A comparison of the chemical compounds in single- and co-boiled Zhi-Zi-Da-Huang decoctions with UPLC-qTOF-MS/MS and UPLC-DAD

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Research

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Abstract

Background

Zhi-Zi-Da-Huang decoction (ZZDHD) is a famous Traditional Chinese Medicine decoction due to its therapeutic effects on clinical hepatobiliary disorders. ZZDHD is composed of *Gardeniae Fructus*, *Rhei Radix et Rhizoma*, *Fructus Aurantii Immaturus*, and *Sojae Semen Praeparatum*. With the development of current technology, dispensing granules have been widely used for convenience. However, limited research has been conducted to determine differences in the chemical compounds between dispensing granules and traditional decoction.

Methods

A strategy based on UPLC-qTOF-MS/MS and UPLC-DAD was established to quantitatively and qualitatively analyze the chemical compounds present in single- and co-boiled ZZDHD. First, we utilized UPLC-qTOF-MS/MS to identify the compounds in single- and co-boiled ZZDHD. Then, 15 compounds were quantitatively analyzed in ZZDHD by UPLC-DAD. Finally, fingerprint and chemometric analyses were adopted to evaluate the difference between single- and co-boiled ZZDHD.

Results

One hundred and forty-seven compounds were authenticated or determined according to their retention behaviors, mass mensuration, and characteristic fragment ions. In addition, 15 analytes of single- and co-boiled ZZDHD indicated a significant difference in the content of many compounds, including geniposide (G2), emodin, chrysophanol, daidzin, and crocin-I. The similarities of single and co-boiled ZZDHD were higher than 0.99, as evaluated by fingerprinting; 30 common peaks were evaluated by the chemometric analysis, which found 13 important variable values (VIP > 1) including C3, A3, crocetin, daidzin, C5, physcion, G2, genipin-1-O- β -D-gentiobioside (G1), chrysophanol, emodin, A2, A5, and C6.

Conclusions

This study provided an experimental basis for explaining the rationality of single-and co-boiled ZZDHD, and a study model for comparing the chemical substances between traditional decoctions and dispensing granules.

1 Background

As combinations of multiple compounds, Traditional Chinese Medicine decoctions are the oldest and most commonly used treatments for certain diseases. During the decoction process, chemical compounds may interact with each other due to the chemical components present in each herb. For example, large changes in the dissolution rate, generation of new chemical compounds, and changes in pharmacological effects and clinical efficacy might occur during decoction[1–3]. Dispensing granules in Chinese medicine consist of individual Chinese medicinals (e.g., plants) obtained by water extraction, concentration, drying, and granulation. Dispensing granules originated from compound granules produced in Japan in the 1970s, which were subsequently used in South Korea and Taiwan, China[4]. Compared with traditional decoctions, dispensing granules are considered to be faster, simpler, and more convenient for clinical application[5]; however, there is a controversy about the effect of co-decoction of dispensing granules that is reflected in the literature. Many studies have reported that the clinical effects of dispensing granules are equivalent to or better than the effects of traditional decoctions[6, 7], indicating that dispensing granules could replace traditional decoctions; however, there are some issues that need to be considered in the development of dispensing granules. Specifically, it is important to determine whether the production process is consistent, whether a quality control standard has been established, and whether significant differences exist in the main active components, pharmacodynamics, and clinical efficacy. However, not all traditional decoctions can be replaced with dispensing granules. Over the past decades, a few studies have revealed differences in the content of marker compounds and chemical composition between dispensing granules and traditional decoctions[8–11]. Therefore, it is essential to evaluate the chemical consistency between traditional decoctions and dispensing granules of Traditional Chinese Medicine recipes by means of modern analytical methods and technologies.

Zhi-Zi-Da-Huang decoction (ZZDHD) is a widely used Traditional Chinese Medicine decoction that contains a combination of *Gardeniae Fructus* (Zhi Zi, in Mandarin Chinese, ZZ), *Rhei Radix et Rhizoma* (Da Huang, in Mandarin Chinese, DH), *Fructus Aurantii Immaturus* (Zhi Shi, in Mandarin Chinese, ZS) and *Sojae Semen Praeparatum* (Dan Dou Chi, in Mandarin Chinese, DDC). The fruit of *Gardenia jasminoides* Ellis is included in the Chinese Pharmacopoeia under the item of ZZ. Similarly, the rhizomes and roots of *Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., and *Rheum officinale* Baill. are recorded under the item of DH. ZS derives from immature fruits of *Citrus aurantium* L. and *Citrus sinensis* (L.) Osbeck. DDC is a fermented product obtained from the ripe seed of *Glycine max* (L.) Merr. As a classical prescription for clearing heat and eliminating dampness and jaundice, ZZDHD was originally recorded in book of 'Jin Gui Yao Lve' by the physician Zhang Zhongjing (in Eastern Han Dynasty of ancient China). With the theory of Traditional Chinese Medicine, the published paper showed that ZZDHT was used in various diseases caused by heat and dampness. For example, in 80 cases of angina pectoris which was caused by heat stagnation and blood stasis, the effective rate of ZZDHT was 92.5%[12]. ZZDHD is appropriate for the treatment of acute or chronic hepatitis, alcoholic liver disease (ALD), jaundice and cholestasis[13–16]. Jaundice was closely related to the disorder of liver in the theory of TCM, and the clinical observation proved that ZZDHT had a good effect on jaundice hepatitis patients[17]. A previous pharmacological study also demonstrated that ZZDHD exhibited a significant hepatoprotective activity by reversing histopathological changes and the biochemical parameters, like aspartate transaminase (AST), alanine transferase (ALT), reduced glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD)[18]. Another investigation indicated that ZZDHD exerted a hepatoprotective effect on ANIT-induced liver injury with cholestasis by reversing the changes in bile flow, the serum and hepatic enzymes, and histopathology

of the liver tissue, and the mechanism of this activity is possibly related to its antioxidant properties[14]. ZZDHD has complex chemical compounds, and its main active ingredients include iridoid glucosides and crocins in ZZ, flavonoids in ZS and DDC, and anthraquinone in DH[19–24]. In order to facilitate clinical applications, Traditional Chinese Medicines have been developed from the decoction into a dispensing granule. However, study on the traditional decoction and dispensing granule of ZZDHD is limited. There is currently controversy about the co-boiled Zhi-Zi-Da-Huang decoction (CB, all plants were boiled together) and the single-boiled Zhi-Zi-Da-Huang decoction (SB, each plant was boiled separately).

Recently, due to the rapid development of multi combination and mixed mass spectrometry (MS) technology, an increasing number of complex Traditional Chinese Medicine decoctions have been characterized globally. Qualitative and quantitative analyses were performed by multi-wavelength ultra-high-performance liquid chromatography (UHPLC) with diode array detection detector and electrospray ionization with quadrupole time-of-flight-tandem mass spectrometry[9, 25]. These technologies have been used to evaluate the consistency of chemical compounds between traditional decoctions and dispensing granules. Liu utilized the method of ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry (UPLC-DAD-ESI-qTOF-MS) to distinguish the differences in chemical composition between the combined and separated decoctions of Shen-Shuai-Ning granules[9]. Shang proposed and validated ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-qTOF-MS/MS) method to rapidly analyze chemical changes between traditional decoction and dispensing granules of San-Ao-Tang[10]. Thus, detecting and identifying more chemical information of traditional decoctions by the methods is worthy of consideration.

The primary goal of the present study was to compare the chemical compounds from single- and double-boiled Zhi-Zi-Da-Huang decoctions. First, chemical compounds were rapidly analyzed by UPLC-qTOF-MS/MS, and the significantly changed components were identified by comparing their mass spectra with the LC-MS/MS library. Second, 15 compounds in ZZDHD were quantified by UPLC-DAD. Finally, based on the qualitative analysis of UPLC fingerprints for comparison of various samples, several chemometric analyses, including principal component analysis (PCA), partial least-squares discrimination analysis (PLS-DA), and hierarchical cluster analysis (HCA), were achieved to further evaluate the differences in quality of these ZZDHD samples. The results obtained from this study could provide a meaningful basis for explaining the rationality of single- and co-boiled ZZDHD.

