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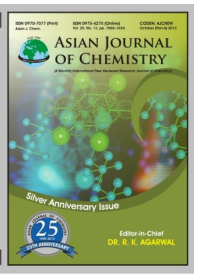

Characterization and Identification of Major Constituents in Baihe Zhimu Decoction by HPLC-MS^n

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Baihe Zhimu decoction is a widely used traditional Chinese medicine recipe which has been proved effective in treating multitudinous diseases. In this paper, by comparing the retention times and MS^n data with those obtained from reference substance and the published data, a total of 39 constituents including xanthones, phenolic glycosides and saponins were detected in Baihe Zhimu decoction. Therefore, this simple and specific HPLC-MS^n method has been developed and applied for the primary investigation of the chemical constituents of this recipe. Besides, the data obtained in this study may be helpful for further pharmacokinetic and metabolic studies of Baihe Zhimu decoction.

Key Words: Baihe Zhimu decoction, Traditional Chinese medicine recipe, HPLC-MS^n.

INTRODUCTION

A traditional Chinese medicine recipe of Baihe Zhimu decoction (BZD) was taken from the Synopsis of Prescriptions of the Golden Chamber (Jinkui Yaolue) by the Chinese therapist Zhang Zhongjing. Baihe Zhimu decoction has been widely used for lily disease, which is related to depression in modern iatrology. The ancient prescription is composed of *Anemarrhena asphodeloides* (Liliaceae) and *Lilium brownii var. viridulum* (Liliaceae). As a traditional Chinese medicine formula, Baihe Zhimu decoction is widely used to clear heat and nourish *yin*[1]. Modern pharmacological research has indicated that Baihe Zhimu decoction is effective in treating asthma[2], Alzheimer's disease[3], memory decay[4,5], diabetes[6], cancer[7,8] and depression[9]. Various analytical techniques currently have been used for identification of crude extracts of *Anemarrhena asphodeloides* and *Lilium brownii var. viridulum*, but only a few researches are related to the constituents in Baihe Zhimu decoction[10].

In this work, 39 constituents including 5 xanthones, 5 phenolic glycosides and 29 saponins were characterized and identified in Baihe Zhimu decoction by HPLC-MS^n. This approach takes the advantage in sensitivity and specificity. In addition, abundant structural information of original constituents from Baihe Zhimu decoction can be acquired by MS^n techniques, which might be helpful for further pharmacokinetic and metabolic studies of Baihe Zhimu decoction.

EXPERIMENTAL

Mangiferin, neomangiferin, timosaponin AII, timosaponin AIII, timosaponin BII, timosaponin BIII and anemarrhenasaponin I were isolated from *A. asphodeloides* in our laboratory. Their structures were identified by NMR data and their purities were higher than 95 % determined by HPLC. HPLC-grade acetonitrile and formic acid were purchased from Dikma Company (Dikma, USA). Triple deionized water was prepared using a Milli-Q system (Millipore, Billerica, MA, USA).

A. asphodeloides (No. ZM061206) was obtained from Shanghai Yanghetang Traditional Chinese Medicine Co. Ltd. (Shanghai, China) and *L. brownii var. viridulum* (No. BH120207) was purchased from Shanghai Kangqiao Pharmaceutical Factory Co. Ltd. (Shanghai, China). They were all authenticated by Prof. C.G. Huang, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. The voucher specimens of these two species were deposited at the Herbarium of Shanghai Institute of Materia Medica.

HPLC-ESI-MS^n analysis was performed on an Agilent series 1200 HPLC instrument coupled with an Agilent 6300 series ion trap mass spectrometer. A Speed Vacplus model vacuum drier (Savant, USA) was used to prepare the samples. HPLC separation was achieved on a reversed-phase Inertsil ODS-3 column (4.6 mm × 150 mm, 5 μm, GL Sciences, Tokyo, Japan) connected to a Dikma EasyGuard Kit C_{18} guard column (4 mm × 2 mm, 5 μm, Dikma Technologies, Beijing, China).

