Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 1157–1165

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Identification of components in Zhi-Zi-Da-Huang decoction by HPLC coupled with electrospray ionization tandem mass spectrometry, photodiode array and fluorescence detectors

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ARTICLE INFO

Article history:
Received 14 November 2008
Received in revised form 27 January 2009
Accepted 21 February 2009
Available online 6 March 2009

Keywords:
Zhi-Zi-Da-Huang decoction
HPLC
Electrospray ionization tandem mass spectrometry
Photodiode array detection
Fluorescence detection

ABSTRACT

High-performance liquid chromatography (HPLC) coupled with electrospray ionization (ESI) tandem mass spectrometry (MS) in positive and negative ion mode and photodiode array (PDA) and fluorescence detectors (FD) was applied to simultaneously characterise thirty active components in Zhi-Zi-Da-Huang decoction (ZZDHD). The analysis was preformed on a Lichrospher C_{18} column (4.6 mm × 250 mm, 5 μm) using a binary eluent (0.1% aqueous acetic acid (A) and methanol (B), 1 ml/min) under gradient conditions. Based on the retention times (t_R), UV spectral data, fluorescence characteristics, results of extracted ion current (EIC), information of molecular weight and mass fragmentation behaviours, 17 flavonoids, five anthraquinones, two coumarins and six iridoids were detected and identified. The ESI-MS/MS fragmentation behaviour of iridoids, flavonoids, anthraquinones, coumarins was also proposed. The fragmentation patterns proposed could be extended to the components mentioned above but in different herbs and prescriptions. The method was useful for the identification of unknown components in the herbal system and other complex samples, especially for which standards are unavailable.

1. Introduction

Traditional Chinese medicine (TCM) preparations have been widely used over a millennium, and are becoming more and more popular worldwide. Comprehensive identification of the chemical components in TCM is important for revealing the material basis of their therapeutic effects, ensuring the safety, as well as optimising the quality control. With the help of advanced analytical method and available reference standards, the simultaneous characterisation of multiple active ingredients in herbal medicines has been reported, e.g. [1–4]. However, due to difficulties of isolation and preparation, it is unrealistic and unpractical to base all studies on reference standards.

Modern analytical technology, such as HPLC–PDA–ESI-MS/MS suitable for achieving high sensitivity and providing more information on the constituents of interest, has been successfully applied in the simultaneous analysis and identification of multiple active components in herbs and other complex system [5–9]. HPLC-FD, on the other hand, possessing the advantages of high sensitivity, selectivity and cost-effectiveness, has been applied in the routine analysis and quality evaluation of fluorescent components in herbs and biological samples [10–13]. A comprehensive analysis method involving both HPLC–PDA–ESI-MS/MS and HPLC-FD would provide more information to characterise the components and appear to be a better approach in identifying the unknown compounds in TCM preparation and other complex sample, especially for which the standards are unavailable.

Zhi-Zi-Da-Huang decoction (ZZDHD) is a classical traditional Chinese formula comprising four crude drugs: *Gardenia jasminoides* Ellis (Zhi-Zi), *Rheum officinale* Baill (Da-Huang), *Citrus aurantium* L. (Zhi-Shi) and Semen Sojae Preparatum (Dan-Dou-Chi), and has been widely used to treat diseases like alcoholic jaundice, alcoholic liver disease and acute hepatitis for nearly 2000 years.

In this study, an integrated approach based on HPLC coupled with electrospray ionization tandem mass spectrometry, photodiode array and fluorescent detectors has been developed to characterise the chemical components in ZZDHD.

2. Experimental

2.1. Materials and reagents

Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Acetic acid (analytical reagent) was purchased from

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doi:10.1016/j.jpba.2009.02.023

Nanjing Chemical Reagent Factory (Nanjing, China). Water was distilled twice before use. Ethanol (analytical grade) was purchased from Nanjing Chemical Co. (Nanjing, China).

Daidzein was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); purity was >98%.

The medicinal plants and materials used in the experiment including the root and bark of *Rheum officinale* Baill, the immature fruit of *Citrus aurantium* L., *Gardenia jasminoides* Ellis and Semen Sojae Preparatum were purchased from a traditional Chinese medicinal store in Nanjing, China and authenticated by Prof. Qin Minjian (Department of Chinese Materia Medica, China Pharmaceutical University, Nanjing, China).

2.2. Preparation of standard solutions

Stock solution was prepared by dissolving the 10 mg daidzein in 10 ml of 70% ethanol, the stock solution was then diluted with 70% ethanol to the concentration of 30 μg/ml and stored at 4 °C.

2.3. Preparation of analytical sample of ZZDHD

Gardenia jasminoides Ellis (1.5 g), *Rheum officinale* Baill (0.5 g), *Citrus aurantium* L. (2 g) and Semen Sojae Preparatum (4 g) were immersed in 80 ml distilled water (1/10, w/v) for 1 h and then decocted by boiling for 30 min. The extract was filtered. This procedure was then repeated twice by adding 80–80 ml water. The three extracts were then combined and concentrated in vacuum to 25 ml to obtain a suspension of ZZDHD. 2.5-ml aliquot of the suspension was then mixed with 7 ml of 95% ethanol. The solution thus obtained was centrifuged at 4000 × *g* for 5 min and filtered through a 0.45-μm membrane filter before introduced to the HPLC analysis. Using this extraction protocol, five other solutions were prepared for the evaluation of the reproducibility of the analytical method. All sample solutions were stored at 4 °C and used at room temperature.

2.4. HPLC–PDA–ESI/MS/MS and FD system

A Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with an electrospray ionization source (San Jose, CA, USA), a Finnigan Surveyor quaternary LC pump, a Finnigan Surveyor auto-sampler and a Finnigan Surveyor photodiode array detector was used. Data processing was carried out using Xcalibur 1.1 software (Thermo Electron Corporation). Fluorescence measurement was carried out using RF-10AXL fluorescence detector (Shimzadu Corporation, Kyoto, Japan). Data acquisition and processing were performed by N2000 software (Zhejiang University, China).

Chromatographic analysis was performed on a LiChrospher-C_{18} column (250 mm × 4.6 mm, 5 μm, Hanbon Science & Technology Co., Jiangsu, China). The mobile phase was 0.1% aqueous acetic acid (A) and methanol (B). A gradient programmer was used according to the following profile: 0 min 5% B, 12.5 min 30% B, 26.8 min 36% B, 50 min 45% B, 58 min 54.6%, 67–75 min 74.3%, 85–100 min 95%, 101–110 min 5% B. A constant flow rate of 1.0 ml/min was maintained. The column temperature was set to 25 °C. UV spectra were recorded from 210 nm to 480 nm, the monitor wavelength was set at 258 nm, and the sample injection volume was 20 μl. The LC effluent was split by the ratio of 4:1 using a valved three-way split, so that approximately 200 μl/min was introduced into the source of the mass spectrometer, the other 800 μl/min was introduced into the photodiode array detector and fluorescence detector successively.