2 Materials And Methods

2.1 Chemicals and reagents

Acetonitrile was of HPLC grade (Merck, Darmstadt, Germany). Formic acid and methanol were of analytical grade. Water was purchased from Hangzhou Wahaha Company. Genipin-1-O- β -D-gentiobioside (G1) and geniposide (G2) standards were isolated and purified from *Gardenia jasminoides* in our laboratory. Crocin-I and crocetin were purchased from the Chengdu Must Biotechnology Co., Ltd. (Chengdu, China) and Chengdu Chroma-Biotechnology Co., Ltd. (Chengdu, China), respectively. Narirutin, naringin, hesperidin, neohesperidin, rhein, emodin, chrysophanol, physcion, daidzin, daidzein, and genistein standards were purchased from the Chengdu Alfa Biotechnology Co., Ltd. (Chengdu, China). The purity of 15 compounds was over 98% as determined by HPLC, and their chemical structures are shown in Fig. 1.

Gardeniae Fructus (ZZ), *Rhei Radix et Rhizoma* (DH), *Aurantii Fructus Immaturus* (ZS), and *Sojae Semen Praeparatum* (DDC) were purchased from Hebei Baicaokangshen Pharmaceutical Co., Ltd. and verified by Professor Yongqing Xiao of the Institute of Chinese Materia Medica China Academy of Chinese Medical Sciences.

2.2 Preparation of standard and sample solutions

2.2.1 Preparation of standard solutions

Standards of fifteen compounds were accurately weighed and separately dissolved in methanol to obtain the stock solution. One mixed stock solution of G2, G1, naringin, and neohesperidin was obtained with a concentration of 4,430 μ g/mL, 1,380 μ g/mL, 4,520 μ g/mL, and 3,700 μ g/mL, respectively. The other mixed stock solution containing daidzin, daidzein, genistein, narirutin, hesperidin, crocin-I, rhein, crocetin, emodin, chrysophanol, and physcion was prepared with concentrations of 64.50 μ g/mL, 41.08 μ g/mL, 39.284 μ g/mL, 514 μ g/mL, 814 μ g/mL, 380 μ g/mL, 71 μ g/mL, 1.502 μ g/mL, 12.45 μ g/mL, 6.645 μ g/mL, and 1.664 μ g/mL, respectively. The stock solutions were all stored in a refrigerator at 4 °C for further analysis.

2.2.2 Preparation of the co-boiled Zhi-Zi-Da-Huang decoction solutions (CB)

The four herbs in ZZDHD, which had the following contents: 18.0 g of ZZ, 24.0 g of ZS, 20.0 g of DDC, and 6.0 g of DH (total 68.0 g), were mixed and macerated in 680 mL of deionized water for 30 min, and then extracted twice by refluxing with the same volume of water for 30 min each. The combined extract was evaporated under a vacuum and freeze-dried to a powder. As shown in Table 1, we calculated the yield of freeze-dried powder of each batch of samples (the yield of freeze-dried powder = the quality of freeze-dried powder obtained / the total quality of crude drug of the Zhi-Zi-Da-Huang decoction) and determined the amount of sample accordingly. A total of 0.2207 g (equivald to 1 g crude drug of the Zhi-Zi-Da-Huang decoction) of freeze-dried powder of ZZDHD was dissolved into 10 mL 75% methanol, followed by ultrasonic extraction for 30 min. The weight lost by the extracted solution was compensated prior to filtration. After centrifuging at 10,000 rpm for 5 min, the supernatant was filtered using a 0.22 μ m membrane and stored in a refrigerator at 4 °C for further analysis.

2.2.3 Preparation of the single-boiled Zhi-Zi-Da-Huang decoction solutions (SB)

Separately, 30.0 g of ZZ, ZS, DDC, and DH was weighed, macerated and extracted twice by refluxing with 300 mL of deionized water for 30 min. After concentrating the samples, the extracts were freeze-dried into powder from water extracts, and the amount of sample was determined according to the yield

(Table 1). In total, 0.2207 g (equivalent to 1.0 g crude drug of the Zhi-Zi-Da-Huang decoction) freeze-dried powder with ZZ, ZS, DDC, and DH in the ratio of (9:12:10:3, W/W/W/W) was weighed and prepared following the method described above.

Table 1
Yield of freeze-dried powder of co-boiled (CB) and single-boiled (SB) Zhi-Zi-Da-Huang decoction.

Sample	Herbs	Freeze-dried Powder/g			Yield/%	RSD/%
		1	2	3		
CB	ZZ, DH, ZS, DDC	15.01	15.69	15.11	22.44 ± 0.52	2.32
SB	ZZ	8.58	8.64	8.42	28.46 ± 0.37	1.31
	DH	8.54	10.45	9.74	31.87 ± 3.25	10.08
	ZS	5.4	6.21	6.37	19.93 ± 1.72	8.68
	DDC	4.71	5.58	5.27	17.28 ± 1.47	8.5

2.3 Qualitative analysis with UPLC-qTOF-MS/MS

2.3.1 Liquid chromatography

UPLC was performed with a Waters Acquity Ultra-High-Performance Liquid Chromatography System, equipped with a DAD detector, a quaternary pump and an autosampler. Chromatographic separation was performed on an Acquity UPLC BEH column (2.1 × 100 mm i.d., 1.7 μm). The mobile phase was composed of solvent A (acetonitrile) and solvent B (0.5% formic acid aqueous solution) with a gradient elution program: 5% A (0–2 min), 5–28% A (2–5 min), 28% A (5–11 min), 28–92% A (11–13 min), 92–100% A (13–16 min), 100% A (16–19 min). The flow rate was set at 0.3 mL/min with the column temperature maintained at 35 °C and the sample volume was 1 μL. The re-equilibration duration was set at 10 min between individual runs.

2.3.2 Mass spectrometry

High-accuracy mass spectrometric data were recorded on a Waters Xevo G2-XS Q-ToF mass spectrometer (AB SCIEX, Foster City, CA, USA). Mass spectrometry conditions for MS/MS were set as follows: MS^e acquisition mode; capillary voltage, 3.0 kV; cone hole voltage, 10 V; cone hole gas flow rate, 50 L/h; ion source compensation voltage, 80 V; ion source temperature, 150 °C; dissolvent gas temperature, 450 °C. A complete scan was performed in the ESI positive and negative ion mode within the m/z 50–1,200 Da with a cumulative time of 200 ms. The collision voltage is 10–50 V under positive ion scanning and the collision voltage is 5–60 V under negative ion scanning. Data acquisition and processing were carried out using the Masslynx 4.1 software.

2.4 Determination of 15 compounds in ZZDHD

2.4.1 UPLC-DAD conditions

The UPLC-DAD was carried out on a Shimadzu LC-30A HPLC system, consisting of a Shimadzu LC-30AT dual pump, a Shimadzu SIL-30A autosampler, a CTO-30A column compartment, and an SPD-M30A photodiode array detector. The quantitative samples were detected at 254 nm, 283 nm, and 440 nm. Other chromatographic conditions, such as the chromatographic column, mobile phase composition, gradient program, flow rate, column temperature, and injection volume, were the same as those mentioned in Sect. 2.3.1. Each sample was measured in parallel three times.

2.4.2 Validation of the quantification method

The system precision was determined by examining six replicate injections of the same sample solution within a day and performing the procedure on three consecutive days. Six independently prepared solutions from the same sample were determined to check the repeatability. The samples were measured at 0, 2, 4, 8, 12, and 24 h to verify for stability. The recovery test was conducted to evaluate the accuracy of this method. The CB sample (0.1103 g, which equated to 0.5 g crude ZZDHD) was weighed independently six times and spiked with a known amount (approximately equivalent to 0.8, 1.0, and 1.2 times the amount that the actual sample contained) of the corresponding standard compounds. Then, the spiked samples were extracted and quantified with the methods mentioned above.

2.5 Fingerprint of single-boiled and co-boiled ZZDHD

2.5.1 UPLC-DAD conditions

The qualitative samples were detected at 254 nm, 280 nm, and 440 nm as described in section '2.4.1'.

2.5.2 Validation of the fingerprint method

Precision, repeatability, and stability were determined as described in section '2.4.2'.

2.5.3 Similarity analysis of UPLC fingerprints

To establish the representative chromatographic fingerprint, a total of 12 samples (six batches of CB (S1–S6), six batches of SB (S7–S12)) were analyzed following the abovementioned methods. The chromatography fingerprint was established using the 'Similarity Evaluation System of Traditional Chinese Medicine Chromatographic Fingerprint' software (China Committee of Pharmacopeia, 2012).