TABLE-1
CHROMATOGRAPHIC RETENTION TIME, MS^n DATA AND RELATIVE ABUNDANCE OF ORIGINAL CONSTITUENTS

No.	Identification	t_R (min)	Precursor ion (m/z)	Data-dependent MS^n data (m/z)
1	Neomangiferin	14.8	583[M-H]	MS^2 [583]: 565, 493, 463, 421, 331, 301, 259;MS^3 [493] : 331, 313, 273, 257; MS^4 [331] : 313, 301, 285, 272, 259
2	Mangiferin	16.3	421[M-H]$^-$	MS^2 [421]: 403, 331, 301, 259;MS^3 [301] : 273, 257,229;MS^4 [257] : 229
3	Regaloside A	17.7	399[M-H]$^-$	MS^2 [399]:273
4	Regaloside D	18.1	399[M-H]$^-$	MS^2 [399]:273
5	Isomangiferin	20.0	421[M-H]$^-$	MS^2 [421]: 403, 331, 301, 259;MS^3 [301] : 273, 257,229;MS^4 [257] : 229
6	Regaloside E	21.2	457[M-H]$^-$	MS^2 [457]: 397; MS^3 [397]: 235,295
7	Mangiferin isomer	22.8	421[M-H]$^-$	MS^2 [421]: 403, 331, 301, 259;MS^3 [301] : 273, 257,229;MS^4 [257] : 229
8	Timosaponin E1	28.2	935[M-H]$^-$	MS^2 [917]: 755, 5 93;MS^3 [755]: 593
9	Regaloside B	28.3	441[M-H]$^-$	MS^2 [441]:381;MS^3 [381] : 279, 219
10	Timosaponin E	28.8	935[M-H]$^-$	MS^2 [935]: 773, 611, 449;MS^3 [773]: 611, 449
11	4-Acetyl derivative of regaloside D	29.1	441[M-H]$^-$	MS^2 [441]:381;MS^3 [381] : 279, 219
12	Timosaponin D isomer	30.4	917[M-H]$^-$	MS^2 [917]: 755, 593; MS^3 [755]: 593; MS^4 [593]: 415
13	Timosaponin N	30.5	935[M-H]$^-$	MS^2 [935]: 773, 611;MS^3 [773]: 611;MS^4 [611]: 449
14	Timosaponin D isomer	31.9	917[M-H]$^-$	MS^2 [917]: 755, 593; MS^3 [755]: 593; MS^4 [593]: 415
15	Timosaponin D isomer	32.5	917[M-H]$^-$	MS^2 [917]: 755, 593; MS^3 [755]: 593; MS^4 [593]: 415
16	Timosaponin BII	33	919[M-H]$^-$	MS^2 [919]: 757, 595; MS^3 [757]: 595, 433; MS^4 [595]: 433
17	26-O-β-D-Glucopyranosyl-3β,26-dihydroxy-5-choleslen-16,22-dioxo-3-O-α-L-rhamnopy-ranosyl-(1→2)-β-D-glucopyranoside or its isomer	34.6	899[M-H]$^-$	MS^2 [899]: 737, 575; MS^3 [737]: 575
18	Timosaponin BII isomer	34.9	919[M-H]$^-$	MS^2 [919]: 757, 595; MS^3 [757]: 595, 433; MS^4 [595]: 433
19	Timosaponin D	35.0	917[M-H]$^-$	MS^2 [917]: 755, 593; MS^3 [755]: 593; MS^4 [593]: 415
20	Timosaponin BII isomer	35.3	919[M-H]$^-$	MS^2 [919]: 757, 595; MS^3 [757]: 595, 433; MS^4 [595]: 433
21	Timosaponin BII isomer	35.9	919[M-H]$^-$	MS^2 [919]: 757, 595; MS^3 [757]: 595, 433; MS^4 [595]: 433
22	Timosaponin BIII isomer	37.3	919[M-H]$^-$	MS^2 [919]: 757, 595; MS^3 [757]: 595, 433; MS^4 [595]: 433
23	26-O-β-D-Glucopyranosyl-3β,26-dihydroxy-5-choleslen-16,22-dioxo-3-O-α-L-rhamnopy-ranosyl-(1→2)-β-D-glucopyranoside or its isomer	37.4	899[M-H]$^-$	MS^2 [899]: 737, 575; MS^3 [737]: 575
24	Timosaponin F (C39)	37.5	771[M-H]$^-$	MS^2 [771]: 609; MS^3 [609]: 591;
25	Timosaponin BIII isomer	37.9	919[M-H]$^-$	MS^2 [919]: 757, 595; MS^3 [757]: 595, 433; MS^4 [595]: 433
26	Mangiferin norathyriol	38	259[M-H]	MS^2 [259]: 215
27	Timosaponin BIII isomer	38.4	919[M-H]$^-$	MS^2 [919]: 757, 595; MS^3 [757]: 595, 433; MS^4 [595]: 433
28	Timosaponin BIII	38.6	901[M-H]$^-$	MS^2 [901]: 739, 577;MS^3 [739]: 577, 457
29	Timosaponin AII isomer	39.8	755[M-H]$^-$	MS^2 [755]: 593;MS^3 [593]: 431
30	Timosaponin BII isomer	39.9	919[M-H]$^-$	MS^2 [919]: 757, 595; MS^3 [757]: 595, 433; MS^4 [595]: 433
31	Timosaponin AII isomer	41.5	755[M-H]$^-$	MS^2 [755]: 593;MS^3 [593]: 431
32	Anemarrhenasaponin Ia	41.7	771[M-H]$^-$	MS^2 [771]: 609; MS^3 [609]: 591;
33	Anemarrhenasaponin I	43.5	757[M-H]$^-$	MS^2 [757]: 595;MS^3 [595]: 577, 465, 433
34	Timosaponin AII isomer	46.0	755[M-H]$^-$	MS^2 [755]: 593;MS^3 [593]: 431
35	Hydroxyl deacylbrownioside	47.6	753[M-H]$^-$	MS^2 [753]: 591;MS^3 [591]: 573, 429, 411
36	Timosaponin AII	48.9	755[M-H]$^-$	MS^2 [755]: 593;MS^3 [593]: 431
37	Timosaponin AIV	52.2	739[M-H]$^-$	MS^2 [739]: 577; MS^2 [577]: 457
38	Timosaponin AIII isomer	54.7	739[M-H]$^-$	MS^2 [739]: 577; MS^2 [577]: 457
39	Timosaponin AIII	55.1	739[M-H]$^-$	MS^2 [739]: 577; MS^2 [577]: 457

