 Da less than that of aconine and benzoylaconine. Their tandem mass spectra were similar to hypaconitine and benzoylhypacoitine, and some fragment ions of compound 53 were 14 Da greater than the fragment ions of hypaconitine. They could be the reported deoxyaconine and benzoyldeoxyaconine. Likewise, compound 44 was identified as benzoyl-3,13-deoxymesaconine. Compounds 50, 58 and 61 were 60 Da less than mesaconitine, aconitine and hypaconitine, respectively. They were presumed as pyromesaconitine, pyroaconitine, and pyrohypaconitine, respectively, attributing to one molecule of acetic acid eliminated from mesaconitine, aconitine and hypaconitine, respectively. Similarly, Compounds 4, 10 and 15 were identified as mesaconine, aconine and hypaconine, respectively. Compounds 2, 9, 12, 26, 27 and 30 could be considered as chuanfumine, isotalatizidine, songorine, guanfu base H, chasmanine and talatizamine, respectively, based on their molecular weight and tandem fragment patterns. Compounds 16, 18 and 22 showed the same $[M+H]^+$ ion at *m/z* 454. Due to the fact that compound 16 was the most abundance of them, it was the reported fuziline. The other two isomers were not distinguished. Compounds 19, 21 and 29 gave the same $[M+H]^+$ ion at *m/z* 438. Owing to its high abundance, compound 19 was the reported neoline. Fragment ions of compound 21 were 16 Da more than the corresponding fragment ions of talatizamine, implying the presence of an additional hydroxy. It was tentatively characterized as 10-OH talatizamine. Compound 29 could be the reported bullatine B. Compounds 3 and 7 had the same molecular weight of 423 Da. Methoxy at C16 position was more easily eliminated than hydroxyl at C15 position. Compound 3 showed fragment ions without $[M+H-2H_2O]^+$, thus it was presumed as senbusine B. Compound 7 could be the reported senbusine A.

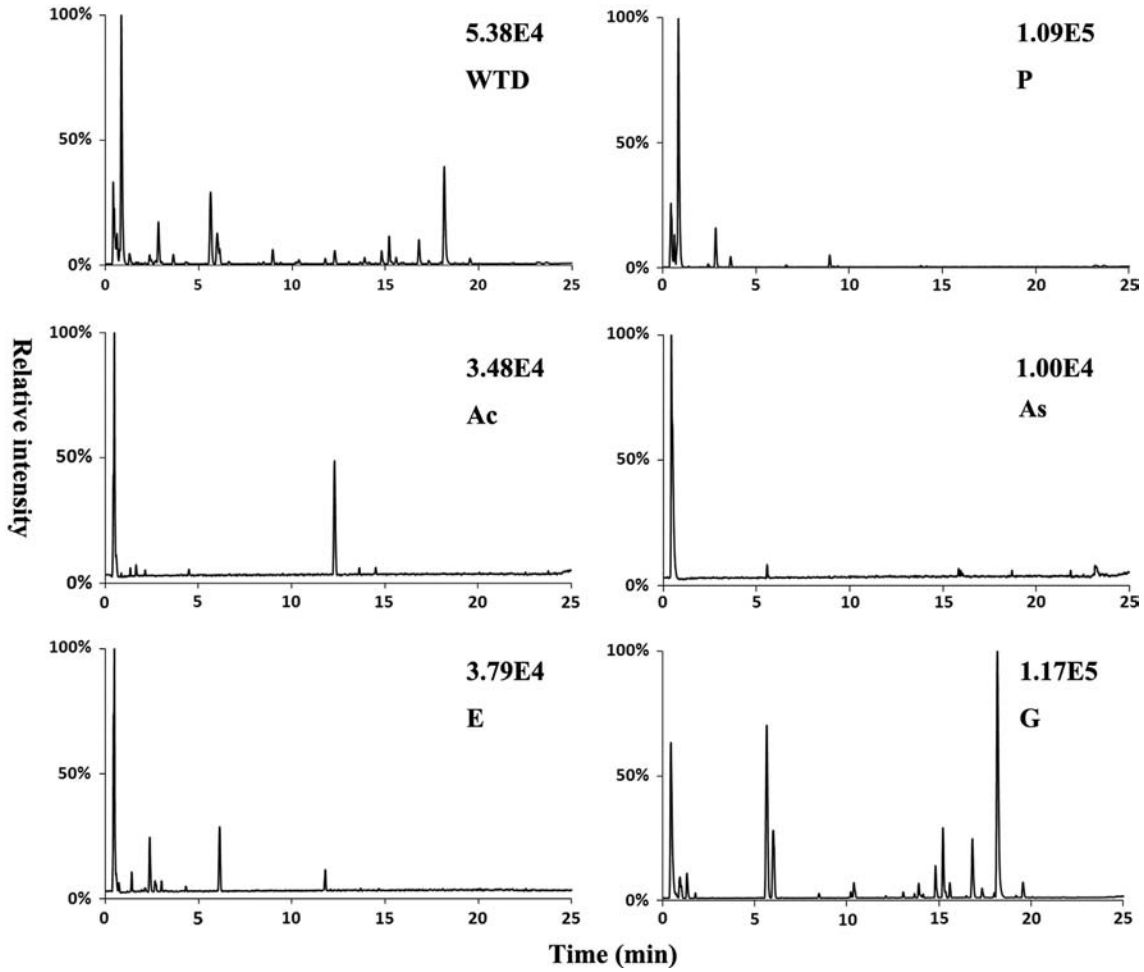


Fig. 2. Base peak chromatograms of WTD and the five individual herbs in negative ion mode. Ac=Aconiti Radix Cocta, E=Ephedrae Herba, P=Paeoniae Radix Alba, As=Astragali Radix, and G=Glycyrrhiza Radix Preparata.