Full scan data acquisition and dependant scan event data acquisition were performed from *m*/*z* 100 to 1000 in both positive and negative MS scan mode. Heated capillary temperature was 350 °C, argon was used as collision gas at a pressure of 1.5 mTorr and collision energy was 20 eV, spray voltage was 4500 V and −3000 V for positive and negative MS scan mode respectively, source CID voltage was set at 10 eV. The fluorescent excitation wavelength and emission wavelength was set at λ_{ex} 329 nm/λ_{em} 446 nm and λ_{ex} 440 nm/λ_{em} 520 nm, respectively.

3. Results and discussion

Fig. 1 shows the structures of components identified in the sample solution of ZZDHD. Other information such as MW of these compounds is summarised in Table 1.

3.1. Optimisation of sample preparation

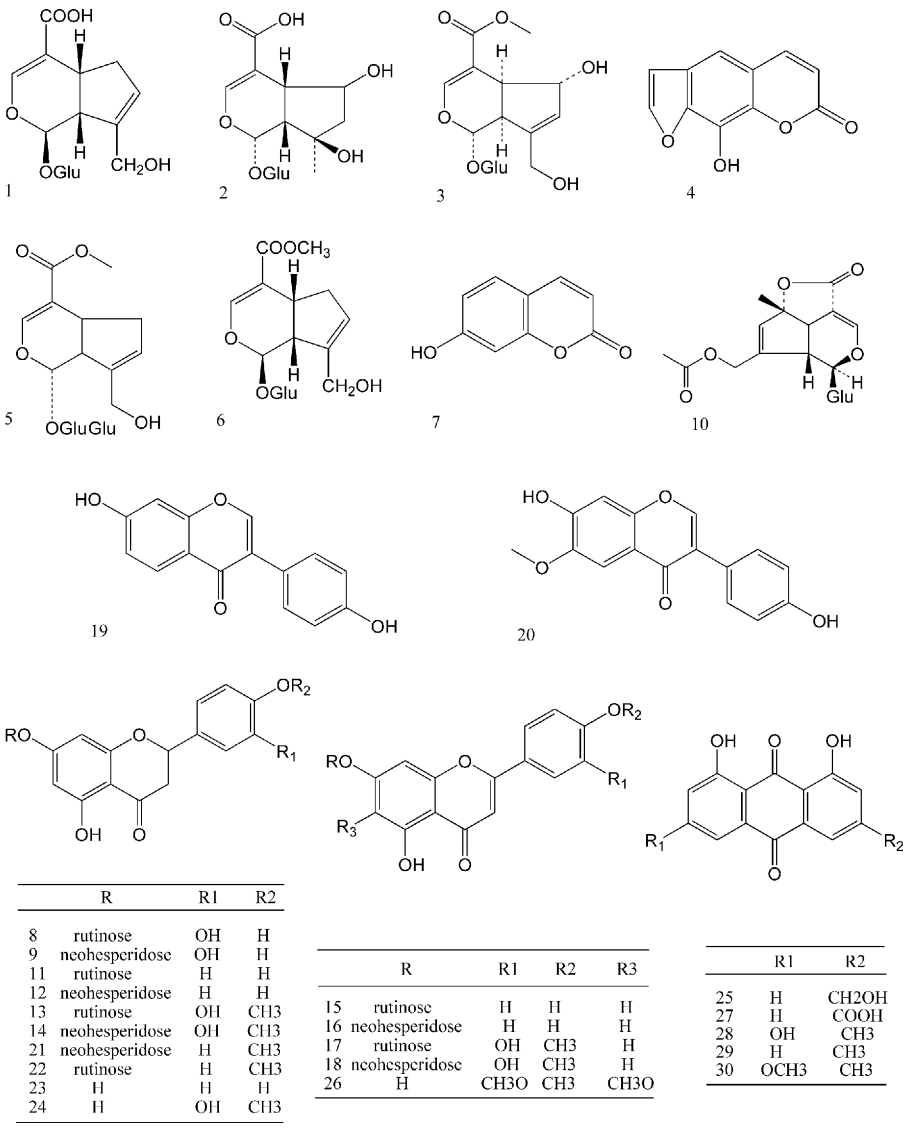
According to the method described in Jin-Kui-Yao-Lue (Synopsis of Golden Chamber), ZZDHD was prepared using water as the extracting solvent. Extraction time and solvent volume of each extraction were optimised on the basis of peak areas of daidzein and five main components (peaks 6, 12, 13, 14, 27 in the chromatograms in Fig. 2a). Best results were obtained when the mixture of the four crude drugs was extracted, evaporated and diluted as described in Section 2.3. As described there, ethanol was used for the dilution. This solvent is often added to the aqueous extract of TCMs [14,15], to increase the solubility of lipophilic substances, and to precipitate polysaccharides and proteins.

3.2. Optimisation of HPLC–PDA–ESI-MS/MS and FD conditions

Mobile phase composition, elution conditions, column temperature and detection wavelength have been optimised to obtain the conditions described in Section 2.4. Addition of acetic acid was necessary to mobile phase A to depress the tailing of the peaks of phenolic compounds. Of the tested concentrations (0.1%, 0.5%, 1%, 2%) 0.1% gave the best results. Since isocratic elution was not suitable for the complete separation of multiple components in ZZDHD, sections of nine slopes were built into the gradient so that it covered the parts of the chromatogram where increased resolution was desired, this gradient of the mobile phase could achieve maximum throughput and optimal resolution. The wavelength for the detection of the components in the sample solution of ZZDHD was selected by using a PDA detector. Seven wavelengths (230, 240, 254, 258, 285, 330 and 435 nm) were tested for the highest sensitivity and largest number of peaks. The optimum wavelength was found to be 258 nm. A representative chromatogram is shown in Fig. 2a.