MS^n analyses were conducted in negative ion mode and operating parameters were optimized as follows: collision gas, ultra high-purity helium; nebulizing gas, high-purity nitrogen; capillary voltage, 3.5 kV; end plate offset, 500 V; nebulizer, 30 psi.; drying gas flow rate, 10 L/min; drying gas temperature, 300 ºC. For full-scan MS analysis, spectra were recorded in the range of 100-1500 Da.

The mobile phase was composed of acetonitrile (A) and 0.1 % formic acid (B) delivered at a flow rate of 0.5 mL/min. A gradient program was used as follows: 5-10 % A at 0-8 min; 10-20 % A at 8-10 min; 20 % A at 10 -20 min; 20-55 % A at 20-50 min and 55-100 % A at 50-70 min. At the end of the run, 100 % acetonitrile was allowed to flush the column for 5 min and an additional 10 min of post-run time was set to allow equilibration of the column with the starting eluant. The system was then reconfigured to initial conditions within 30 s and the column was reconditioned for 4.5 min. The column temperature was maintained at 25 ºC and the sample injection volume was 10 μL.

Decoction preparation: *A. asphodeloides* (200 g) and *L. brownii var. viridulum* (100 g) were immersed in 3 L (10 times their total weight) of deionized water for 0.5 h. The mixture was boiled for 2 h and then filtered. The decoction preparation was repeated twice. The two extracts were merged and evaporated to 100 mL under reduced pressure at 65 ºC with a rotary evaporator. The solution was obtained at 0.3 g/mL of Baihe Zhimu decoction and filtered through a 0.45 μm membrane for LC/MS analysis.

RESULTS AND DISCUSSION

Mass spectrometry analysis of reference compounds: It was valuable for identification of the components to realize the retention time and MS spectral data of the reference compounds. These data provided a scientific basis for identification of other compounds in Baihe Zhimu decoction. Typical MS^n spectra of seven reference compounds studied in the experiment is shown in Fig. 1 (Table-1).