3.2.2. *Monoterpene glycosides*

Monoterpene glycosides from Paeoniae Radix Alba showed quasi-molecular ion $[M+H]^+$ and $[M+Na]^+$in positive ion mode, or $[M-H]^-$ and $[M+HCOO]^-$ in negative ion mode. The monoterpene glycosides in Radix Paeoniae were usually esterified with benzoic acid, p-hydroxybenzoic acid, gallic acid and substituted by sulfuric acid [19,20]. The neutral losses of a benzoic acid or a glucosyl group, and aglycone ions at *m/z* 195 or 197, or their fragmentations of losing H_2O and CO were usually detected in these compounds [21,22]. Paeoniflorin was identified by comparing retention time and accurate mass with the pure standard. The tandem mass spectra and possible fragmentation pathways of paeoniflorin are illustrated in Fig. 5. The ion at *m/z* 179 was the base peak in positive ion mode corresponding to the glucosyl residue. In negative ion mode, the ion at *m/z* 449 corresponding to the loss of CH_2O was the feature fragment ion. In that case, elimination of a benzoic acid and a glucosyl residue happened successively. The ion at *m/z* 121 was generated by benzoic acid.

Compound 13 and paeoniflorin had the same molecular weight, but there was no ion at *m/z* 449 observed in MS^2 of compound 13 in negative ion mode. It was identified as albiflorin. Compound 1 was 64 Da greater than paeoniflorin, showing similar fragment pathways as paeoniflorin such as successively elimination of a benzoic acid and a glucosyl residue. It was presumed as paeoniflorin sulfonate. Compound 43 showed $[M+H]^+$ and $[M+Na]^+$ ions at *m/z* 585 and 607, respectively, and it gave an fragment ion at *m/z* 503. It was indicated that compound 43 had an additional benzoyl than paeoniflorin. It could be the reported benzoylpaeoniflorin. Compound 72 gave $[M-H]^-$ at *m/z* 631, 152 Da greater than that of paeoniflorin. Its fragment ion at *m/z* 313 was generated by a galloyl glucose residue. It was tentatively assigned as galloyl paeoniflorin. Compound 74 showed $[M-H]^-$ at *m/z* 647, 104 Da greater than that of paeoniflorin sulfonate. And the fragment ion at *m/z* 259 was the feature fragment in tandem mass spectra of paeoniflorin sulfonate analogs. It could be the reported benzoylpaeoniflorin sulfonate.

3.2.3. *Flavones and their glycosides*

Flavones and their glycosides detected in WTD came from Astragali Radix and Glycyrrhiza Radix Preparata. Flavones only showed $[M+H]^+$ in positive ion mode, but flavones glycosides showed both $[M+H]^+$ and $[M+Na]^+$. In negative ion mode, only $[M-H]^-$ was observed for flavones and their glycosides. The main MS/MS behavior of aglycones described previously was retro Diels-Alder (RDA) fragmentation pathway. Losses of small molecules and/or radicals like $CH_3\cdot$ and CO were also discussed [23–25]. For flavones glycosides, in both positive and negative ion mode, the product ions were detected due to the cleavage at the glycosidic linkages [26], and fragment ions with low *m/z* were the same as the fragment ions obtained in their aglycones, such as liquiritin (Fig. 6). The loss of 162 Da was the characteristic neutral loss of flavonoid O-glycosides in both positive and negative ion mode. RDA fragments of *m/z* 137 and 119, *m/z* 135 and 119 were the feature fragments in positive and negative ion mode, respectively. Retrocyclisation cleavage also happened in positive ion mode.

Table 1
Compounds identified in WTD by UPLC–MS in positive ion mode.