Anthraquinones and coumarins in ZZDHD are strongly fluorescent substances with λ_{ex} 420–460 nm/λ_{em} 500–540 nm and λ_{ex} 300–350 nm/λ_{em} 410–480 nm, respectively [16,17]. The appropriate excitation and emission wavelength couple (λ_{ex}/λ_{em}) was established manually by scanning the maximum absorption wavelength of ZZDHD, 500 nm was presumed as the initial excitation wavelength. After recording the fluorescence spectrum, the emission wavelength was obtained, then the opposite process was carried out in order to optimise the excitation wavelength, and this process was repeated until the final optimum wavelength couple was selected. For anthraquinones, λ_{ex}/λ_{em} is 440 nm/520 nm and for coumarins λ_{ex}/λ_{em} is 329 nm/446 nm. Fig. 2b and c is HPLC-FD chromatograms recorded at the two wavelengths.

The MS spectra were detected in both negative and positive ion mode and their total ion current (TIC) chromatograms are shown in Fig. 2d and e. In MS spectra, most of the constituents exhibited their quasi-molecular ions $[M+H]^+$, adduct ions $[M+Na]^+$ and $[M+NH_4]^+$ in positive ion mode, while exhibited $[M-H]^-$, $[M+Cl]^-$ and $[M+CH_3COO]^-$ in negative ion mode. In positive ion mode, flavonoids were found to be more sensitive in yielding the prominent $[M+H]^+$ ions in the first order mass spectra, but in negative ion mode, flavonoid aglycones and coumarins yielded more fragments



	R	R1	R2
8	rutinose	OH	H
9	neohesperidose	OH	H
11	rutinose	H	H
12	neohesperidose	H	H
13	rutinose	OH	CH3
14	neohesperidose	OH	CH3
21	neohesperidose	H	CH3
22	rutinose	H	CH3
23	H	H	H
24	H	OH	CH3

	R	R1	R2	R3
15	rutinose	H	H	H
16	neohesperidose	H	H	H
17	rutinose	OH	CH3	H
18	neohesperidose	OH	CH3	H
26	H	CH3O	CH3	CH3O

	R1	R2
25	H	CH2OH
27	H	COOH
28	OH	CH3
29	H	CH3
30	OCH3	CH3

Fig. 1. Chemical structures of the thirty identified components in Zhi-Zi-Da-Huang decoction. **1** Geniposidic acid; **2** shanzhiside; **3** scandoside methyl ester; **4** xanthotoxol; **5** genipingentiobioside; **6** geniposide; **7** umbelliferone; **8** eriocitrin; **9** neoeriocitrin; **10** asperuloside; **11** isonaringin; **12** naringin; **13** hesperidin; **14** neohesperidin; **15** isorhoifolin; **16** rhoifolin; **17** diosmin; **18** neodiosmin; **19** daidzein; **20** glycitein; **21** poncirin; **22** neoponcirin; **23** naringenin; **24** hesperetin; **25** aloe-emodin; **26** eupatilin; **27** rhein; **28** emodin; **29** chrysophanol; **30** physcion.

and exhibited higher signal to noise ratio (S/N) compared with positive ion mode. Anthraquinones were found to be more efficiently ionized in negative ion mode ESI-MS than positive mode. Spray voltage was an important factor in negative ion mode, −3000 V, −4000 V, and −4500 V were tried for negative MS scan mode and −3000 V was found to give higher S/N for anthraquinones. Fragmentation collision energy set at 20 eV resulted in high sensitivity and furnished a complete fragmentation pattern for most components.

Peak identification was performed at first on the basis of the information from the total ion current (TIC) chromatograms. However, due to the poor S/N of certain components in TIC chromatograms, a commonly used method for extracting MS data: extracted ion current (EIC) technology, was employed. Based on *m*/*z* value of detected ions, retention time of chromatographic peak, UV spectra and fluorescent characteristics of the compound corresponding to a chromatographic peak and comparison with literature data, 17 flavonoids, five anthraquinones, two coumarins and six iridoids were identified. The integrated information of the thirty components is summarised in Table 1. The structures are shown in Fig. 1.

3.3. Method validation

In the course of the method validation the following detection modes were selected to evaluate the retention times and peak areas of peaks in Fig. 1: FD for peaks 4, 7 (λ_{ex} 329 nm/λ_{em} 446 nm), 25, 27, 28, 29 and 30 (λ_{ex} 440 nm/λ_{em} 520 nm), MS-TIC for peaks 5, 6, 8, 9, 11, 12, 13, 14, 16, 17, 19, 21, 23, 24 and 26, MS-EIC for peaks 1, 2, 3, 10, 15, 18, 20 and 22.

3.3.1. Repeatability

The repeatability was assessed by analyzing six independently prepared sample solutions of ZZDHD. The relative standard deviation (R.S.D.) of retention time and peak area of the thirty components were below 1.46% and 4.70%, respectively.

3.3.2. Injection precision

The injection precision of peaks was evaluated by successive analysis of the same sample solution six times. The R.S.D. of retention time and peak area of the thirty components were less than 1.55% and 3.81%, respectively.

Table 1
Characterisation of components in Zhi-Zi-Da-Huang decoction.