In the negative ion mode, high abundance of quasi-molecular ion $[M-H]^-$ at *m/z* 421 was firstly exhibited by peak 2 in reference substances and it produced three characteristic product ions at *m/z* 403 ($[M-H-H_2O]^-$), *m/z* 331 ($[M-H-90]^-$) and *m/z* 301 ($[M-H-120]^-$). **S2** was unambiguously identified as mangiferin. According to the literature[11-13], the proposed fragmentation pathway for **S2** is shown in Fig. 2(a).

S1 existed in the extracted ion chromatogram (EIC) of *m/z* 583 showed an increase of 162 Da in molecular weight than that of mangiferin. Moreover, their product ion at *m/z* 421 *via* loss of galactose (Glc) in the MS^2 spectral underwent similar sequential fragmentation with the parent ion of mangiferin. Thus **S1** was ascertained to be neomangiferin. The information above was important for identification and characterization of other xanthones.

Negative ESI analysis of **S3** gave the $[M-H]^-$ ion at *m/z* 919. The MS^2 experiment of the *m/z* 919 ion yielded two prominent ions at *m/z* 757 and 595 through neutral loss of Glc (162 Da) and an additional Gal (324 Da), respectively. The ion at *m/z* 757 was subjected to MS^3 analysis to yield an ion at *m/z* 595 by loss of Gal (162 Da). Then MS^4 spectra of *m/z* 595 resulted in the ion of *m/z* 433 *via* loss of Glc (162 Da). Based on the above data, **S3** was confirmed as timosaponin BII. According to the literature[14-16], the proposed fragmentation pathway for **S3** is shown in Fig. 2(b). Uniformly, **S4**, **S5**, **S6** and **S7** were unambiguously identified as timosaponin BIII, anemarrhenasaponin I, timosaponin AII and timosaponin AIII.