2.6 Statistical analysis

The qTOF-MS/MS data of 12 samples from two methods of ZZDHD preparation were processed using Masslynx 4.1 software. HCA, PCA, and PLS-DA were implemented to characterize the dissimilarities in chemical components among different samples with regard to the processing method. These were operated by SIMCA-P software 14.0 (Umetrics, Umeå, Sweden). Nonparametric tests were used to evaluate the significant differences between the different groups by GraphPad Prism software (GraphPad Prism Software Inc., CA, USA). $P < 0.05$ was considered to be significant.

3 Results

3.1 UPLC-qTOF-MS/MS qualitative analysis of chemical constituents in ZZDHD

The MS spectra were obtained in both positive and negative ion modes. Total ion chromatograms of samples were obtained using the optimized UPLC-qTOF-MS/MS system (Fig. 2). In the full scan mass spectra, most authentic compounds exhibited $[M-H]^-$ in negative mode or $[M+H]^+$ in positive mode. The chemical formula of an unknown structure was deduced based on the high accuracy $[M-H]^-/[M+HCOO]^-$ (in the negative ion mode) or $[M+H]^+/[M+Na]^+$ (in the positive ion mode) precursor ion. Information on the characteristic constituents, such as t_R (min), formula, observed m/z values, mass error (in ppm), and MS/MS product fragment ions, is summarized in Table 2. In total, 147 compounds were identified in negative and positive ion modes, including 77 known compounds and 70 unknown compounds. Among these compounds, 11, including gallic acid-4-O- β -D-glucopyranoside, glucosylsyringic acid, Unknown 4, gallate-O-diglucoside, glycerinmonogallate, Unknown 15, 3', 4', 5, 5', 6, 7-Hexamethoxyflavone, Unknown 59, Unknown 61, Unknown 66, and Unknown 69, were found only in the co-boiled ZZDHD.

Table 2 Characterization compounds of Zhi-Zi-Da-Huang decoction by UPLC-qTOF-MS/MS.

Peak NO.	t _R /min	Formula	Negative ion m/z			Positive ion m/z			Identifier
			Calcd. mass (m/z)	Measured mass (m/z)	MS/MS	Calcd. mass (m/z)	Measured mass (m/z)	MS/MS	
1	0.80	C12H22O11	341.1084	341.1088	387.1143[M+HCOO] ⁻			365.1028[M+Na] ⁺ 707.1824[2M+Na] ⁺	Unknown
2	0.83	C7H12O6	191.0497	191.0562	127.039[M+H-2H2O-CO] ⁻ 109.0290[M+H-3H2O-CO] ⁻ 93.0342[M+H-3H2O-CO2] ⁻			215.0492[M+Na] ⁺	Quinone
3	1.04	C6H8O7	191.0192	191.0195				215.0162[M+Na] ⁺	Citric acid
4	1.05	C12H16O8	287.0767	287.0772	333.0831[M+HCOO] ⁻ 125.0232[M+H-Glc] ⁻			311.0742[M+Na] ⁺ 127.0388[M+H-Glc] ⁺	Unknown
5	1.16	C13H16O10	331.0665	331.0673	271.0462[M+H-C2H4O2] ⁻ 211.0232[M+H-2C2H4O2] ⁻ 169.0137[M+H-2C2H4O2-C2H2O] ⁻			355.0575[M+Na] ⁺	Gallate glucuronide
6	1.27	C31H47O24				803.2457	803.2443	825.2187[M+Na] ⁺	Unknown
7	1.32	C13H16O10	331.0665	331.0669	169.0137[M+H-Glc] ⁻ 151.0023[M+H-Glc-H2O] ⁻				Glucuronide
8	1.43	C7H6O5	169.0137	169.0136	125.0239[M+H-CO2] ⁻	171.0293	171.0285	127.0387[M+H-CO2] ⁺	Gallate
9	1.47	C30H22O7	493.1287	493.1289				517.1169[M+Na] ⁺	Unknown
10	1.48	C16H22O11	389.1084	389.1091	227.0175[M+H-Glc] ⁻			359.0906[M+Na] ⁺ 229.1367[M+H-Glc] ⁺	Deacetylated acid
11	1.52	C25H32O16	587.1765	587.1773		589.1921	589.1773	611.1543[M+Na] ⁺	Unknown
12	1.52	C16H20O11	387.0927	387.0937	775.18451[M+HCOO] ⁻ 225.0363[M+H-Glc] ⁻			411.0834[M+Na] ⁺	Unknown
13	1.56	C19H26O15	493.1193	493.1200	169.0132[M+H-2Glc] ⁻ 125.0233[M+H-2Glc-CO2] ⁻			517.1115[M+Na] ⁺ 127.0354[M+H-2Glc-CO2] ⁺	Gallate
14	1.66	C14H16O11	359.0614	359.0616	315.0681[M+H-CO2] ⁻ 153.0173[M+H-CO2-Glc] ⁻ 125.0232[M+H-CO2-Glc-CO] ⁻			383.0541[M+Na] ⁺ 127.0378[M+H-CO2-Glc-CO] ⁺	Unknown
15	1.76	C16H22O12	405.1033	405.1039	287.0750[M+H-C8H8O] ⁻ 125.0232[M+H-C8H8O-Glc] ⁻			429.0963[M+Na] ⁺ 245.09784[M+H-Glc] ⁺	Unknown
16	1.84	C16H22O10	373.1135	373.1190	211.0629[M+H-Glu] ⁻ 193.0534[M+H-Glu-H2O] ⁻			397.1061[M+Na] ⁺ 229.1398[M+H-C8H8O2] ⁺	Gallate
17	1.89	C16H22O12	405.1033	405.1039	287.0716[M+H-C8H8O] ⁻ 125.0232[M+H-C8H8O-Glc] ⁻			429.0963[M+Na] ⁺	Unknown
18	1.97	C16H24O11	391.1240	391.1241	229.0721[M+H-Glc] ⁻ 211.0659[M+H-Glc-H2O] ⁻ 167.0694[M+H-Glc-H2O-CO2] ⁻			415.1135[M+Na] ⁺	Carboxylic acid
19	2.11	C10H12O7	243.0505	243.0502	169.0124[M+H-C3H6O2] ⁻ 125.0237[M+H-C3H6O2-CO2] ⁻				Glycolic acid
20	2.36	C16H26O9	361.1499	361.1499	407.1550[M+HCOO] ⁻ 199.0966[M+H-Glc] ⁻ 181.0858[M+H-Glc-H2O] ⁻ 137.0957[M+H-Glc-H2O-CO2] ⁻			385.1436[M+Na] ⁺ 201.0429[M+H-Glc] ⁺	Unknown
21	2.43	C16H24O11	391.1240	391.1240	783.2554[2M+H] ⁻			415.1177[M+Na] ⁺	Shikimate