Peak	t_R (min)	λ_{max} (nm)	MW	ESI-MS$^+$ m/z	ESI-MS$^-$ m/z	Identification
1	11.04	236	374	375[M+H]$^+$, 415[M+Na+H_2O]$^+$, 397[M+Na]$^+$, 392[M+NH_4]$^+$, 213[M+H−162]$^+$, 195[M+H−162−H_2O]$^+$, 177[M+H−162−2H_2O]$^+$, 167[M+H−162−H_2O−CO]$^+$	373[M−H]$^-$, 433[M+CH_3COO]$^-$, 409[M+Cl]$^-$, 211[M−H−162]$^-$, 193[M−H−162−H_2O]$^-$, 167[M−H−162−CO_2]$^-$, 149[M−H−162−H_2O−CO_2]$^-$	Geniposidic acid
2	12.80	236	392	415[M+Na]$^+$, 410[M+NH_4]$^+$, 397[M+Na−H_2O]$^+$, 375[M−H_2O+H]$^+$, 357[M−2H_2O+H]$^+$	391[M−H]$^-$, 229[M−H−162]$^-$, 211[M−H−162−H_2O]$^-$, 185[M−H−162−CO_2]$^-$, 167[M−H−162−H_2O−CO_2]$^-$	Shanzhiside
3	14.16	238	404	427[M+Na]$^+$, 422[M+NH_4]$^+$, 404[M+NH_4−H_2O]$^+$, 225[M+H−162−H_2O]$^+$, 207[M+H−162−2H_2O]$^+$	403[M−H]$^-$, 463[M+CH_3COO]$^-$, 439[M+Cl]$^-$, 241[M−H−162]$^-$, 223[M−H−162−H_2O]$^-$	Scandoside methyl ester
4	17.47		202		201[M−H]$^-$, 375[2M−H−CO]$^-$, 173[M−H−CO]$^-$, 157[M−H−CO_2]$^-$	Xanthotoxol
5	18.31	239	550	551[M+H]$^+$, 573[M+Na]$^+$, 568[M+NH_4]$^+$, 533[M+H−H_2O]$^+$, 389[M+H−162]$^+$, 371[M+H−162−H_2O]$^+$, 227[M+H−162−162]$^+$, 209[M+H−162−162−H_2O]$^+$	549[M−H]$^-$, 609[M+CH_3COO]$^-$, 585[M+Cl]$^-$, 225[M−H−162−162]$^-$, 207[M−H−162−162−H_2O]$^-$, 179 [M−H−162−162−H_2O−CO]$^-$	Genipingentiobioside
6	21.37	239	388	799[2M+Na]$^+$, 777[2M+H]$^+$, 427[M+K]$^+$, 411[M+Na]$^+$, 406[M+NH_4]$^+$, 227[M+H−162]$^+$, 209[M+H−162−H_2O]$^+$, 181[M+H−162−H_2O−CO]$^+$	387[M−H]$^-$, 447[M+CH_3COO]$^-$, 225[M−H−162]$^-$, 207[M−H−162−H_2O]$^-$	Geniposide
7	26.32		162	163[M+H]$^+$	161[M−H]$^-$, 133[M−H−CO]$^-$, 117[M−H−CO_2]$^-$, 105[M−H−2CO]$^-$	Umbelliferone
8	30.09	228, 284	596	597[M+H]$^+$, 451[M+H−146]$^+$, 435[M+H−162]$^+$, 619[M+Na]$^+$, 579[M+H−H_2O]$^+$, 289[M+H−308]$^+$	595[M−H]$^-$, 631[M+Cl]$^-$, 287[M−H−308]$^-$	Eriocitrin
9	32.09	227, 285	596	597[M+H]$^+$, 451[M+H−146]$^+$, 435[M+H−162]$^+$, 619[M+Na]$^+$, 579[M+H−H_2O]$^+$, 289[M+H−308]$^+$	595[M−H]$^-$, 631[M+Cl]$^-$, 459[M−H−136]$^-$, 287[M−H−308]$^-$	Neoeriocitrin
10	33.94		428	446[M+NH_4]$^+$, 451[M+Na]$^+$	427[M−H]$^-$, 463[M+Cl]$^-$, 265[M−H−162]$^-$, 247[M−H−162−H_2O]$^-$	Asperuloside
11	38.03	227, 284	580	581[M+H]$^+$, 619[M+K]$^+$, 603[M+Na]$^+$, 563[M+H−H_2O]$^+$, 545[M+H−2H_2O]$^+$, 435[M+H−146]$^+$, 419[M+H−162]$^+$, 401[M+H−162−H_2O]$^+$, 273[M+H−308]$^+$,	579[M−H]$^-$, 615[M+Cl]$^-$, 271[M−H−308]$^-$	Isonaringin
12	40.16	227,283	580	581[M+H]$^+$, 603[M+Na]$^+$, 563[M+H−H_2O]$^+$, 545[M+H−2H_2O]$^+$, 435[M+H−146]$^+$, 419[M+H−162]$^+$, 401[M+H−162−H_2O]$^+$, 273[M+H−308]$^+$,	579[M−H]$^-$, 615[M+Cl]$^-$, 271[M−H−308]$^-$, 459[M−H−120]$^-$	Naringin
13	42.19	227, 285	610	611[M+H]$^+$, 633[M+Na]$^+$, 593[M+H−H_2O]$^+$, 465[M+H−146]$^+$, 449[M+H−162]$^+$, 431[M+H−162−H_2O]$^+$, 303[M+H−308]$^+$, 345[M+H−266]$^+$	609[M−H]$^-$, 645[M+Cl]$^-$, 301[M−H−308]$^-$, 286[M−H−308−CH_3]$^-$(	Hesperidin
14	45.71	284	610	611[M+H]$^+$, 633[M+Na]$^+$, 593[M+H−H_2O]$^+$, 465[M+H−146]$^+$, 449[M+H−162]$^+$, 431[M+H−162−H_2O]$^+$, 303[M+H−308]$^+$, 345[M+H−266]$^+$	609[M−H]$^-$, 645[M+Cl]$^-$, 301[M−H−308]$^-$,489[M−H−120]$^-$, 403[M−H−206]$^-$, 343[M−H−266]$^-$, 325[M−H−284]$^-$	Neohesperidin
15	46.19		578	579[M+H]$^+$, 601[M+Na]$^+$, 433[M+H−146]$^+$	577[M−H]$^-$, 269[M−H−308]$^-$,	Isorhoifolin
16	48.57	234, 314	578	579[M+H]$^+$, 601[M+Na]$^+$, 433[M+H−146]$^+$,	577[M−H]$^-$, 269[M−H−308]$^-$,	Rhoifolin
17	50.02		608	609[M+H]$^+$, 631[M+Na]$^+$, 301[M+H−308]$^+$	607[M−H]$^-$, 643[M+Cl]$^-$, 299[M−H−308]$^-$	Diosmin
18	51.32	255,268, 345	608	609[M+H]$^+$, 631[M+Na]$^+$, 301[M+H−308]$^+$	607[M−H]$^-$, 643[M+Cl]$^-$, 299[M−H−308]$^-$	Neodiosmin
19	56.90	249	254	255[M+H]$^+$, 277[M+Na]$^+$, 237[M+H−H_2O]$^+$, 227[M+H−CO]$^+$, 199[M+H−2CO]$^+$, 137	253[M−H]$^-$, 225[M−H−CO]$^-$	Daidzein
20	59.30		284	285[M+H]$^+$, 270[M+H−CH_3]$^{+\cdot}$, 242[M+H−CH_3−CO]$^{+\cdot}$	283[M−H]$^-$, 268[M−H−CH_3]$^{\bullet -}$, 240[M−H−CH_3−CO]$^{\bullet -}$	Glycitein
21	60.01		594	595[M+H]$^+$, 617[M+Na]$^+$, 433[M+H−162]$^+$	593[M−H]$^-$, 285[M−H−308]$^-$	Poncirin

Table 1 (*Continued*)