Peak no.	t_R (min)	Measured mass $[M+H]^+$/ $[M+Na]^+$	Molecular formula	Indentify	Error (ppm)[a]	Source[b]	MS^2
1	0.84	−/567.1153	$C_{23}H_{28}O_{13}S$	Paeoniflorin sulfonate	1.8	P	261.0455, 179.0713
2	0.90	394.2592/−	$C_{22}H_{35}NO_5$	Chuanfumine	1.0	Ac	376.2487, 358.2401, 340.2285, 328.2221, 322.2172
3	0.96	424.2666/−	$C_{23}H_{37}NO_6$	Senbusine B	−6.5	Ac	406.2636, 374.2288,
4	1.41	486.2691/−	$C_{24}H_{39}NO_9$	Mesaconine	−1.4	Ac	468.2589, 454.2482, 436.2307, 422.2160, 404.2086
5	1.47	152.1067/−	$C_9H_{13}NO$	Norephedrine or Norpseudoephedrine	−2.0	E	134.0996, 117.0718, 115.0563
6	1.54	152.1069/−	$C_9H_{13}NO$	Norephedrine or Norpseudoephedrine	−0.6	E	134.0996, 117.0718, 115.0563
7	1.67	424.2644/−	$C_{23}H_{37}NO_6$	Senbusine A	−11.7	Ac	406.2636, 388.2494, 374.2373, 356.2195
8	1.82	166.1236/−	$C_{10}H_{15}NO$	Ephedrine	1.8	E	148.1110, 133.0868, 132.0829, 117.0718, 115.0563
9	1.82	408.2775/−	$C_{23}H_{37}NO_5$	Isotalatizidine	7.6	Ac	390.2635, 378.2656, 360.2581, 358.2401, 342.2437
10	2.21	500.2842/−	$C_{25}H_{41}NO_9$	Aconitine	−2.4	Ac	482.2611, 468.2599, 450.2510, 436.2307, 418.2192
11	2.27	180.1385/−	$C_{11}H_{17}NO$	Methylephedrine	1.1	E	162.1261, 147.1050, 146.0970, 117.0718, 115.0563
12	2.50	358.2374/−	$C_{22}H_{31}NO_3$	Songorine	−0.8	Ac	340.2285, 330.2413, 322.2172, 312.2290
13	2.84	481.1695/503.1498	$C_{23}H_{28}O_{11}$	Albiflorin	−1.9	P	319.1181, 197.0799, 175.0718, 133.0666
14	3.02	−/357.1868	$C_{16}H_{30}O_7$	Not indentified	−4.5	E	317.0630, 203.0539
15	3.52	470.2744/−	$C_{24}H_{39}NO_8$	Hypaconine	−0.9	Ac	438.2462, 406.2195, 388.2149, 374.2288
16	3.61	454.2799/−	$C_{24}H_{39}NO_7$	Fuziline	0.0	Ac	436.2672, 418.2551, 404.2438, 386.2319, 372.2143, 354.2047
17	3.65	481.1697/503.1498	$C_{23}H_{28}O_{11}$	Paeoniflorin	−1.8	P	179.0713, 151.0744
18	4.12	454.2799/−	$C_{24}H_{39}NO_7$	Delcosine or Bullatine F	0.0	Ac	436.2672, 404.2438
19	4.28	438.2852/−	$C_{24}H_{39}NO_6$	Neoline	0.5	Ac	420.2736, 402.2642, 388.2494, 370.2390, 362.2317, 356.2195, 338.2111, 324.1942
20	4.70	342.1696/−	$C_{20}H_{23}NO_4$	Not indentified	−1.2	Ac	297.1109, 265.0089
21	5.00	438.2852/−	$C_{24}H_{39}NO_6$	10-OH talatizamine	0.5	Ac	406.2548, 388.2494
22	5.01	454.2788/−	$C_{24}H_{39}NO_7$	Delcosine or Bullatine F	−2.4	Ac	436.2672, 404.2438
23	5.09	484.2903/−	$C_{25}H_{41}NO_8$	Pseudaconine	−0.4	Ac	452.2613, 420.2736
24	5.57	285.0738/−	$C_{16}H_{12}O_5$	Not indentified	−6.7	As	253.0518, 225.0527, 137.0226
25	5.62	419.1315/441.1125	$C_{21}H_{22}O_9$	Liquiritin	−5.2	G	257.0823, 147.0465, 137.0226, 119.0474
26	5.93	344.2554/−	$C_{22}H_{33}NO_2$	Guanfu base H	−8.7	Ac	326.2486
27	5.96	422.2885/−	$C_{24}H_{39}NO_5$	Talatizamine	−3.8	Ac	390.2635, 372.2581, 358.2401
28	5.98	551.1821/573.1538	$C_{26}H_{30}O_{13}$	Isoliquiritin apioside	−7.2	G	257.0823, 147.0465, 137.0226, 119.0474
29	6.52	438.2837/−	$C_{24}H_{39}NO_6$	Bullatine B	−3.0	Ac	420.2736, 388.2408
30	7.80	452.2990/−	$C_{25}H_{41}NO_6$	Chasmanine	−3.8	Ac	420.2736, 388.2494, 356.2195
31	8.78	576.2803/−	$C_{30}H_{41}NO_{10}$	Not indentified	0.0	Ac	558.2643, 526.2488
32	9.05	576.2783/−	$C_{30}H_{41}NO_{10}$	Not indentified	−3.5	Ac	558.2643, 526.2488
33	9.35	464.2951/−	$C_{26}H_{41}NO_6$	14-Acetyl talatizamine	−12.1	Ac	432.2773
34	9.81	606.2906/−	$C_{31}H_{43}NO_{11}$	10-OH benzoylmesaconine	−0.5	Ac	588.2817, 574.2646, 556.2546, 542.2388, 524.2305
35	10.28	269.0809/−	$C_{16}H_{12}O_4$	Formononetin	0.3	As	254.0564, 237.0527, 197.0615
36	10.43	257.0806/−	$C_{15}H_{12}O_4$	Liquiritigenin	−0.8	G	147.0465, 137.0226, 119.0474
37	11.50	285.0722/−	$C_{16}H_{12}O_5$	Calycosin	−12.3	As	270.0555, 253.0518, 225.0527, 197.0615, 137.0226
38	11.51	620.3065/−	$C_{32}H_{45}NO_{11}$	10-OH benzoylaconine	0.0	Ac	602.2960, 588.2817, 570.2712, 556.2649, 538.2468
39	12.60	590.2972/−	$C_{31}H_{43}NO_{10}$	Benzoylmesaconine	2.0	Ac	572.2924, 558.2714, 540.2614, 526.2488, 508.2337, 494.2170, 482.2226, 476.2089
40	13.66	1001.4581/1023.4315	$C_{48}H_{72}O_{22}$	24-OH Licorice saponin A3	−0.7	G	825.4297, 663.3834, 649.3859, 631.3058, 487.3450, 469.3008, 451.3207
41	13.89	897.4210/919.3862	$C_{44}H_{64}O_{19}$	22-Acetoxyl licorice saponin G2	10.6	G	721.3832, 545.3448, 527.3349, 509.3307, 375.0514
42	14.00	604.3123/−	$C_{32}H_{45}NO_{10}$	Benzoylaconine	1.2	Ac	586.3068, 572.2924, 554.2723, 522.2462, 508.2238
43	14.14	585.2040/607.1688	$C_{30}H_{32}O_{12}$	Benzoylpaeoniflorin	−16.1	P	503.3365, 485.3256, 341.1018
44	14.19	558.3033/−	$C_{31}H_{43}NO_8$	Benzoyl-3,13-deoxymesaconine	−4.8	Ac	526.279
45	14.81	985.4648/1007.4456	$C_{48}H_{72}O_{21}$	Licorice saponin A3	0.9	G	809.4334, 647.3778, 615.3877, 453.3335
46	14.92	574.3026/−	$C_{31}H_{43}NO_9$	Benzoylhypaconine	2.6	Ac	542.2797, 524.2707, 510.2507, 492.2417
47	15.20	881.4171/903.3950	$C_{44}H_{64}O_{18}$	22-Acetoxyl glycyrrhizic acid	0.7	G	705.3865, 529.3493, 511.3399, 375.0514
48	15.51	516.2956/−	$C_{29}H_{41}NO_7$	Not indentified	0.0	Ac	484.2714 ,442.2635, 414.2683
49	15.58	839.4063/861.3860	$C_{42}H_{62}O_{17}$	Isomer of licorice saponin G2	0.4	G	663.3721, 469.3302, 451.3207, 375.0514
50	15.84	572.2854/−	$C_{31}H_{41}NO_9$	Pyromesaconitine	0.0	Ac	554.2723, 540.2614, 522.2462, 508.2337, 490.2219
51	16.10	558.3057/−	$C_{31}H_{43}NO_8$	Benzoyl-3,13-deoxyaconine	−0.7	Ac	526.279
52	16.32	648.3030/−	$C_{33}H_{45}NO_{12}$	10-OH mesaconitine	2.3	Ac	616.2684, 602.2960, 598.2704, 588.2817, 570.2712, 556.2546, 550.2465
53	16.58	588.3169/−	$C_{32}H_{45}NO_9$	Benzoyldeoxyaconine	0.3	Ac	556.2959, 524.2707, 496.2377
54	16.81	839.4046/861.3860	$C_{42}H_{62}O_{17}$	Licorice saponin G2	−1.7	G	663.3721, 487.3450, 469.3302, 451.3207, 375.0514
55	17.12	785.4807/807.4547	$C_{41}H_{68}O_{14}$	Astragaloside IV	5.7	As	605.4075, 587.4001, 569.3823, 473.3640, 455.3507, 437.3386, 419.3310,
56	17.32	865.4205/887.4004	$C_{44}H_{64}O_{17}$	22-Acetoxyl licorice saponin C2	−1.3	G	689.3850, 513.3531, 495.3475, 435.3252, 417.3148, 375.0514
57	17.36	839.4063/861.3860	$C_{42}H_{62}O_{17}$	Isomer of licorice saponin G2	0.4	G	663.3721, 487.3450, 469.3302, 451.3207, 375.0514
58	17.84	586.3041/−	$C_{32}H_{43}NO_9$	Pyroaconitine	5.1	Ac	554.2827, 536.2265, 504.2511
59	18.17	823.4109/845.3873	$C_{42}H_{62}O_{16}$	Glycyrrhizic acid	−0.2	G	647.3778, 471.3452, 453.3335, 435.3252, 407.3329, 375.0514