					229.0708[M-H-Glc]-			253.0679[M+Na-Glc]+ 229.1410[M+Na-Glc-H2O]+ 211.9930[M+Na-Glc-2H2O]+	
22	2.52	C16H26O9	361.1499	361.1497	407.1522[M+HCOO]- 723.3071[2M-H]- 199.0963[M-H-Glc]- 181.0859[M-H-Glc-H2O]- 137.0958[M-H-Glc-H2O-CO2]-			385.1395[M+Na]+ 747.2789[2M+Na]+	Unk
23	2.58	C11H12O7	255.0505	255.0505	211.0600[M-H-CO2]- 93.0343[M-H-Glc]-			279.0455[M+Na]+ 229.1398[M+H-CO]+	Unk
24	2.84	C16H24O9	359.1342	359.1346	405.1399[M+HCOO]- 197.9606[M-H-Glc]-			383.1262[M+Na]+ 743.2716[2M+Na]+	Unk
25	3.23	C17H24O11	403.1240	403.1246	449.1295[M+HCOO]- 241.0705[M-H-Glc]-			427.1147[M+Na]+ 831.2433[2M+Na]+ 265.0281[M+Na-Glc]+	Dea asp met
26	3.44	C13H14O9	313.0560	313.0558				337.0496[M+Na]+ 651.1091[2M+Na]+ 319.0424[M+Na-H2O]+ 301.0753[M+Na-2H2O]+	Unk
27	3.44	C16H18O9	353.0873	353.0869	179.0336[M-H-C7H10O5]- 135.0437[M-H-C7H10O5-CO2]-			377.0836[M+Na]+	Chk
28	3.52	C16H22O11	389.1084	389.1074	345.1171[M-H-CO2]- 209.0306[M-H-Glc-H2O]- 191.0183[M-H-Glc-2H2O]-			413.1022[M+Na]+	Moi
29	3.59	C27H22	345.1643	345.1616				369.1491[M+Na]+ 715.1443[2M+Na]+	Unk
30	3.66	C17H24O11	403.1240	403.1242	449.1311[M+HCOO]- 241.0710[M-H-Glc]- 223.0605[M-H-Glc-H2O]-			427.1232[M+Na]+ 831.2433[2M+Na]+ 409.1067[M+Na-H2O]+ 265.0664[M+Na-Glc]+ 247.0527[M+Na-Glc-H2O]+	Gar
31	3.69	C16H26O8	345.1549	345.1554	391.1607[M+HCOO]- 691.3181[2M-H]- 165.0913[M-H-Glc-H2O]-	347.1706	347.1704	369.1530[M+Na]+ 207.0651[M+Na-Glc]+ 189.0544[M+Na-Glc-H2O]+	Jas
32	3.78	C17H26O11	405.1397	405.1397	451.1242[M+HCOO]-			427.1147[M+Na]+	Unk
33	3.83	C17 H24 O11	403.1240	403.1238	449.1300[M+HCOO]- 241.0700[M-H-Glc]-			427.1190[M+Na]+ 831.2433[2M+Na]+ 265.0686[M+Na-Glc]+ 247.0582[M+Na-Glc-H2O]+	Sca este
34	3.98	C21H34O12	477.1972	477.1975	523.1696[M+HCOO]-				Unk
35	4.01	C16H26O8	345.1549	345.1553	391.1608[M+HCOO]-	347.1706	347.1700	369.1491[M+Na]+ 715.3159[2M+Na]+ 229.1398[M+H-C8H8O2]+	Jas
36	4.06	C13H14O9	313.0560	313.0562	627.1202[2M-H]-			337.0496[M+Na]+ 651.1177[2M+Na]+	Unk
37	4.13	C17H28O10	391.1604	391.1604					Unk
38	4.16	C15H14O6	289.0716	289.0708	245.0807[M-H-CO2]-	291.0869	291.0863		Cat
39	4.19	C16H18O9	353.0873	353.0874	707.1819[M+HCOO]- 191.0522[M-H-Glc]	355.1029	355.1022	377.0781[M+Na]+ 731.1740[2M+Na]+	5-O- acic
40	4.23	C13H14O9	313.0560	313.0578	627.1203[2M-H]-			337.0496[M+Na]+	Unk
41	4.33	C23H34O15	549.1819	549.1922	595.1924[M+HCOO]- 225.0763[M-H-2Glc]- 207[M-H-2Glc-H2O]-			573.1796[M+Na]+ 1123.3578[2M+Na]+	Gen gen
42	4.39	C11H12O6	239.0556	239.0562	195.0656[M-H-CO2]- 177.0546[M-H-CO2-H2O]- 149.0589[M-H-CO2-H2O-CO]-			263.0513[M+Na]+	Unk
43	4.48	C21H26O13	485.1295	485.1306	531.1357[M+HCOO]- 971.2648[2M-H]-	487.1452	487.1457	509.1280[M+Na]+ 995.2667[2M+Na]+ 373.1830[M+Na-C8H8O2]+	Fab

					177.0186[M-H-C11H18O9-CH2]-			341.0872[M+H-C5H8O4-CH2]+ 179.0339[M+H-C11H18O9-CH2]+	
44	4.54	C27H30O15	593.1506	593.1514	473.1095[M-H-C4H8O4]-	595.1663	595.1671	617.1490[M+Na]+	Lon
45	4.56	C17H22O10	385.1135	385.1136	223.0606[M-H-Glc]- 205.0501[M-H-Glc-H2O]-			409.1067[M+Na]+ 795.2334[2M+Na]+ 247.0560[M+Na-Glc]+	Unk
46	4.68	C17H24O10	387.1296	387.1296	433.1408[M+HCOO]- 225.0763[M-H-Glc]- 207.0660[M-H-Glc-H2O]-	389.1448	389.1443	411.1290[M+Na]+ 799.2553[2M+Na]+ 227.0914[M+H-Glc]+ 209.0808[M+H-Glc-H2O]+ 191.0703[M+H-Glc-2H2O]+	Gen
47	4.80	C33H42O19	741.2242	741.2246	787.2296[M+HCOO]- 579.1746[M-H-Glc]-			765.2151[M+Na]+	Nar gluc
48	4.86	C21H20O9	415.1029	415.1042	461.1094[M+HCOO]- 253.0505[M-H-Glc]-	417.1186	417.1183	439.1006[M+Na]+ 255.0612[M+H-Glc]+	Dai
49	4.93	C16H26O7			375.1661[M+HCOO]- 167.0323[M-H-Glc]-	331.1757	331.1747	353.1586[M+Na]+ 683.3264[2M+Na]+ 169.1223[M+H-Glc]+	Jas
50	4.94	C22H22O10				447.1291	447.1293	469.1173[M+Na]+ 285.0735[M+H-Glc]+ 167.1212[M+H-Glc-C8H6O]+	Glyc
51	4.96	C37H40O16	739.2238	739.2234				763.2059[M+Na]+	Unk
52	5.08	C33H42O9	741.2242	741.2242	787.2291[M+HCOO]- 271.0672[M-H-2Glc-Rha]-	743.2399	743.2404	765.2227[M+Na]+ 273.0718[M+H-2Glc-Rha]+	Nar gluc neo
53	5.09	C27H30O16	609.1456	609.1465	300.0272[M-H-Rha-Glc]-	611.1612	611.1621	633.1438[M+Na]+ 303.0504[M+H-Rha-Glc]-	Ruti
54	5.16	C27H32O15	595.1663	595.1668	641.1731[M+HCOO]- 287.0577[M-H-Rha-Glc]- 151.0027[M-H-Rha-Glc-C8H8O2]-	597.1819	597.1827	619.1581[M+Na]+ 435.1237[M+H-Rha]+ 289.0676[M+H-Rha-Glc]+ 153.0166[M+H-Rha-Glc-C8H8O2]+	Erio
55	5.18	C20H28O10	427.1604	427.1605	855.3271[2M-H]- 265.1075[M+H-Glc]-			451.1581[M+Na]+ 879.3251[2M+Na]+ 289.1024[M+Na-Glc]+	Unk
56	5.25	C21H20O12	463.0877	463.0884	301.0385[M-H-Glc]-	465.1033	465.1036	487.2151[M+Na]+ 303.0503[M+H-Glc]+	Isoc
57	5.27	C27H32O15	595.1663	595.1688	1191.3396[2M-H]- 459.1149[M-H-C8H8O2]- 151.0030[M-H-Rha-Glc-C8H8O2]-	597.1819	597.1827	619.1652[M+Na]+ 435.1237[M+H-Rha]+ 289.0676[M+H-Rha-Glc]+ 153.0166[M+H-Rha-Glc-C8H8O2]+	Neoeriocitrin
58	5.32	C27H34O15	597.1819	597.1829		599.1976	599.1974	621.1761[M+Na]+	Unk
59	5.37	C22H22O11	461.1084	461.1094	507.1164[M+HCOO]-	463.1240	463.1246	485.1065[M+Na]+ 301.0711[M+H-Glc]+	Unk
60	5.39	C21H20O10	431.0978	431.0983	477.1039[M+HCOO]- 269.0457[M+H-Glc]-	433.1135	433.1139	455.0952[M+Na]+ 271.0568[M+H-Glc]+ 243.0613[M+H-Glc-CO]+	Gen
61	5.40	C32H42O14	649.2496	649.2505	605.2639[M-H-CO2]- 443.2107[M+H-CO2-Glc]-			673.2403[M+Na]+ 629.2582[M+Na-CO2]+ 467.1535[M+Na-CO2-Glc]+	Unk
62	5.46	C22H24O11	463.1240	463.1248	509.1304[M+HCOO]- 927.2535[2M-H]- 301.0740[M+H-Glc]-	465.1397	465.1388	487.1222[M+Na]+ 951.2556[2M+Na]+	Hes
63	5.52	C27H32O14	579.1714	579.1740	625.1776[M+HCOO]- 1159.3480[2M-H]- 459.1146[M-H-C8H8O]- 271.0616[M-H-Rha-Glc]- 151.0028[M-H-Rha-Glc-C8H8O]-	581.1870	581.1884	603.1710[M+Na]+ 1183.3512[2M+Na]+ 419.1342[M+H-Rha]+ 273.0764[M+H-Rha-Glc]+ 153.0179[M+H-Rha-Glc-C8H8O]+ 147.0414[M+H-Rha-Glc-C6H6O3]+	Nar
64	5.56	C27H30O14	577.1557	577.1567	269.0460[M-H-Rha-Glc]- 151.0027[M-H-Rha-Glc-C8H6O]-	579.1714	579.1724	271.0568[M+H-Rha-Glc]+ 153.0166[M+H-Rha-Glc-C8H6O]+	Rho