Peak	t_R (min)	λ_{max} (nm)	MW	ESI-MS$^+$ *m/z*	ESI-MS$^-$ *m/z*	Identification
22	61.12		594	595[M+H]$^+$, 617[M+Na]$^+$, 433[M+H−162]$^+$, 287[M+H−308]$^+$	593[M−H]$^-$, 653[M+CH_3COO]$^-$, 629[M+Cl]$^-$, 473[M−H−120]$^-$, 431[M−H−162]$^-$, 387[M−H−206]$^-$, 285[M−H−308]$^-$	Neoponcirin
23	61.80	230,289	272	273[M+H]$^+$, 153	271[M−H]$^-$, 227[M−H−CO_2]$^-$, 177, 165, 151, 119, 107	Naringenin
24	63.94	230,288	302	303[M+H]$^+$, 325[M+Na]$^+$	301[M−H]$^-$, 361[M+Cl]$^-$, 286[M−H−CH_3]$^{\bullet-}$, 257[M−H−CO_2]$^-$, 242[M−H−CO_2−CH_3]$^{\bullet-}$, 164, 151	Hesperetin
25	67.25	430	270		269[M−H]$^-$, 251[M−H−H_2O]$^-$, 223[M−H−H_2O−CO]$^-$	Aloe-emodin
26	71.38		344	345[M+H]$^+$	343[M−H]$^-$, 328[M−H−CH_3]$^{\bullet-}$, 313[M−H−2CH_3]$^{\bullet-}$	Eupatilin
27	77.70	259, 431	284		283[M−H]$^-$, 255[M−H−CO]$^-$, 239[M−H−CO_2]$^-$, 211[M−H−CO_2−CO]$^-$, 183[M−H−CO_2−2CO]$^-$	Rhein
28	86.27	249,288, 431,443	270		269[M−H]$^-$, 241[M−H−CO]$^-$, 225[M−H−CO_2]$^-$	Emodin
29	89.22	255,431	254		253[M−H]$^-$, 225[M−H−CO]$^-$	Chrysophanol
30	92.49	277, 432	284		283[M−H]$^-$, 268[M−CH_3]$^{\bullet-}$, 240[M−CH_3−CO]$^{\bullet-}$	Physcion

3.3.3. Sample stability test

The analysis of the same sample solution at different times (0, 2, 4, 6, 8, 12, and 24 h) was used to evaluate the stability of sample solutions within 24 h, stored at room temperature. The R.S.D. of retention time and peak area of the thirty components were less than 1.48% and 4.76%, respectively.

All results indicated that the HPLC method for the identification of components in ZZDHD was valid and satisfactory.

3.4. Identification of flavonoids in Zhi-Zi-Da-Huang decoction

The UV spectra of peaks 8, 9, 11, 12, 13, 14 in the HPLC chromatogram determined by PDA showed two absorption maxima at about 227 nm and 284 nm, consistent with the characteristic absorption of flavonoids. Neutral loss scan of 308, 146, 162 mass unit revealed the possible presence of flavonoid glycoside with a neohesperidose or a rutinose for compounds **8**, **9**, **11**–**18**, **21** and **22**.

Compounds **8** *vs.* **9**, **11** *vs.* **12**, and **13** *vs.* **14**, **15** *vs.* **16**, **17** *vs.* **18** and **21** *vs.* **22** gave the ions at *m/z* 595, 579, 609, 577, 607 and 593, respectively, in negative mode and gave the ions at *m/z* 597, 581, 611, 579, 609 and 595, respectively, in positive mode, suggesting that the MW of these six pairs of compounds were 596, 580, 610, 578, 608, 594 and the possible compounds were eriocitrin *vs*. neoeriocitrin, isonaringin *vs*. naringin, hesperidin *vs*. neohesperidin, isorhoifolin *vs.* rhoifolin, diosmin *vs*. neodiosmin, poncirin *vs*. neoponcirin (Table 1). The common features of ESI-MS/MS data of six pairs of compounds observed in negative mode were the [M−H]$^-$, [M+Cl]$^-$ and [M−H−308]$^-$, in positive mode were [M+H]$^+$, [M+Na]$^+$, [M+H−H_2O]$^+$, [M+H−2H_2O]$^+$, [M+H−146]$^+$, [M+H−162]$^+$, [M+H−162−H_2O]$^+$ and [M+H−308]$^+$, indicating that the fragmentation pathways of each isomer were very similar. All these information suggested that they were six pairs of isomers. The structural difference between each isomer was the substitution of different glycone in the same aglycone. According to literature [2,18], the relative abundance of [M+H−146]$^+$ and [M+H−308]$^+$ ions in isomers of flavonoid glycoside with rutinose and with neohesperidose are strikingly different. The neohesperidose favours the elimination of the disaccharide residue to yield a [M+H−308]$^+$, however, rutinose favours the elimination of the dehydrated glucose residue to yield [M+H−146]$^+$. Besides, the isomeric pair has different eluting speeds in the RP-HPLC and the retention time of the one with rutinose is shorter. The above features of MS and HPLC of isomeric pair were used to distinguish six isomeric pairs. Peaks 8, 11, 13, 15, 17 and 21 were identified as eriocitrin, isonaringin, hesperidin, isorhoifolin, diosmin, poncirin with rutinose, respectively. Peaks 9, 12, 14, 16, 18 and 22 were identified as neoeriocitrin, naringin, neohesperidin, rhoifolin, neodiosmin and neoponcirin with neohesperidose.

The UV spectra of compounds corresponding to peaks 23 and 24 in the HPLC chromatogram showed double maximum absorption bands at about 230 nm and 288 nm, consistent with the characteristic absorption of flavonoids. The two compounds gave [M−H]$^-$ ions at *m/z* 271 and 301, and [M−H−44]$^-$ ion through the loss of CO_2 from the quasi-molecular ions respectively. In positive mode, compounds 23 and 24 yielded [M+H]$^+$ at *m/z* 273 and 303, respectively. The information above suggested that the compounds 23 and 24 were the aglycones of compounds 12 and 13 with MW at 272 and 302. The ion at *m/z* 151 was produced from a retro-Diels-Alder (RDA) fragmentation reaction cleaved at the C-ring of flavonoid aglycones (see Fig. 3). By referring to the reported data [19], peaks 23 and 24 were identified as naringenin and hesperetin. Fig. 3 showed the proposed fragmentation pattern of naringenin.

Compound **19** was identified as daidzein by comparing it with the reference standard. It exhibited absorption maxima at 249 nm and the ESI$^-$-MS^2 spectrum yielded a product ion at *m/z* 225 which was from the loss of CO from the ion of [M−H]$^-$ at *m/z* 253. The ESI$^+$-MS^2 spectrum of daidzein yielded a series of product ions at *m/z* 237, 227 and 137, which were result from the losses of H_2O, CO and cleavage of the B-ring from [M+H]$^+$ ion at *m/z* 255, product ion at *m/z* 199 originated from the successive loss of CO from *m/z* 227.