Table 1 (*continued*)

Peak no.	t_R (min)	Measured mass $[M+H]^+$/ $[M+Na]^+$	Molecular formula	Indentify	Error (ppm)[a]	Source[b]	MS^2
60	18.33	632.3056/–	$C_{33}H_{45}NO_{11}$	Mesaconitine	−1.4	Ac	600.2761, 572.2924, 522.2361
61	18.44	556.2903/–	$C_{31}H_{41}NO_8$	Pyrohypaconitine	−0.4	Ac	524.2606, 492.2320
62	18.72	662.3161/–	$C_{34}H_{47}NO_{12}$	10-OH aconitine	−1.5	Ac	612.2831, 602.2960, 570.2712, 556.2627
63	18.74	827.4828/849.4619	$C_{43}H_{70}O_{15}$	Astragaloside II	1.4	As	647.389, 629.4005, 473.3640, 455.3507, 437.3386
64	19.57	823.4112/845.3873	$C_{42}H_{62}O_{16}$	Licorice saponin H2 or K2	0.1	G	647.3778, 471.3452, 453.3335, 375.0514
65	20.96	616.3114/–	$C_{33}H_{45}NO_{10}$	Hypaconitine	−0.3	Ac	584.2823, 556.2959, 524.2606, 496.2464, 492.2417, 464.2476, 338.1788
66	23.48	630.3294/–	$C_{34}H_{47}NO_{10}$	Deoxyaconitine	3.3	Ac	598.3026, 570.3026, 538.2773, 510.2804