65	5.58	C44H64O24	975.3709	975.3696	1021.3752[M+HCOO] ⁻ 651.2679[M-H-2Glc] ⁻ 327.1615[M-H-2Glc-2Glc] ⁻ 283.1700[M-H-2Glc-2Glc-CO2] ⁻ -			999.3718[M+Na] ⁺ 675.2645[M+Na-2Glc] ⁺ 351.1546[M+Na-2Glc-2Glc] ⁺	Cro
66	5.62	C27H32O14	579.1714	579.1788	1159.3497[2M-H] ⁺ 459.1168[M-H-C8H8O] ⁻ 271.0622[M-H-Rha-Glc] ⁻ 151.0038[M-H-Rha-Glc-C8H8O] ⁻	581.1870	581.1890	603.1709[M+Na] ⁺ 435.1293[M+H-Rha] ⁺ 273.0752[M+H-Rha-Glc] ⁺ 153.0166[M+H-Rha-Glc-C8H8O] ⁺	Nar
67	5.73	C32H40O17	695.2222	695.2214	741.2259[M+HCOO] ⁻	697.2344	697.2365	719.222[M+Na] ⁺	Unk
68	5.73	C28H34O15	609.1819	609.1841	301.0722[M-H-Rha-Glc] ⁻ 286.0485[M-H-Rha-Glc-CH3] ⁻	611.1976	611.1992	633.1818[M+Na] ⁺ 303.0872[M+H-Rha-Glc] ⁺ 153.0191[M+H-Rha-Glc-C9H10O2] ⁺	Hes
69	5.81	C31H32O16	659.1612	659.1771	497.1339[M-H-Glc] ⁻	661.1769	661.1788	683.1613[M+Na] ⁺	3'-4-hyd glut
70	5.91	C38H54O19	813.3181	813.3193	859.3226[M+HCOO] ⁻ 651.2784[M-H-Glc] ⁻ 489.2144[M-H-2Glc] ⁻ 327.1611[M-H-3Glc] ⁻ 283.1705[M-H-3Glc-CO2] ⁻			837.3183[M+Na] ⁺ 675.2648[M+Na-Glc] ⁺ 513.2100[M+Na-2Glc] ⁺ 351.1546[M+Na-3Glc] ⁺	Cro
71	5.92	C15H10O5	269.0450	269.0454	151.0027[M-H-C8H6O] ⁻	271.0606	271.0606	293.1542[M+Na] ⁺ 153.0166[M+H-C8H6O] ⁻	Api
72	5.98	C22H24O11	463.1240	463.1254	301.0740[M-H-Glc] ⁻	465.1430	465.1430	487.1227[M+Na] ⁺ 303.0869[M+H-Glc] ⁺	Hes isor
73	5.98	C27H36O12	551.2129	551.2136	597.2201[M+HCOO] ⁻ 1103.4235[2M-H] ⁻			575.2907[M+Na] ⁺	Unk
74	6.02	C27H36O12	551.2129	551.2150	597.2205[M+HCOO] ⁻ 1103.4350[2M-H] ⁻	553.2285	553.2296	575.2097[M+Na] ⁺ 1127.4340[2M+Na] ⁺ 207.0644[M+H-2Glc-CO] ⁺	Unk
75	6.08	C27H28O13	559.1452	559.1465	397.1143[M-H-C9H7O3] ⁻ 223.0609[M-H-C9H7O3-Rha-CO] ⁻	561.1633	561.1633	583.1441[M+Na] ⁺ 1143.2992[2M+Na] ⁺	4-Si cafl
76	6.08	C22H22O11	461.1084	461.1095	923.2242[2M-H] ⁻ 313.0531[M-H-C9H8O2] ⁻			485.1050[M+Na] ⁺ 315.0816[M+H-C9H8O2] ⁺	2-O-gluc
77	6.17	C28H34O14	593.1870	593.1887	639.1938[M+HCOO] ⁻ 285.0767[M-H-Rha-Glc] ⁻	595.2027	595.2145	617.1866[M+Na] ⁺	6'-O Sin
78	6.23	C22H36O13	461.1143	461.2225	507.218[M+HCOO] ⁻			485.1997[M+Na] ⁺ 301.1031[M+H-Glc] ⁺	Unk
79	6.32	C22H22O11	461.1084	461.1092	299.0593[M-H-Glc] ⁻			485.1031[M+Na] ⁺ 301.1031[M+H-Glc] ⁺	1-O-cinr gluc
80	6.35	C55H74O28	1181.4228	1181.4275	857[M-H-2Glc] ⁻ 651.2679[M-H-3Glc-CO2] ⁻			881.3163[M+Na-2Glc] ⁻ 675.2595[M+Na-3Glc-CO2] ⁻	Unk
81	6.36	C32H42O13	633.2547	633.2558	427.2139[M-H-Glc-CO2] ⁻			657.2484[M+Na] ⁺	Unk
82	6.41	C15H10O4	253.0501	253.0503		255.0657	255.0659	277.0447[M+Na] ⁺	Dai
83	6.60	C16H12O5				285.0763	285.0763	307.0584[M+Na] ⁺	Glyc
84	6.77	C38H47O10				663.3169	663.3191	685.3001[M+Na] ⁺	Unk
85	6.84	C11H6O4	201.0188	201.0183	173.0263[M-H-CO] ⁻ 145.0292[M-H-2CO] ⁻ 117.0343[M-H-3CO] ⁻	203.0344	203.0324		Ber
86	7.00	C28H34O14	593.1870	593.1867	639.1920[M+HCOO] ⁻ 1187.3767[2M-H] ⁻ 285.0767[M-H-Rha-Glc] ⁻ 151.0219[M-H-Rha-Glc-C9H10O] ⁻	595.2027	595.1996	617.1820[M+Na] ⁺ 471.2157[M+Na-Rha] ⁺ 287.0899[M+H-Rha-Glc] ⁺ 153.0166[M+H-Rha-Glc-C9H10O] ⁺	Pon