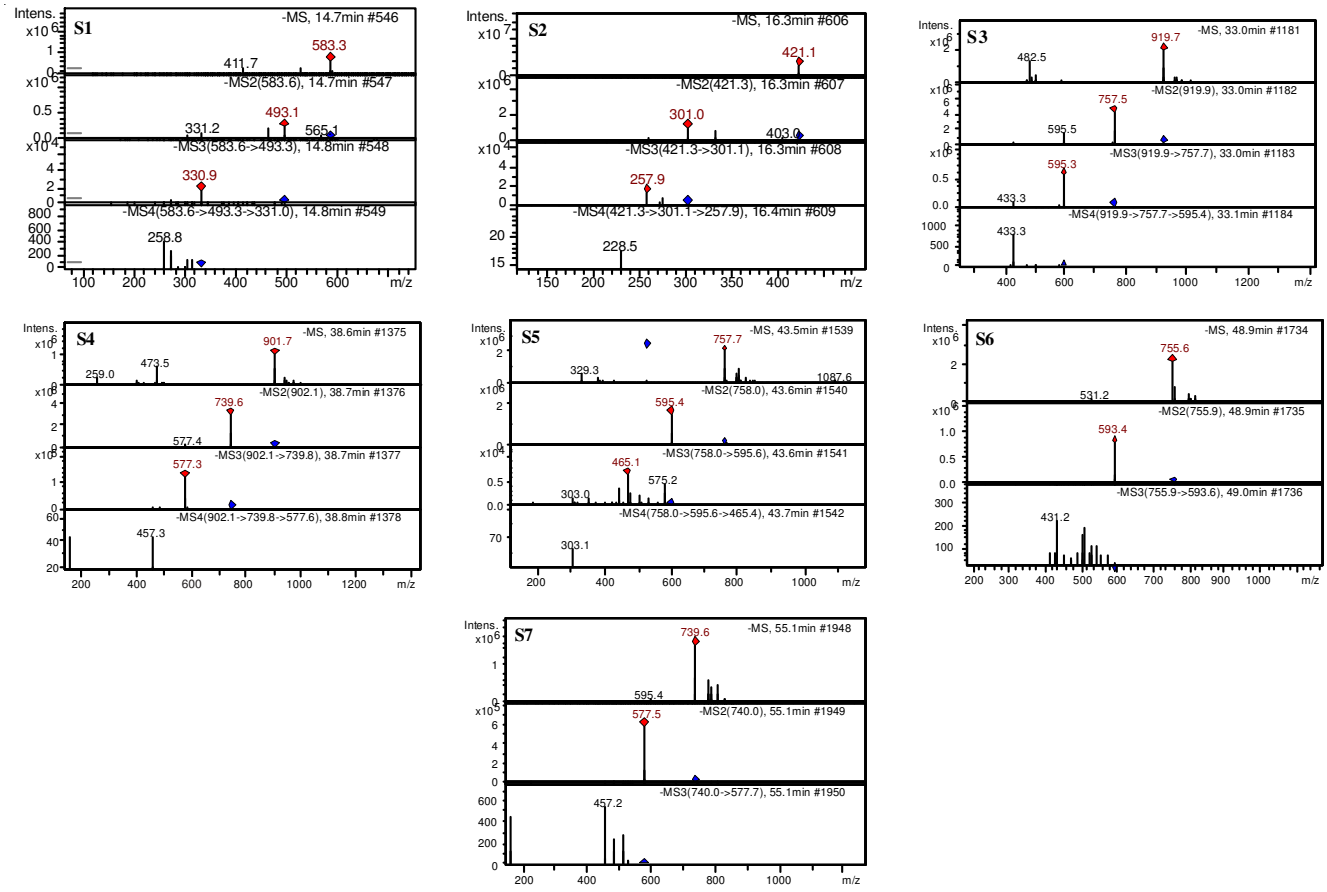

Fig. 1. ESI-MS and MS^n spectra for **S1**-**S7** in negative-ion mode

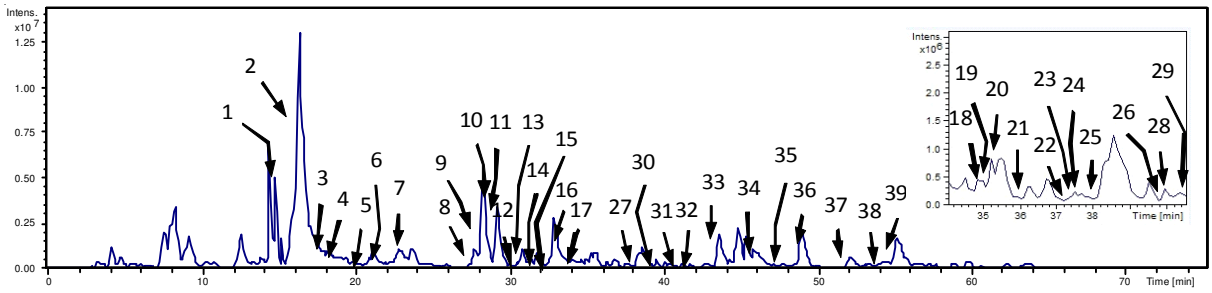

Fig. 2. Total ion chromatograms (TICs) in negative ion mode of Baihe Zhimu decoction extract

In conclusion, the common fragmentation behavoiur of timosaponins were the successive or simultaneous losses of sugar units substituted at O-C (3), O-C (26) in the negative MS experiments. The aglycone ions with one sugar units at *m/z* 595 or 577 could be observed usually. The objective laws of these fragmentations discussed above were effectively used for characterization and identification of other steroid saponins in Baihe Zhimu decoction.

Characterization of the original drugs in Baihe Zhimu decoction: The original constituents in Baihe Zhimu decoction were characterized by HPLC-ESI-MS^n. TICs of them are presented in Fig. 3. Based on the direct comparison with chromatograms of Baihe Zhimu decoction, a total of 39 compounds were identified from Baihe Zhimu decoction and their structures are shown in Fig. 3.

Characterization of xanthones: Based on its retention behavoiur and MS spectrum obtained on-line, compound **1** was characterized as **S1** by comparing it with the reference compound, so it was confirmed as neomangiferin.

By comparing the retention time, molecular weight and fragment ions with that of standard **S2**, compound **2** was confirmed as mangiferin. Compounds **5** and **7** underwent similar sequential fragmentation with the parent ion of mangiferin. According to the literature[10], compound **5** was tentatively identified as isomangiferin and compound **7** was the isomer of mangiferin. Likewise, compound **26** might be mangiferin norathyriol.

Characterization of phenolic glycosides: The pseudomolecular ions of compounds **3** and **4** were *m/z* 399. Ion at *m/z* 237 was attributed to the loss of a glucose residue. According to the polarity and the literature[11], compounds **3** and **4** were tentatively identified as regaloside A and regaloside D, orderly. Likewise, compound **6** could be regaloside E.