Compound **20** gave a [M+H]$^+$ ion at *m/z* 285 and a [M+H−CH_3]$^{\bullet+}$ ion at *m/z* 270 indicating the presence of a methyl group, [M+H−CH_3−CO]$^{\bullet+}$ ion at *m/z* 242 also appeared. In negativmode, compound **20** gave the ions of [M−H]$^-$ at *m/z* 283, [M−H−CH_3]$^{\bullet-}$ at *m/z* 268 and [M−H−CH_3−CO]$^{\bullet-}$ at *m/z* 240. All of the fragments above coincided with those reported [20,21]; peak 20 was identified as glycitein.

3.5. Identification of anthraquinones in Zhi-Zi-Da-Huang decoction

Anthraquinones might exist in Zhi-Zi-Da-Huang decoction as they are important compounds found in the crude drug *Rheum offic-*

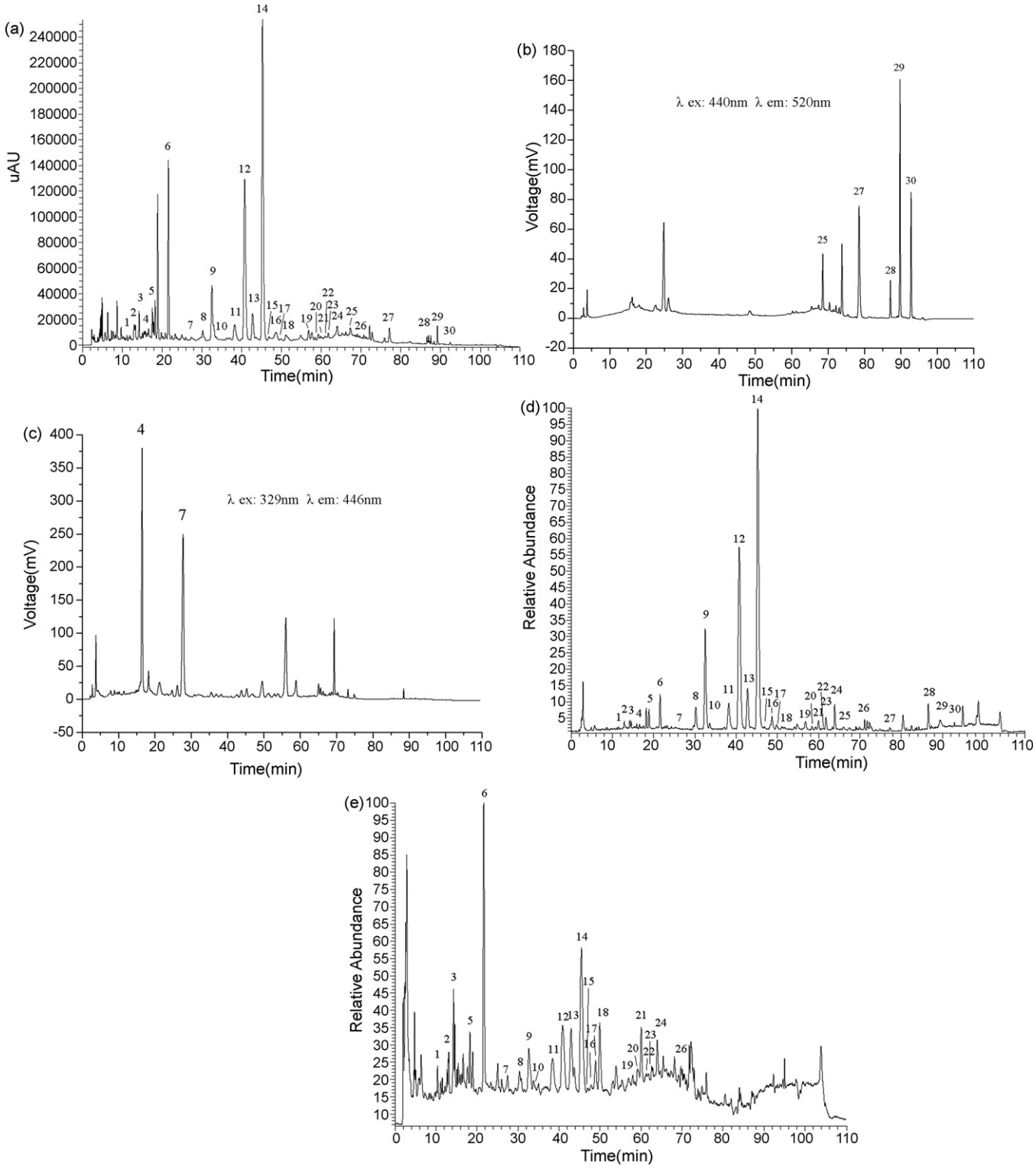


Fig. 2. Chromatograms of the Zhi-Zi-Da-Huang decoction with: (a) HPLC–UV chromatogram monitored at 258 nm; (b) HPLC-FD (λ_{ex} 440 nm/λ_{em} 520 nm); (c) HPLC-FD (λ_{ex} 329 nm/λ_{em} 446 nm); (d) MS-TIC (negative ion mode); (e) MS-TIC (positive ion mode). For peak numbering see Fig. 1.

inale Baill. Compounds corresponding to peak 25, 27, 28, 29 and 30 exhibited two maximum absorption bands at 240–290 nm and 430–440 nm. Peaks of the five compounds in the FD chromatogram (λ_{ex} 440 nm/λ_{em} 520 nm) showed higher selectivity and sensitivity compared with those in PDA and MS-TIC (negative ion mode) chromatograms. The information above was consistent with the FD characteristics and UV spectra of anthraquinones.