87	7.08	C21H20O10	431.0975	431.0975	285.0767[M-H-C6H10O4]-			455.0920[M+Na]+ 287.0913[M+H-C6H10O4]+	Alc gluc
88	7.15	C21H20O10	431.0987	431.0968	269.0444[M-H-Glc]- 225.0544[M-H-Glc-CO2]-			455.0941[M+Na]+ 271.9568[M+H-Glc]-	Emc Gluc
89	7.24	C27H36O11	535.2179	535.2169		537.2330	537.2336	559.2155[M+Na]+	6'-O jasr
90	7.35	C42H45O7	661.3346	661.3346		663.3169	663.3190	355.0999[M+H-Rha-Glc]+	Unk
91	7.60	C20H18O10	417.0822	417.0811				441.0772[M+Na]+	Unk
92	7.65	C28H34O14	593.1870	593.1860	431.1326[M-H-Glc]-				6'-O gen
93	7.91	C15H10O5	269.0450	269.0443		271.0606	271.0597	541.0952[2M+H]+	Gen
94	7.97	C15H12O5	271.0599	271.0606	151.0024[M-H-C8H8O]- 107.0127[M-H-C8H8O-CO2]-	273.0763	273.0755	153.0166[M-H-C8H8O]+	Nar
95	8.32	C48H59O22	987.3498	987.3456	825.3241[M-H-Glc]- 663.2418[M-H-2Glc]- 501.2146[M-H-3Glc]-			1011.3468[M+Na]+ 849.2996[M+Na-Glc]+ 687.2340[M+Na-2Glc]+	Unk
96	8.70	C15H16O4				261.1127	261.1117	283.0938[M+Na]+ 243.1011[M+H-H2O]+	Mer
97	8.71	C21H24O6	371.1495	371.1490	743.3033[2M-H]-			395.1471[M+Na]+	Unk
98	8.85	C32H44O14	651.2653	651.2648	697.2690[M+HCOO]-				Unk
99	9.01	C32H44O14	651.2653	651.2650	697.2705[M+HCOO]- 327.1615[M-H-Glc]- 283.1715[M-H-Glc-CO2]- 239.1821[M-H-Glc-2CO2]-			675.2297[M+Na]+ 351.1546[M+Na-2Glc]+	Cro
100	9.29	C21H24O6	371.1494	371.1495	743.3068[2M-H]-			395.1430[M+Na]+	Unk
101	9.50	C44H64O24	975.3709	975.3678	1021.3727[M+HCOO]- 651.2679[M-H-2Glc]- 327.1615[M-H-2Glc-2Glc]- 283.1715[M-H-2Glc-2Glc-CO2]-			999.3626[M+Na]+ 675.2595[M+Na-Glc]+	Cro
102	10.16	C21H22O5				355.1545	355.1528	203.0178[M+H-C10H16O]+ 147.0430[M+H-C10H16O-2CO]+	Isor
103	10.23	C20H18O9	401.0873	401.0868	357.0953[M-H-CO2]- 313.0723[M-H-2CO2]-				Unk
104	11.44	C21H23O6						395.1467[M+Na]+ 767.3038[2M+Na]+	Unk
105	11.79	C15H16O4				261.1127	261.1121	283.0938[M+Na]+ 243.0996[M+H-H2O]+	Hes
106	12.20	C16H10O6	297.0399	297.0394	253.0583[M-H-CO2]- 225.0548[M-H-CO2-CO]-	299.0556	299.0589		Unk
107	12.34	C20H20O7				373.1287	373.1281	395.1101[M+Na]+ 767.2318[2M+Na]+ 343.0810[M+H-2CH3]+ 271.0599[M+H-4CH3O]+	Tan
108	12.38	C38H54O19	813.3181	813.3162	859.3215[M+HCOO]- 651.2628[M-H-Glc]- 489.2126[M-H-2Glc]- 327.1615[M-H-3Glc]- 283.1715[M-H-3Glc-CO2]-	815.3338	815.3289	837.3161[M+Na]+ 675.2595[M+Na-Glc]- 513.2100[M+Na-2Glc]- 351.1546[M+Na-3Glc]-	Cro
109	12.52	C55H74O28	1181.4228	1181.4275	857.3233[M-H-2Glc]- 651.2679[M-H-3Glc-CO2]-				Unk
110	12.52	C21H24O6	371.1495	371.1490					Unk
111	12.52	C19H18O6				343.1182	343.1177	365.0988[M+Na]+ 707.2096[2M+Na]+ 313.0688[M+H-2CH3]+ 285.0688[M+H-2CH3-CO]+ 153.0166[M+H-C9H8O-CO-2CH3]+	3', 4 Tetr

112	12.55	C26H3008	469.1862	469.1862	515.1919[M+HCOO]-	471.2019	471.2014	493.1789[M+Na]+ 963.3779[2M+Na]+	Lim
113	12.59	C17H1406	313.0712	313.0712	269.0457[M-H-CO2]-	315.0869	315.0863		Unk
114	12.71	C48H60022	987.3498	987.3476	825.3182[M-H-Glc]- 663.3836[M-H-2Glc]- 501.2146[M-H-3Glc]-			1011.3468[M+Na]+ 849.3115[M+Na-Glc]+ 687.2340[M+Na-2Glc]+	Unk
115	12.75	C28H36010	531.2230	531.2228		533.2387	533.2380	555.2204[M+Na]+	Nor
116	12.78	C48H78019	957.5059	957.5042	1003.509[M+HCOO]-	959.5216	959.5223	981.4979[M+Na]+	Unk
117	12.79	C15H806	283.0243	283.0239	239.0342[M-H-CO2]- 183.0439[M-H-CO2- 2CO]-	285.0399	285.0393		Rhe
118	12.79	C18H1607	343.0818	343.0816		343.0974	343.0967		Unk
119	12.80	C32H44014	651.2653	651.2646	327.1689[M-H-2Glc]- 283.1749[M-H-2Glc- CO2]-			675.2632[M+Na]+	Cro
120	12.83	C48H78018	941.5110	941.5092	987.514[M+HCOO]-	943.5266	943.5270	965.5088[M+Na]+	Unk
121	12.83	C21H2208				403.1399	403.1393	425.1209[M+Na]+ 827.2540[2M+Na]+ 388.1130[M+H-CH3]+ 373.0912[M+H-2CH3]+ 358.0676[M+H-3CH3]+	Not
122	12.85	C32H44014	651.2653	651.2658	327.1598[M-H-2Glc]- 283.0238[M-H-2Glc- CO2]-			675.2595[M+Na]+	Cro
123	12.91	C47H76017	911.5004	911.4994	957.5037[M+HCOO]-			935.4932[M+Na]+	Unk
124	13.05	C22H2409				433.1499	433.1501	418.1250[M+H-CH3]+	3', 4 Hep
125	13.07	C57H6203	793.4621	793.4614	839.4663[M+HCOO]-	795.4777	795.4777	817.4599[M+Na]+	Unk
126	13.08	C17H2604	293.1753	293.1755	264.9948[M-H-CO]- 221.1583[M-H-CO- CO2]-				Unk
127	13.13	C21H2209				419.1342	419.1342	441.1161[M+Na]+ 859.2434[2M+Na]+ 404.1098[M+H-CH3]+ 389.0582[M+H-2CH3]+	3', 4 Hex
128	13.17	C13H24012				373.1346	373.1352	395.1116[M+Na]+ 767.2371[2M+Na]+	Unk
129	13.22	C17H2605	309.1702	309.1706	146.9664[M-H-Glc]-				Unk
130	13.22	C30H50012	601.3224	601.3227	657.0352[M+HCOO]-			625.3192[M+Na]+	Unk
131	13.24	C13H2004	239.1283	239.1286	195.1385[M-H-CO2]-				Unk
132	13.33	C20H2008				389.1236	389.1232	411.1052[M+Na]+ 799.2224[2M+Na]+ 374.1299[M+H-CH3]+ 359.0751[M+H-2CH3]+	5-H 5'- pen
133	13.33	C30H4805	487.3423	487.3433	469.0117[M-H-H2O]-				Erul
134	13.38	C24H3006				415.2121	415.2118	437.1937[M+Na]+ 851.3989[2M+Na]+	Unk
135	13.46	C15H1005	269.0450	269.0453	241.0499[M-H-CO]- 225.0549[M-H-CO-O]-	271.0606	271.0601		Emc
136	13.84	C13H2808	311.1706	311.1686					Unk
137	13.90	C24H4008				457.2776	457.2801		Unk
138	14.08	C27H54013	585.3486	585.3472	233.1061[M-H-Glc- Rha-CO2]-				Unk
139	14.11	C57H9806				879.7442	879.7438	901.7264[M+Na]+ 301.1427[M+Na-Glc-3Rha]+	Unk
140	14.16	C21H2802	311.2011	311.2020	149.0965[M-H-Glc]-				Unk
141	14.22	C15H2203	249.1491	249.1494	205.1611[M-H-CO2]-				Unk
142	14.29	C20H3408				403.2332	403.2324	425.2149[M+Na]+	Unk

					279.0934[M+Na-Rha]+	
143	14.32	C14H30O8	325.1862	325.1846		Unk
144	14.86	C24H34O2	353.2481	353.2481	325.1841[M-H-CO]- 281.2469[M-H-CO-CO2]-	Unk
145	14.98	C57H98O6			879.7442 879.7438 901.7277[M+Na]+	Unk
146	15.01	C15H32O8	339.2019	339.1994		Unk
147	15.49	C21H24O5	355.1545	355.1574	311.2009[M-H-CO2]-	Unk

* represented the standard compound.

3.1.1 Identification of compounds in Gardeniae Fructus

A total of 61 compounds were obtained from ZZ, of which were 30 known compounds. ZZ contained a large number of iridoid glucosides, including gardsoside, shanzhiside, genipin-1-β-D-gentiobioside, geniposide, and gardenoside. Crocins, including crocin-I and crocin-II, were deemed the main active compounds in ZZ[30]. Geniposide, the most abundant iridoid glucosides in ZZDHD, and crocin-I, the most abundant crocin, were used to characterize the fragmentation pathways, which are shown in Fig. A1.