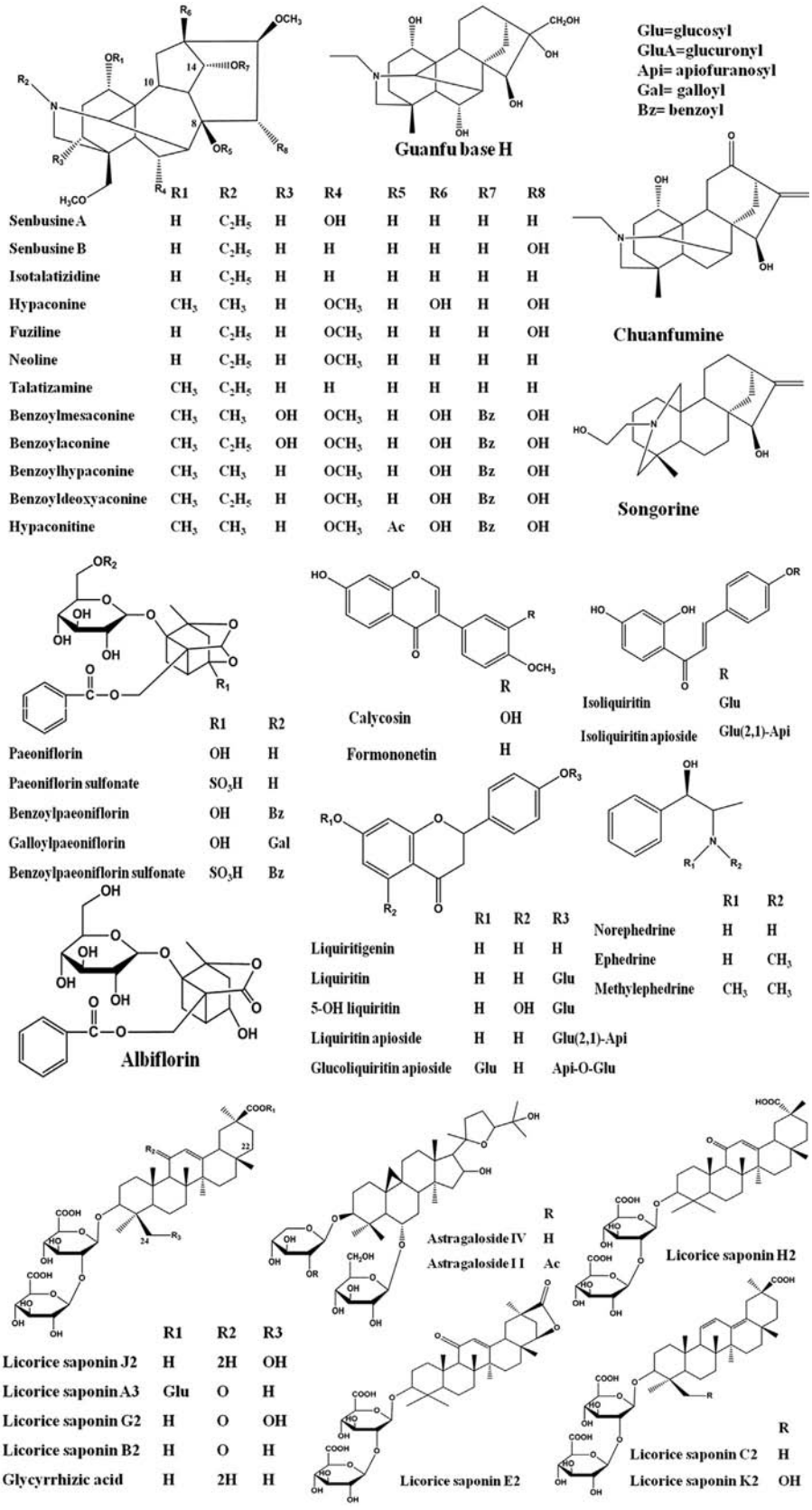
[a] Error was calculated by $[M+H]^+$, if there is no $[M+H]^+$, $[M+Na]^+$ was used.
[b] Ac, E, P, As, G were shorten for Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata.

Table 2
Compounds identified in WTD by UPLC–MS in negative ion mode.

Peak no.	t_R (min)	Measured mass $[M-H]^-$/ $[M-HCOO]^-$	Molecular formula	Indentify	Error (ppm)[a]	Source[b]	MS^2
67	0.62	169.0141/–	$C_7H_6O_5$	Gallic acid	5.9	P	125.0221
1	0.86	543.1193/–	$C_{23}H_{28}O_{13}S$	Paeoniflorin sulfonate	4.8	P	497.1121, 421.0795, 375.0743, 259.0279, 213.0206, 121.0294
68	1.78	417.1210/–	$C_{21}H_{22}O_9$	Isoliquiritin	7.2	G	255.0631, 135.0094, 119.0502
69	2.08	711.2150/–	$C_{32}H_{40}O_{18}$	Glucoliquiritin apioside	2.7	G	549.1949, 417.1210, 255.0631
70	2.38	280.1187/–	$C_{14}H_{19}NO_5$	Not indentified	0.7	E	262.1067, 234.1120
13	2.86	479.1525/525.1632	$C_{23}H_{28}O_{11}$	Albiflorin	−4.8	P	357.1222, 327.1061, 121.0294
17	3.65	479.1525/525.1632	$C_{23}H_{28}O_{11}$	Paeoniflorin	−4.8	P	449.1453, 327.1061, 165.0526, 121.0294
71	5.57	283.0601/–	$C_{16}H_{12}O_5$	Not indentified	0.0	As	268.0317, 211.0414
25	5.62	417.1210/–	$C_{21}H_{22}O_9$	Liquiritin	7.2	G	255.0631, 135.0094, 119.0502
28	6.00	549.1615/–	$C_{26}H_{30}O_{13}$	Isoliquiritin apioside	2.2	G	417.1210, 255.0631, 135.0094, 119.0502
71	6.12	577.1555/–	$C_{27}H_{39}O_{14}$	Not indentified	0.5	E	413.0831, 293.0465
72	6.62	631.1662/–	$C_{30}H_{32}O_{15}$	Galloylpaeoniflorin	0.8	P	509.1302, 313.0575
73	8.48	433.1109/–	$C_{21}H_{22}O_{10}$	5-OH liquiritin	−4.6	G	271.0623, 151.0008
74	8.97	647.1409/–	$C_{30}H_{32}O_{14}S$	Benzoylpaeoniflorin sulfonate	−3.1	P	259.2079
75	10.21	549.1649/–	$C_{26}H_{30}O_{13}$	Liquiritin apioside	8.4	G	417.1210, 255.0631, 135.0094, 119.0502
35	10.28	267.0638/–	$C_{16}H_{12}O_4$	Formononetin	−5.2	As	252.0400, 251.0317, 223.0423, 195.0438
36	10.43	255.0631/–	$C_{15}H_{12}O_4$	Liquiritigenin	−8.2	G	135.0094, 119.0502
37	11.52	283.0603/–	$C_{16}H_{12}O_5$	Calycosin	0.7	As	268.0388, 267.0276, 239.0351, 211.0414
76	11.79	361.1843/–	$C_{17}H_{30}O_8$	Not indentified	−3.9	E	315.1970
39	12.59	–/634.2870	$C_{31}H_{43}NO_{10}$	Benzoylmesaconine	1.9	Ac	–
77	13.05	823.4149/–	$C_{42}H_{64}O_{16}$	Licorice saponin J2	4.6	G	351.0580
40	13.67	999.4459/–	$C_{48}H_{72}O_{22}$	24-OH licorice saponin A3	2.8	G	837.3892, 485.3244, 351.0850
41	13.90	895.3937/–	$C_{44}H_{64}O_{19}$	22-Acetoxyl licorice saponin G2	−2.3	G	351.0580
45	14.81	983.4461/–	$C_{48}H_{72}O_{21}$	Licorice saponin A3	−2.1	G	821.4030, 469.3272, 351.0580
47	15.21	879.4042/–	$C_{44}H_{64}O_{18}$	22-Acetoxyl glycyrrhizic acid	3.8	G	351.0580, 193.0344
78	15.28	835.3786/–	$C_{42}H_{60}O_{17}$	24-OH Licorice saponin E2	4.7	G	351.0580
49	15.58	837.3948/–	$C_{42}H_{62}O_{17}$	Isomer of licorice saponin G2	5.4	G	351.0580
79	16.47	881.4231/–	$C_{44}H_{66}O_{18}$	22-Acetoxyl licorice saponin J2	7.5	G	351.0580
80	16.76	819.3837/–	$C_{42}H_{60}O_{16}$	Licorice saponin E2	4.8	G	351.0580
54	16.80	837.3931/–	$C_{42}H_{62}O_{17}$	Licorice saponin G2	3.3	G	351.0580
55	17.12	–/829.4594	$C_{41}H_{68}O_{14}$	Astragaloside IV	0.5	As	–
81	17.34	863.4081/–	$C_{44}H_{64}O_{17}$	22-Acetoxyl licorice saponin C2	2.4	G	351.0580
57	17.34	837.3921/–	$C_{42}H_{62}O_{17}$	Isomer of licorice saponin G2	2.1	G	351.0580
59	18.17	821.3981/–	$C_{42}H_{62}O_{16}$	Glycyrrhizic acid	3.3	G	351.0580
82	19.17	807.4207/–	$C_{42}H_{64}O_{15}$	Licorice saponin B2	5.7	G	351.0580
64	19.53	821.3965/–	$C_{42}H_{62}O_{16}$	Licorice saponin H2 or K2	1.3	G	351.0580
83	20.02	821.3999/–	$C_{42}H_{62}O_{16}$	Licorice saponin H2 or K2	5.5	G	351.0580