Compounds **9** and **11** showed the $[M-H]^-$ at *m/z* 441 in full scan mass spectra and their corresponding MS^2 spectrum showed dominant ion at *m/z* 279 ($[M-H-Glc]^-$). In literature[11], compound **9** was identified as regaloside B, while compound **11** was identified as 4-acety derivative of regaloside D.

Characterization of saponins: By comparison with the standard and referring to the literature[14-16], compound **16** was unambiguously identified as timosaponin BII which has been described to possess various pharmacological activities[3]. Compounds **18**, **20**, **21** and **30** were tentatively identified as its isomers. Compounds **22**, **25** and **27** displayed similar characteristic fragment ions with **S4**, according to the retention time and literature[14], compound **28** could be unambiguously identified as timosaponin BIII, while other five compounds might be its isomers. Similarly, compound **33** was confirmed as anemarrhenasaponin I. In addition, compounds **36** and **39** were unambiguously identified as timosaponin AII and timosaponin AIII by comparison with the standards and referring to the literature[14,17]. Compounds **29**, **31** and **34** were proposed to be the isomers of timosaponin AII, compounds **37** and **38** were deduced to be the isomers of timosaponin AIII.

Three compounds coexisted in the extracted ion chromatogram (EIC) of *m/z* 935 showed different fragment ions in the MS spectra. Compounds **8**, **10** and **13** gave major product ions on MS^2 were $[M-H-18]^-$ (*m/z* 917) and $[M-H-18-162]^-$ (*m/z* 755). Its MS^3 fragmentation was predominated by the successive elimination of galactose residue from $[M-H-18-162]^-$ (*m/z* 755) to give ion at *m/z* 593. Moreover, compounds **10** and **13** successively eliminated a series of hexoses. According to the polarity and the literature[10], it is concluded that compounds **8**, **10** and **13** tentatively identified as timosaponin E1, timosaponin E and timosaponin N, orderly.