The [M-H]⁻ ion and the typical ions in the MS² spectra of peak 46 (t_R=4.68), namely, m/z 387.1296 [M-H]⁻, 433.1408 [M + HCOO]⁻, and 225.0763 [M-H-Glc]⁻ were all 162 Da less compared to those of peak 41 (t_R=4.33), demonstrating that they are cleaved at the same site. By comparing the retention time values and mass data with those of the reference[26], peak 46 was therefore characterized as geniposide, and peak 41 was regarded as genipin-1-O-β-D-gentiobioside.

Peak 65 (t_R=5.58) showed the [M-H]⁻ ion at m/z 975.3709, which was the correlative elemental composition of C₄₄H₆₃O₂₄⁻. After losing two molecules of glucose and four molecules of glucose, the MS² fragment ions were generated as 651.2679 [M-H-2Glc]⁻ and 327.1615 [M-H-2Glc-2Glc]⁻, respectively. Resulting from loss of one molecule of CO₂, the [M-H-2Glc-2Glc-CO₂]⁻ ion at m/z 283.1700 was observed. Based on the molecular weight and multi-stage information provided by MSⁿ and previously published literature[27], peak 65 was inferred as crocin-I. Based on these fragmentation patterns, other crocins were accurately identified.

3.1.2 Identification of compounds in Fructus Aurantii Immaturus

In total, 51 compounds derived from ZS were detected in ZZDHD, including 26 known compounds. ZS contained large number of flavonoids, including dihydroflavones and isoflavones, such as narirutin, naringin, hesperidin, and neohesperidin. Their fragmentation patterns were in Fig. A2.

The [M-H]⁻ ion of peak 63 (t_R=5.52) was m/z 579.1740 (C₂₇H₃₁O₁₄⁻). The MS² spectra exhibited characteristic product ions, such as [M-H-C₈H₈O]⁻ (m/z 459.1146), [M-H-Rha-Glc]⁻ (m/z 271.0616), and [M-H-Rha-Glc-C₈H₈O]⁻ (m/z 151.0028), thus the compounds were deduced as narirutin. Naringin had a similar cleavage pattern to narirutin, and according to the literature[23], peak 66 (t_R=5.62) was identified as naringin.

Peak 68 (t_R=5.73) displayed [M-H]⁻ ion at m/z 609.1841 (C₂₈H₃₄O₁₅⁻) and its MS² spectra exhibited at m/z 301.0722 [M-H-Rha-Glc]⁻ and 151.0031 [M-H-Rha-Glc-C₉H₁₀O₂]⁻. Based on the fragmentation information and previously published studies[23], peak 68 was primarily identified as hesperidin.

3.1.3 Identification of compounds in Sojae Semen Praeparatum

The major active constituents of DDC were isoflavones[24]. It contained 24 compounds, including eight known compounds, such as daidzin, daidzein, and genistein[31].

In the positive mode, peak 48 (t_R=4.86) displayed the [M + H]⁺ ion at m/z 417.1214 and the [M + Na]⁺ ion at m/z 439.1006. The MS² spectrum provided a diagnostic fragment ion at m/z 255.0612, resulting from the loss of one molecule of glucose. It displayed the [M-H]⁻ ion at m/z 417.1183 and the [M + HCOO]⁻ ion at m/z 461.1094. In addition, a main MS/MS pattern at m/z 253.0505 was observed for peak 48, corresponding to the loss of a glucose residue. Consequently, peak 48 was unambiguously identified as daidzin.

Peak 82 (t_R=6.41) exhibited the [M-H]⁻ ion at m/z 253.0500 with a molecular formula of C₁₅H₉O₄⁻; it showed the quasi-molecular ion [M + H]⁺ ion at m/z 255.0659 and the sodium adduct ion [M + Na]⁺ ion at m/z 277.0447, which was definitely assigned as daidzein.

Peak 93 (t_R=7.91) had the characteristic fragment of ion [M-H]⁻ at m/z 269.0443, representing the corresponding elemental composition of C₁₅H₁₀O₅⁻, and its positive ion was 271.0597 [M + H]⁺ and 541.0952 [2M + H]⁺. It was tentatively identified as genistein.

3.1.4 Identification of compounds in Rhei Radix et Rhizoma

DH contained 25 compounds, including 14 known constituents. Anthraquinones, such as rhein and emodin, were likely detected in ZZDHD as they are compounds typically found in DH[19]. Anthraquinones generally have strong MS response in negative ion mode and weak MS response in positive ion mode.

Peak 117 ($t_R=12.79$) and 135 ($t_R=13.46$) were recognized as rhein and emodin, respectively, and they were used to characterize the fragmentation pathways (Fig. A3). In the negative ion mode, peak 117 showed [M-H]⁻ at m/z 283.0239, and then generated a fragment ion at m/z 239.0342, owing to the loss of a CO₂ residue. Subsequently, the fragment m/z 211.0391 was yielded due to the loss of CO from 239.0342. Lastly the ion further lost one molecule of CO to produce an ion at m/z 183.0441. Based upon these data and the literature[28], the compound was rhein. Peak 135 showed [M-H]⁻ at m/z 269.0453, and then generated fragments with m/z 241.0499 and m/z 225.0549, which was due to the loss of CO and CO₂. Based upon these data and previously published research[29], peak 135 was determined to be emodin. Other constituents of anthraquinones in DH were identified using this fragment information (Table 2).

3.2 UPLC-DAD quantification

3.2.1 Method validation

Quantitative method validation for the established UPLC-DAD analysis was performed for linearity, precision, stability, repeatability, and recovery, as shown in Table 3. All correlation coefficient values ($r > 0.999$) demonstrated a good linear relationship between the analyte concentrations and their peak areas within the relatively wide test ranges. The relative standard deviation (RSD) of precision with 15 analytes ranged from 0.29–3.97%. The RSDs for stability were less than 4.22%. As for repeatability, the RSDs were not more than 4.36%. The developed method also had suitable accuracy with spike recovery of 95.35–107.95% for all analytes. These results demonstrated that the proposed quantitative UPLC-DAD method was linear, precise, stable, repeatable, and sufficiently accurate for simultaneous determination of fifteen compounds in ZZDHD samples.

Table 3
Method validation for quantitative determination of fifteen compounds.

Reference Standards	Working Standard Cruve			Precision	Stability	Repeatability	Spike Recovery		
	Equation	r	Linear Range (mg/mL)	(RSD, %, n = 6)	(24 h, RSD, %)	(RSD, %, n = 6)	(Mean (RSD), %, n = 3)		
							Low	Middle	High
G1	$y = 1027.7x + 11477$	0.9998	21.56 ~ 1380.00	1.43	1.61	1.45	100.56(1.31)	102.68(1.08)	94.35(1.78)
G2	$y = 2446x + 157377$	0.9996	69.22 ~ 4430.00	0.81	0.34	1.67	101.26(0.62)	98.20(0.64)	104.39(0.42)
daidzin	$y = 12059x + 13005$	0.9998	1.01 ~ 64.50	1.46	1.07	1.36	100.34(0.57)	101.48(1.77)	100.87(0.17)
narirutin	$y = 5640.8x + 32911$	0.9998	8.03 ~ 514.00	1.40	0.97	0.65	99.92(2.47)	99.01(2.10)	107.07(1.88)
naringin	$y = 7126.7x - 255950$	0.9991	70.63 ~ 2260.00	1.11	1.74	1.72	100.44(4.71)	99.49(0.64)	106.38(0.36)
crocin-I	$y = 22492x + 41294$	1.0000	5.94 ~ 380.00	0.40	0.80	1.91	102.76(1.22)	100.40(5.31)	105.58(1.28)
hesperidin	$y = 5206.5x + 18349$	0.9996	12.72 ~ 814.00	0.73	1.44	1.60	95.24(0.40)	105.36(2.56)	99.15(2.07)
neohesperidin	$y = 8828.7x - 243244$	0.9992	57.81 ~ 1387.50	1.14	0.60	1.91	104.65(7.11)	101.19(0.38)	104.66(2.30)
daidzein	$y = 18858x + 7509.3$	0.9998	0.64 ~ 41.08	1.95	1.54	0.99	99.17(2.49)	98.66(1.94)	105.20(1.83)
genistein	$y = 23742x + 8741.3$	0.9998	0.61 ~ 39.28	1.64	1.79	1.10	102.75(0.32)	102.06(0.73)	106.41(2.35)
rhein	$y = 5393x + 4241.3$	0.9996	1.11 ~ 71.00	0.92	0.89	1.37	95.19(1.97)	107.94(1.79)	107.94(0.35)
crocetin	$y = 27114x + 401.9$	0.9998	0.02 ~ 1.50	3.97	4.22	4.36	101.38(0.97)	103.16(1.07)	100.01(3.60)
emodin	$y = 5871.2x + 626.5$	0.9999	0.19 ~ 12.45	1.71	0.95	1.46	99.47(1.48)	100.17(0.73)	107.09(0.99)
chrysophanol	$y = 8476x + 514.26$	0.9999	0.10 ~ 6.64	0.29	0.52	1.05	106.69(0.52)	107.72(2.45)	96.96(2.50)
physcion	$y = 8423.2x + 108.53$	0.9998	0.03 ~ 1.66	0.68	0.73	1.03	101.16(1.68)	98.19(1.30)	102.91(2.80)