[a] Error was calculated by $[M-H]^-$, if there is no $[M-H]^-$, $[M+HCOO]^-$ was used.
[b] Ac, E, P, As, G were shorten for Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata.

Calycosin, formononetin and liquiritin were identified by reference compounds. Compound 36 gave $[M+H]^+$ ion at *m/z* 257 and $[M–H]^-$ ion at *m/z* 255, 162 Da less than that of liquiritin. Its fragment ions were as same as liquiritin in low *m/z* region. It was presumed as liquiritigenin. Compound 68 showed the same MS and MS^2 pattern as liquiritin. It could be the reported isoliquiritin. Compound 73 showed $[M–H]^-$ ion at *m/z* 433 and fragment ion at *m/z* 271, suggesting that it had an additional hydroxyl than



	R1	R2	R3	R4	R5	R6	R7	R8
Senbusine A	H	C_2H_5	H	OH	H	H	H	H
Senbusine B	H	C_2H_5	H	H	H	H	H	OH
Isotalatizidine	H	C_2H_5	H	H	H	H	H	H
Hypaconine	CH_3	CH_3	H	OCH_3	H	OH	H	OH
Fuziline	H	C_2H_5	H	OCH_3	H	H	H	OH
Neoline	H	C_2H_5	H	OCH_3	H	H	H	H
Talatizamine	CH_3	C_2H_5	H	H	H	H	H	H
Benzoylmesaconine	CH_3	CH_3	OH	OCH_3	H	OH	Bz	OH
Benzoylaconine	CH_3	C_2H_5	OH	OCH_3	H	OH	Bz	OH
Benzoylhypaconine	CH_3	CH_3	H	OCH_3	H	OH	Bz	OH
Benzoyldeoxyaconine	CH_3	C_2H_5	H	OCH_3	H	OH	Bz	OH
Hypaconitine	CH_3	CH_3	H	OCH_3	Ac	OH	Bz	OH

	R1	R2
Paeoniflorin	OH	H
Paeoniflorin sulfonate	SO_3H	H
Benzoylpaeoniflorin	OH	Bz
Galloylpaeoniflorin	OH	Gal
Benzoylpaeoniflorin sulfonate	SO_3H	Bz

	R
Calycosin	OH
Formononetin	H

	R
Isoliquiritin	Glu
Isoliquiritin apioside	Glu(2,1)-Api

	R1	R2	R3
Liquiritigenin	H	H	H
Liquiritin	H	H	Glu
5-OH liquiritin	H	OH	Glu
Liquiritin apioside	H	H	Glu(2,1)-Api
Glucoliquiritin apioside	Glu	H	Api-O-Glu

	R1	R2
Norephedrine	H	H
Ephedrine	H	CH_3
Methylephedrine	CH_3	CH_3

	R
Astragaloside IV	H
Astragaloside I I	Ac

	R1	R2	R3
Licorice saponin J2	H	2H	OH
Licorice saponin A3	Glu	O	H
Licorice saponin G2	H	O	OH
Licorice saponin B2	H	O	H
Glycyrrhizic acid	H	2H	H