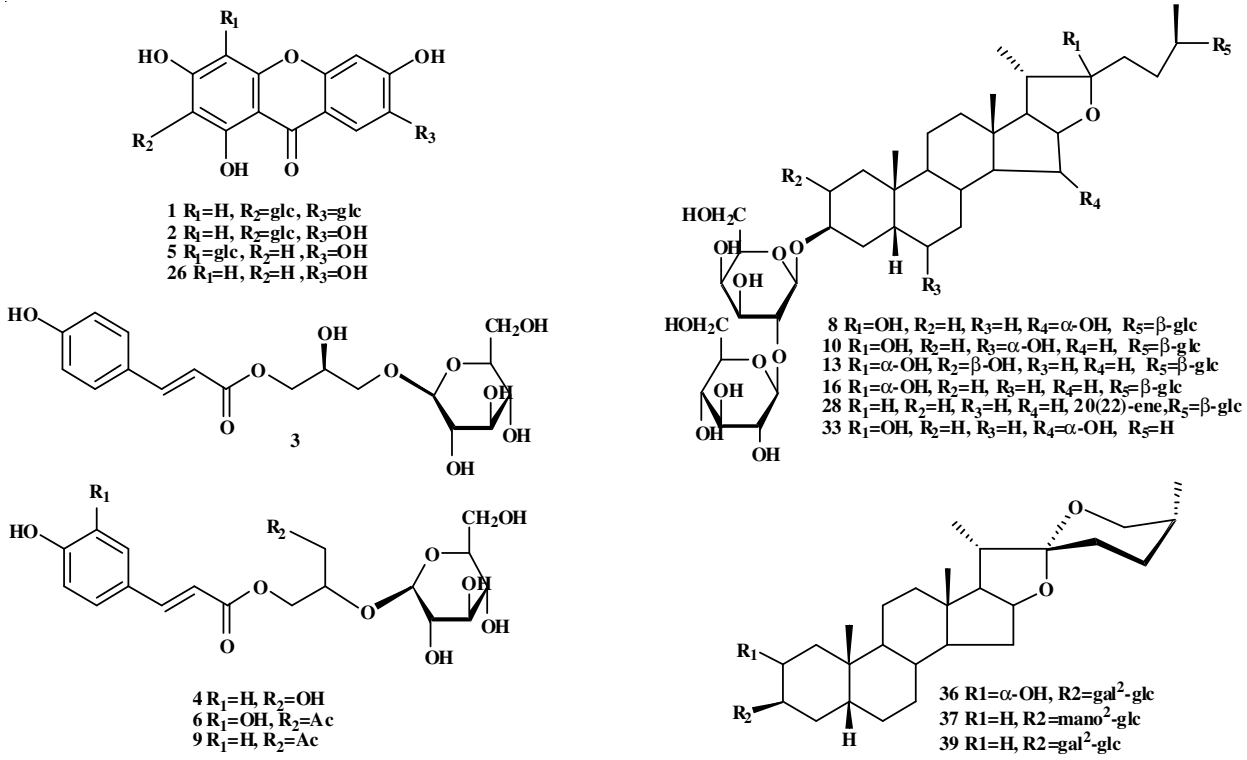

Fig. 3. Chemical structures of some identified compounds in Baihe Zhimu decoction

Compounds **12**, **14**, **15** and **19** showed $[M-H]^-$ ion at *m/z* 917. The fragment ion at *m/z* 755 on MS^2 may be formed by the loss of a glucose residue. The product ion at *m/z* 593 on MS^3 corresponded to successive loss of a galactose residue, while the product ion at *m/z* 451 on MS4 corresponded to successive loss of a galactose residue and a H_2O molecule. On the basis of previous findings[10], the retention time of timosaponin D was between timosaponin BII and timosaponin BIII, compound **19** proposed to be timosaponin D. And compounds **12**, **14** and **15** were tentatively identified as its isomers.

Two peaks with different retention times appeared in the extracted ion chromatogram (EIC) of *m/z* 771. All successive MS/MS fragmentations showed decrease of 162 and 18 Da in molecular weight ascribed to loss of a hexose and H_2O unit. According to the polarity and the literature[10], we concluded that compounds **24** and **32** could be timosaponin F and anemarrhenasaponin Ia.

Compound **35** had a $[M-H]^-$ at *m/z* 753 and the MS^2 spectrum had a fragment at *m/z* 591 (M-162 Da, loss of a Glc) and a fragment at *m/z* 573 (M-162 Da -18 Da, loss of a Glc and a H_2O). The fragments at *m/z* 591 and 573 further lost 162 Da and produced fragments at 429 and 411 on MS^3, respectively. This suggested the existences of hydroxyl and hexoses. This compound had not previously been reported form natural sources or synthesis from *Lilium brownii var. viridulum*. In literature[11], it was tentatively identified as hydroxyl deacylbrownioside, which might be oxidized from deacylbrownioside during boiling.

Compounds **17** and **23** had the [M-H] at *m/z* 899 that fragmented on MS^2 to produce ions at *m/z* 737 (M-146-16 Da, loss of a rha and a O). The fragment at *m/z* 737 further lost 162 Da and produced fragment at 575 on MS^3. Thus, compounds **17** and **23** might be 26-O-β-D-glucopyranosyl-3β,26-dihydroxy-5-choleslen-16,22-dioxo-3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside and its isomer.

Conclusion

In this study, a HPLC-ESI-MS^n method was developed to analyze the constituents of Baihe Zhimu decoction. As a result, a total of 39 compounds were identified or tentatively characterized from Baihe Zhimu decoction extract. The fragmentation patterns of these reference substances, which could be classified into three types including, xanthones phenolic glycosides, observed in an HPLC-ESI-MS^n were analyzed to further identify these structures. In brief, the major fragmentations of phenolic glycosides and saponins corresponded to the losses of branched glycoside chains. The major fragmentations of xanthones norathyriol corresponded to the losses of small molecules such as Me and COO. Moreover, losses of 120 and 90 Da were observed, corresponding to cross-ring cleavages in the sugar moiety, which is the major characteristic of C-C linked β-D-glucopyranosides. Owing to the structural complexity of constituents in Baihe Zhimu decoction, the origins and structures of some compounds could not be definitely elucidated under the current analytical conditions. But the similarities of constituents detected in Baihe Zhimu decoction will be very helpful in revealing the therapeutic basis of Baihe Zhimu decoction.

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