Compounds 27 and 30 both gave the ions at *m*/*z* 283 and suggested that they might be a pair of isomer. The MS/MS fragmentation behaviours of the two compounds were different. Compound **27** gave a $[M-H]^-$ ion at m/z 283, which then lose CO_2 and CO to form the fragments with *m*/*z* 239 and 255, respectively. A further loss of CO from *m*/*z* 239 gave rise to the fragment at *m*/*z* 211. The ion of *m*/*z* at 211 could further lose one molecule of CO to produce ion

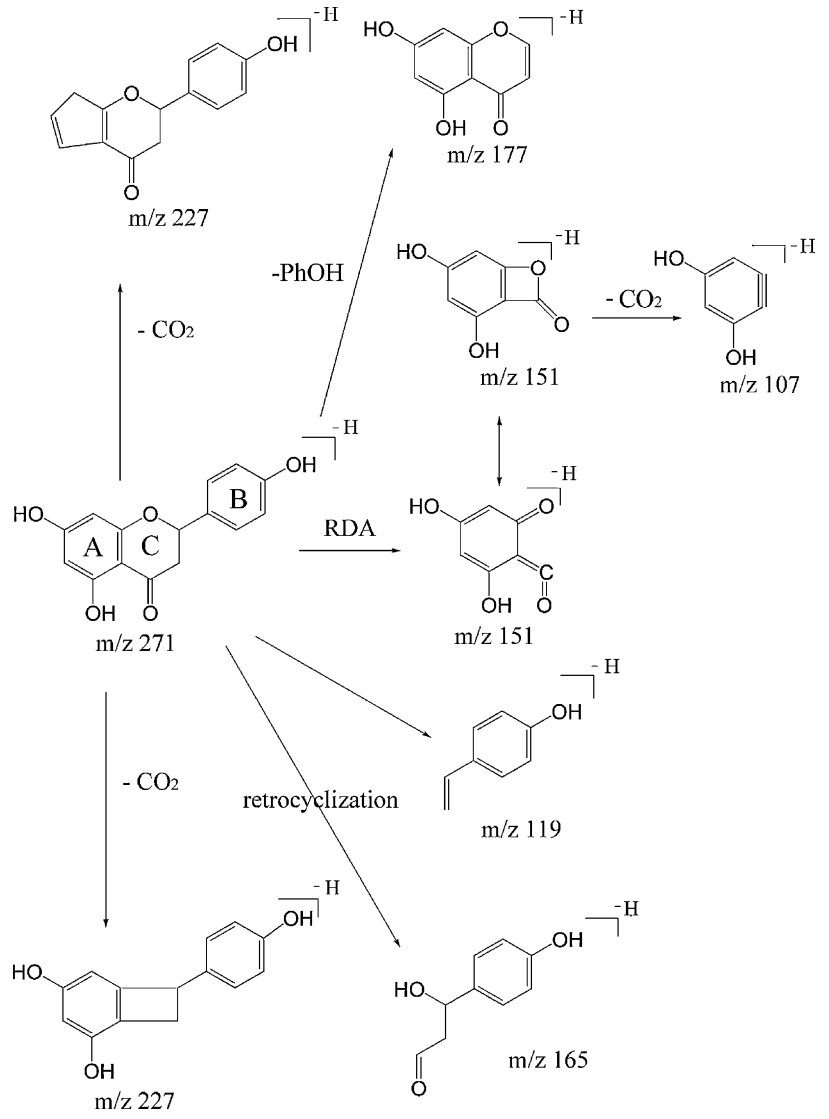


Fig. 3. The proposed fragmentation pattern of naringenin in negative mode.

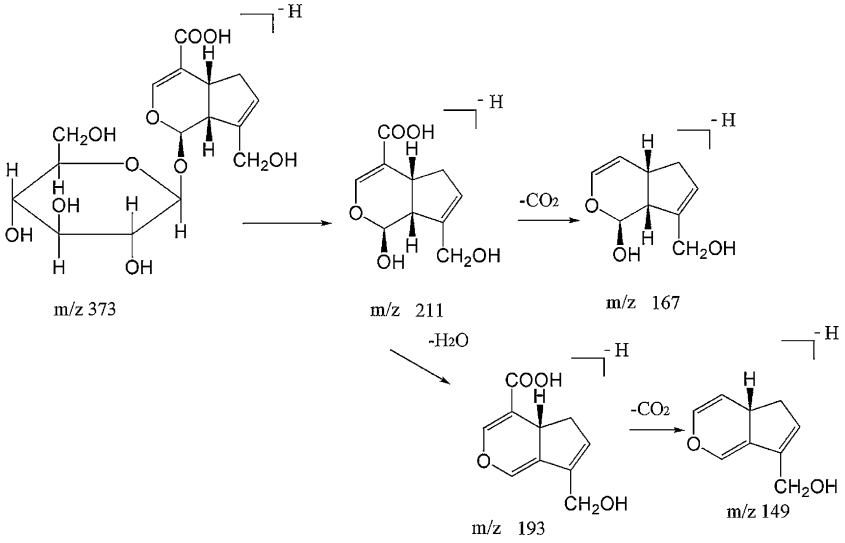


Fig. 4. The proposed fragmentation pattern of geniposidic acid in negative mode.

with *m/z* 183. Compound **30** gave rise the ion of *m/z* at 268, which was proposed generated through the loss of CH_3 from $[M-H]^-$ at *m/z* 283. A further loss of CO from *m/z* 268 gave the ion of *m/z* at 240. By referring to the reported literature [23], compound **27** was identified as rhein, compound **30** was identified as physcione.

Compounds **25** and **28** both gave negative ions at *m/z* 269 suggesting that they were also a pair of isomers. Compound **25** gave a $[M-H]^-$ ion at *m/z* 269; the fragments at *m/z* 251 and 223 were generated through the elimination of H_2O and further loss of CO from *m/z* 269. While for compound **28**, $[M-H]^-$ ion at *m/z* 269 gave two major fragments at *m/z* 241 and 225 through the losses of CO and CO_2, respectively. By referring to the literature [22], compound **25** was identified as aloe-emodin, compound **28** was identified as emodin.

Compound **29** was tentatively identified as chrysophanol. The MS spectra in negative mode gave the ion at *m/z* 225 from its $[M-H]^-$ at *m/z* 253, which was proposed generated through the loss of CO, consistent with the literature data [4].

3.6. Identification of coumarins in Zhi-Zi-Da-Huang decoction

Coumarins might exist in Zhi-Zi-Da-Huang decoction due to their occurrence in *Citrus aurantium* L. The peaks of coumarins have not been found in PDA and TIC chromatograms of ZZDHD due to the low contents and interferences from other co-ingredients. Considering the fluorescent properties of coumarins [23–25] and the high selectivity of HPLC-FD, fluorescence detector combined with HPLC–PDA–ESI/MS/MS can be applied to characterise coumarins in ZZDHD. Four strongly fluorescent compounds were eluted in FD chromatogram (λ_{ex} 329 nm/λ_{em} 446 nm), and two of them were tentatively identified as follows: Peak 7 was identified as umbelliferone, a simple coumarin. The MS spectra in negative mode exhibited an abundant parent ion $[M^{**}-H]^-$ at *m/z* 161, a fragment ion at *m/z* 133 was also observed, which could be attributed to the loss of CO from the parent ion. The loss of 44 mass units from *m/z* 161 (loss of CO_2) occurred with a peak observed at *m/z* 117. These fragments were in accordance with previous data [26].