	R
Licorice saponin C2	H
Licorice saponin K2	OH

Fig. 3. Chemical structures of main constitutions found in WTD.

liquiritin. It was tentatively identified as 5-OH liquiritin. Compounds 28 and 69 had the same molecular weight at 550 Da, and fragment ions at *m*/*z* 417, 255, 135 and 119 implying that they might derive from liquiritin or isoliquiritin with an additional apiosyl substitution. The retention time of isoliquiritin was shorter than liquiritin, thus the retention time of apiosyl substituted

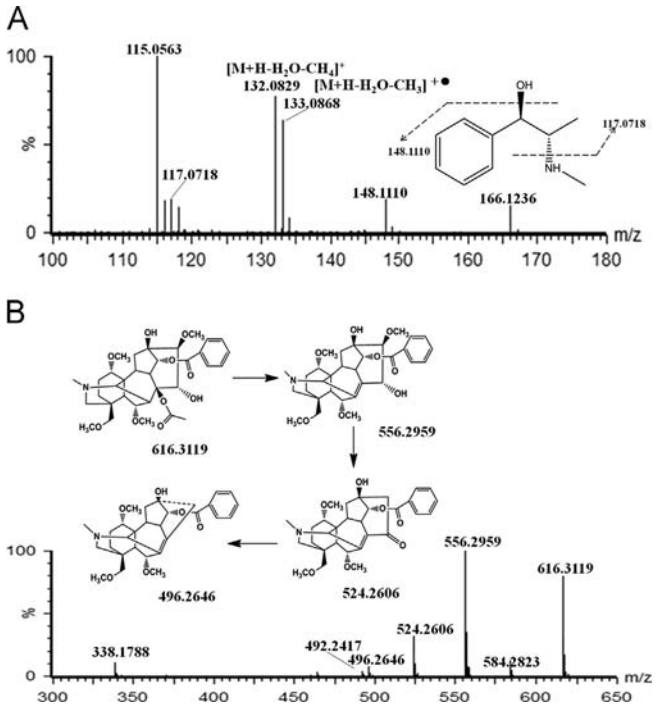


Fig. 4. Tandem mass spectra and possible fragment pathways of ephedrine (A) and hypaconine (B) in positive ion mode.

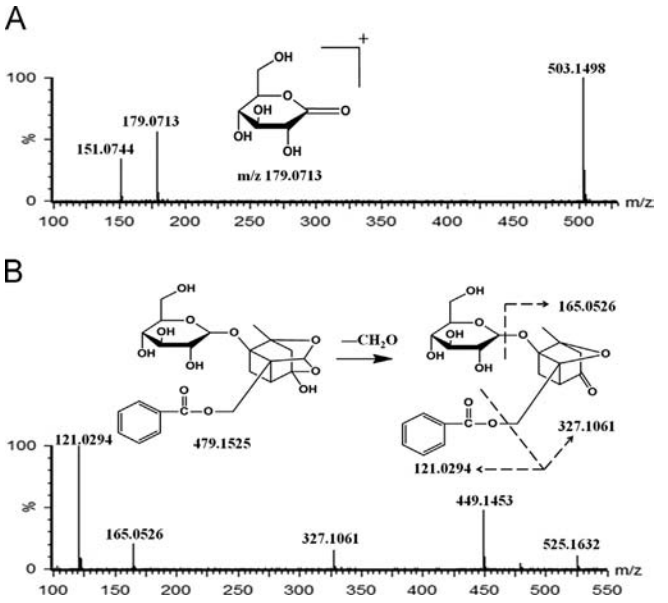


Fig. 5. Tandem mass spectra and possible fragment pathways of paeoniflorin in positive (A) and negative (B) ion mode.

isoliquiritin should be also shorter than apiosyl substituted liquiritin in this study. Compounds 28 and 69 were presumed as isoliquiritin apioside and liquiritin apioside, respectively. Compound 69 showed $[M–H]^-$ ion at *m/z* 711, and fragment ions at *m/z* 549 and 417 corresponding to $[M–Glu–H]^-$ and $[M–Glu–Api–H]^-$, respectively. It could be the reported glucoliquiritin apioside.

3.2.4. Triterpene saponins

The major triterpene saponins in WTD were acidic saponins from Glycyrrhiza Radix Preparata and neutral saponins from Astragali Radix. All triterpene saponins in WTD were observed by $[M+H]^+$ and $[M+Na]^+$ in positive ion mode. In negative ion

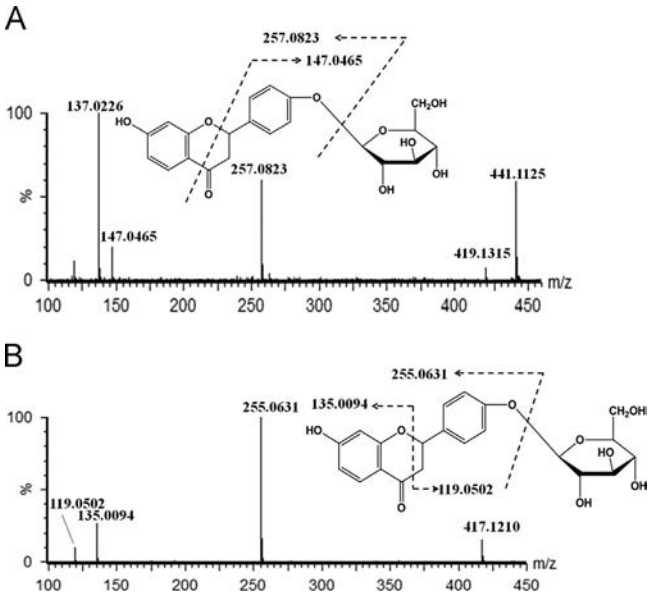


Fig. 6. Tandem mass spectra and possible fragment pathways of liquiritin in positive (A) and negative (B) ion mode.