Peak 4 gave a $[M-H]^-$ ion at *m/z* 201 and a dimer ion $[2M-H-CO]^-$ at *m/z* 375 in negative mode. The ion at *m/z* 173 and *m/z* 157 resulted from the loss of CO and CO_2 from the ion at *m/z* 201, respectively. By searching the known coumarins in four herbs, peak 4 was tentatively identified as xanthotoxol.

3.7. Identification of iridoids in Zhi-Zi-Da-Huang decoction

The UV spectra of five compounds (peaks 1, 2, 3, 5, and 6 in Fig. 1) in the HPLC chromatogram showed maximum absorption bands at 236–239 nm, consistent with the characteristic absorption of iridoids reported in *Gardenia jasminoides* Ellis [27].

Compound **1** showed $[M-H]^-$ at *m/z* 373, $[M+Cl]^-$ at *m/z* 409 and $[M+CH_3COO]^-$ at *m/z* 433 in negative mode. The ion at *m/z* 211 given by MS^2 spectrum suggesting the loss of one hexose (162 Da) from the precursor ion $[M-H]^-$. A series of ions at *m/z* 167 $[M-H-162-CO_2]^-$, 193 $[M-H-162-H_2O]^-$ and 149 $[M-H-162-H_2O-CO_2]^-$ were also observed, which strongly suggest the structure of a C–COOH and a C–OH. In positive mode, compound **1** yielded a series of ions including $[M+H]^+$ ion at *m/z* 375, $[M+Na+H_2O]^+$ ion at *m/z* 415, $[M+Na]^+$ ion at *m/z* 397, $[M+NH_4]^+$ ion at *m/z* 392, $[M+H-162]^+$ ion at *m/z* 213, $[M+H-162-H_2O]^+$ ion at *m/z* 195, $[M+H-162-2H_2O]^+$ ion at *m/z* 177 and $[M+H-162-H_2O-CO]^+$ ion at *m/z* 167. Peak 1 was thus tentatively identified as geniposidic acid. Fig. 4 shows the proposed fragmentation pattern of geniposidic acid in negative mode.

Compound **2** showed a $[M-H]^-$ ion at *m/z* 391. Its MS^2 spectrum gave a series of ions including $[M-H-162]^-$ ion at *m/z* 229, $[M-H-162-H_2O]^-$ ion at *m/z* 211, $[M-H-162-CO_2]^-$ ion at *m/z* 185 and $[M-H-162-H_2O-CO_2]^-$ ion at *m/z* 167. In positive mode, a series of ions including $[M+Na]^+$ ion at *m/z* 415, $[M+NH_4]^+$ ion at *m/z* 410, $[M+Na-H_2O]^+$ ion at *m/z* 397, $[M-H_2O+H]^+$ ion at *m/z* 375 and $[M-2H_2O+H]^+$ ion at *m/z* 357 were observed. Thus peak 2 was plausibly characterised as shanzhiside.

Compound **3** exhibited a $[M-H]^-$ ion at *m/z* 403, a $[M+CH_3COO]^-$ ion at *m/z* 463, a $[M+Cl]^-$ ion at *m/z* 439 and a $[M-H-162]^-$ ion at *m/z* 241 in the MS^2 spectrum, suggesting the presence of one hexose, the $[M-H-162-H_2O]^-$ ion at *m/z* 223 indicated the presence of a hydroxyl group. Thus it was preliminarily identified as scandoside methyl ester.

Compound **5** yielded the ions of $[M+CH_3COO]^-$ at m/z 609, $[M-H]^-$ at *m/z* 549 and $[M+Cl]^-$ at *m/z* 585 in negative mode. In positive mode, its $[M+Na]^+$ at *m/z* 573, $[M+H]^+$ at *m/z* 551, $[M+NH_4]^+$ at *m/z* 568 and $[M+H-H_2O]^+$ at *m/z* 533 were observed, the ions at *m/z* 389 and 371 were obtained through the losses of one hexose (162 Da) and one glucose (180 Da) from the precursor ion $[M+H]^+$ at *m/z* 551, respectively. With successive losses of one hexose (162 Da) and one molecule of water (18 Da) from the ion of m/z 389, the fragment ions at *m/z* 227 and 209 were produced. All of the information above coincided with those reported [28] and suggested the presence of disaccharide. Thus compound **5** was characterised as genipingentiobioside.

Peak 6 was identified as geniposide. Adduct ions of the compounds in positive mode were $[M+Na]^+$ at *m/z* 411, $[M+NH_4]^+$ at *m/z* 406, $[M+K]^+$ at *m/z* 427, $[2M+H]^+$ at *m/z* 777 and $[2M+Na]^+$ at *m/z* 799, the quasi-molecular ions and adduct ions of geniposide in negative mode were $[M-H]^-$ at *m/z* 387 and $[M+CH_3COO]^-$ at *m/z* 447. In ESI-MS^- and ESI-MS^+ patterns, the ions at *m/z* 225 and 227 were obtained through the losses of a hexose (162 Da) from the precursor ion $[M-H]^-$ (*m/z* 387) and $[M+H]^+$ (*m/z* 389), respectively. With successive loss of one molecule of water (18 Da) from the ion of *m/z* 225 and 227, the fragment ions at *m/z* 207 and 209 were obtained, respectively. These fragments were in good agreement with the literature [29].

Compound **10** gave $[M-H]^-$ and $[M+Cl]^-$ at *m/z* 427 and 463, respectively. $[M-H-162]^-$ at *m/z* 265 and $[M-H-162-H_2O]^-$ at *m/z* 247 were also observed. By searching the known iridoids in four herbs, compound **10** was preliminarily identified as asperuloside.

4. Conclusion

In this study, a total of thirty active components, namely six iridoids, seventeen flavonoids, five anthraquinones and two coumarins were identified in Zhi-Zi-Da-Huang decoction by using high-performance liquid chromatography coupled with photodiode array detector, fluorescence detector and electrospray ionization tandem mass spectrometry in both positive and negative ion mode. Different components could be characterised by their UV absorption patterns, fluorescent characteristics, specific molecular ions and fragment ions, along with their chromatographic retention times. The tandem mass spectrometric fragmentation behaviour of the compounds was also investigated extensively. Constituents such as coumarins, which could not be found by single analytical tools, were tentatively identified in this paper. Our present method could reduce the necessity to isolate each individual constituent from ZZDHD utilizing tedious conventional phytochemical procedures. It gave a good example for the rapid identification of bioactive constituents in the complex system.

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