mode neutral saponins were detected by $[M–H]^-$ and $[M+HCOO]^-$, but only $[M–H]^-$ for acidic saponins was observed. Like other saponins, the main fragmentation pattern was the cleavage at the glycosidic linkages. Glycyrrhizic acid and astragaloside IV were identified by comparing retention time and accurate mass with the standards. The MS^2 spectrum and possible fragmentation pathways of glycyrrhizic acid are depicted in Fig. 7. In positive ion mode, $[M–2GlcA–H_2O+H]^+$ was the base peak. The neutral losses of 176 Da, 2×176 Da and their corresponding sodium adjunction peaks and $[2GlcA+Na]^+$ were also observed. In negative ion mode only $[2GlcA–H]^-$ was detected. Based on the cleavage patterns discussed above, 18 triterpene saponins were indentified with accurate mass and similar tandem MS behavior.

Compounds 64 and 83 showed the same molecular weight at 822 Da and almost consistent tandem MS behavior with glycyrrhizic acid. They may be the reported licorice saponin H2 or K2. Due to the limited information, these isomers could not be distinguished by MS. Compound 77 gave $[M–H]^-$ ion at *m/z* 823 and fragment ion at *m/z* 351, thus it was presumed as licorice saponin J2. Compounds 49, 54 and 57 showed the same MS and MS^2 spectra, and their quasi-molecular ions and fragment ions were 16 Da more than these of glycyrrhizic acid except $[2GlcA+Na]^+$ and $[2GlcA–H]^-$. Because compound 54 was the most abundant among them, it had more possibility to be licorice saponin G2. The other two could be isomers of saponin G2. Compounds 41, 47 and 79 were 58 Da greater than compounds 54, 59 and 77, respectively, implying that they had an additional acetoxyl substituent. Acetoxyl group at C22 position was commonly found in licorice saponins. From these points above, compounds 41, 47 and 79 were identified as 22-acetoxyl licorice saponin G2, 22- acetoxyl glycyrrhizic acid and 22-acetoxyl licorice saponin J2, respectively. Compound 82 was 14 Da less than glycyrrhizic acid. It could be the reported licorice saponin B2. Compound 80 gave $[M–H]^-$ ion at *m/z* 819, and compound 78 showed $[M–H]^-$ ion at *m/z* 835 which was 16 Da greater than that of compound 80. They were considered as licorice saponin E2 and 24-OH licorice saponin E2, respectively. Compound 81 gave $[M–H]^-$ ion at *m/z* 863 to be presumed as 22-acetoxyl licorice saponin C2. Compound 63 gave 42 Da greater $[M+H]^+$ and $[M+Na]^+$ ions than these of astragaloside IV, suggesting that it might have an additional acetyl compared with astragaloside IV. Fragment ions at *m/z* 647

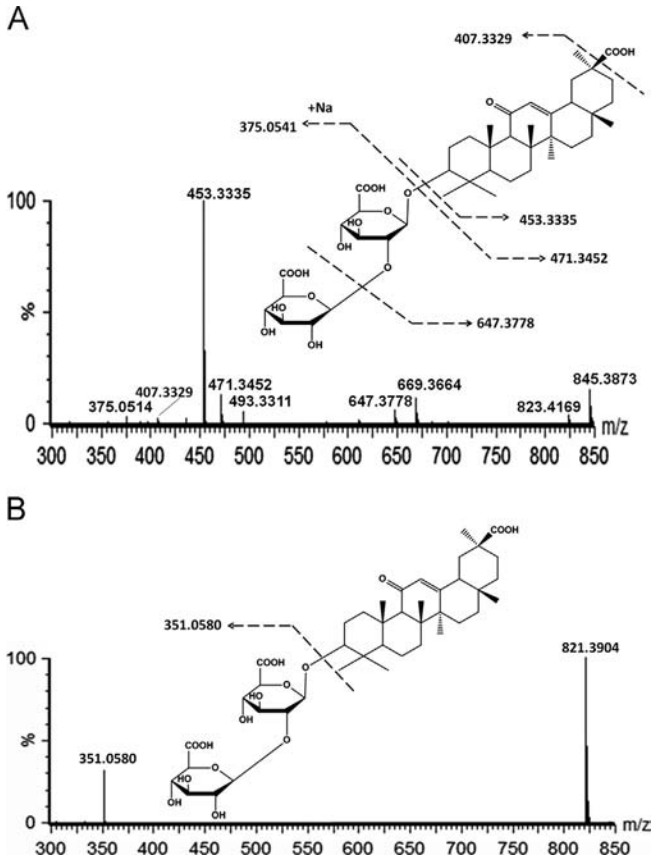


Fig. 7. Tandem mass spectra and possible fragment pathways of glycyrrhizic acid in positive (A) and negative (B) ion mode.

and 473 corresponding to $[M–Glc+H]^+$ and $[M–Glc–Acetyl\ Xyl+H]^+$ showed that compound 63 was astragaloside II.

4. Conclusion

Chemical profiling of WTD were obtained by a simple and effective UPLC–Q-TOF-MS method in both positive and negative ion mode. 74 components including alkaloids, monoterpene glycosides, triterpene saponins, flavones and flavone glycosides were identified or presumed based on their accurate mass and fragment patterns. All the constitutions were also detected in the corresponding individual herbs. The UPLC-Q-TOF-MS method established in this study could also be used for quality control of WTD and provide reference method for quality control of individual herbs. It could also provide a basis for further study *in vivo* of WTD.

Acknowledgments

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