3.2.2 Analysis of content determination

This study investigated the differences in the chemical compounds of ZZDHD prepared following two different boiling methods. Representative chromatograms of one sample (S1) and reference standards at 254 nm, 283 nm, and 440 nm are presented in Fig. 3. A total of 12 batches of ZZDHD samples

from the preparation methods were collected, prepared, and analyzed as described above. Fifteen chemical substances. Specifically, G1, G2, daidzin, narirutin, naringin, crocin-I, hesperidin, neohesperidin, daidzein, genistein, rhein, crocetin, emodin, chrysophanol, and physcion (Fig. 1) were chosen as the reference standards for quantitation. The content of each marker compound was determined at its maximum absorption wavelength, using the external standard method. The quantitative data for the marker compounds are presented in Table 4. A slightly variation in the content of other marker compounds (RSD < 30%) was observed, except for the physcion content. A relatively high content of G2, naringin, and neohesperidin were present in both the single- co-boiled samples. As shown in Fig. 4, the difference was statistically significant between the contents of G2, emodin, and chrysophanol in CB and SB ($P < 0.05$), and the difference was highly statistically significantly different between daidzin, physcion ($p < 0.01$), and crocin-I ($p < 0.001$). There were no significant differences among the other nine compounds.

Table 4
Quantitative determination results of 15 reference standards in Zhi-Zi-Da-Huang decoction ($\mu\text{g/g}$, $n = 6$).

Reference Standards	t_{R}/min	CB	SB	Source	RSD/%
G1	7.53	3940.75 ± 640.79	3804.15 ± 260.04	ZZ	5.89
G2	8.03	10946.08 ± 516.87	10186.12 ± 283.51	ZZ	5.32
daidzin	8.45	169.2 ± 5.69	183.4 ± 8.76	DDC	9.05
narirutin	9.29	1001.79 ± 151.21	923.96 ± 75.49	ZS	12.18
naringin	9.44	11492.88 ± 349.95	11580.58 ± 942.6	ZS	10.24
crocin-I	9.45	950.99 ± 48.56	994.84 ± 79.12	ZZ	12.56
hesperidin	9.59	2078.45 ± 73.72	1903.24 ± 260.08	ZS	6.85
neohesperidin	9.76	9585.49 ± 369.15	9340.64 ± 1201	ZS	16.31
daidzein	10.54	104.82 ± 2.49	106.7 ± 16.27	DDC	5.80
genistein	12.85	97.66 ± 4.31	102.54 ± 13.37	DDC	10.53
rhein	16.29	206.1 ± 49.21	203.28 ± 4.99	DH	9.80
crocetin	16.69	3.93 ± 0.28	1.16 ± 0.28	ZZ	18.22
emodin	17.02	36.94 ± 8.36	45.92 ± 2.68	DH	13.84
chrysophanol	17.72	18.69 ± 2.81	22.12 ± 1.6	DH	12.40
physcion	18.02	4.65 ± 0.41	5.64 ± 0.37	DH	57.73

3.3 UPLC-DAD fingerprint

3.3.1 Method validation

A comprehensive validation of the established UPLC fingerprint method was carried out, including assessments of precision, repeatability, and stability, and the results were summarized in Table 5. The intra- and inter-day variations of all 23 common peaks (RSDs) were within 0.02–0.19% and 0.05–0.32% for the relative retention time (RRT), as well as 1.62–4.73% and 1.83–4.87% for the relative peak area (RPA), respectively.

In the precision, repeatability, and stability tests, RSDs of the RPA from all 30 common peaks were less than 3.80%, 2.74%, and 2.54%, respectively (Table 5). These results showed that the UPLC fingerprinting method was applicable for the qualitative analysis of ZZDHD.

Table 5
Method validation of common peaks in Zhi-Zi-Da-Huang decoction.

No.	t_R /min	RSD/%			No.	t_R /min	RSD/%		
		Precision	Repeatability	Stability			Precision	Repeatability	Stability
A1 ^d	1.058	2.21	2.02	2.04	B4 ^e	9.569	0.73	1.60	1.30
A2 ^d	1.979	1.41	2.17	1.35	B5 ^e	9.720	1.14	1.91	0.60
A3 ^d	2.659	1.98	1.42	0.85	B6 ^e	11.58	0.42	1.51	0.66
A4 ^d	3.292	1.19	1.61	2.54	C1 ^f	3.639	0.46	2.20	0.67
A5 ^d	5.682	3.80	1.52	1.39	C2 ^f	9.439	0.40	1.91	0.71
A6 ^d	7.525	1.43	1.53	1.11	C3 ^f	9.542	1.63	2.31	2.15
A7 ^d	7.747	2.49	1.67	1.52	C4 ^f	9.984	2.23	1.48	1.28
A8 ^d	8.030	0.81	1.67	0.54	C5 ^f	14.853	2.75	2.55	1.33
A9 ^d	8.431	1.46	1.36	0.69	C6 ^f	15.175	0.34	1.57	0.67
A10 ^d	10.253	1.57	1.65	1.48	C7 ^f	15.396	0.42	2.06	0.59
A11 ^d	10.487	1.95	0.99	1.30	C8 ^f	16.283	0.92	1.60	0.91
A12 ^d	12.591	1.64	1.10	1.35	C9 ^f	16.693	0.55	1.75	1.68
B1 ^e	1.339	2.91	2.74	2.42	C10 ^f	17.018	1.71	1.55	1.09
B2 ^e	9.281	1.40	0.65	0.72	C11 ^f	17.716	0.29	1.05	0.45
B3 ^e	9.426	1.11	1.72	1.71	C12 ^f	18.020	0.69	1.03	0.91

d represented the peak recorded at 254 nm; e represented the peak recorded at 280 nm; f represented the peak recorded at 440 nm; t_R was retention times; RPA was relative peak areas.

3.3.2 Fingerprinting analysis

Twelve batches of samples were analyzed under optimized conditions. As shown in Fig. 5, all samples showed similar chromatographic profiles. Peaks existing in all samples were designated as common peaks, and 30 common peaks were found, including 12 common peaks identified at 254 nm, six common peaks identified at 280 nm, and 12 common peaks identified at 440 nm. According to the representative standard peak area of the fingerprint calculated by the Similarity Evaluation System Chromatographic Fingerprint of Traditional Chinese Medicine Chromatographic Fingerprint software, the RPA of the ZZDHD samples was calculated. The peak of the representative standard fingerprint was designated as the reference peak (RPA = peak area of characteristic peak/peak area of reference peak). As shown in Table A1, changes in the contents of single- and co-boiled ZZDHD decoctions were analyzed by RPA. The results indicated that the RPA varied dramatically between samples at 254 nm and 440 nm, and there were two and seven common peaks with an RPA > 15%, respectively, which demonstrated that the content of the single- and the co-boiled was different.

Table A1 Relative peak areas of samples of Zhi-Zi-Da-Huang decoction.

No.	t _R /min	Representative Peak Area	RPA												RSD%
			S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	
A1 ^d	1.058	429846	1.069	1.010	0.999	0.916	1.005	0.982	0.915	0.925	1.061	1.057	1.016	1.045	5.58
A2 ^d	1.982	168667	1.026	1.024	0.929	0.944	0.953	0.926	1.067	1.026	1.079	1.080	0.970	0.977	5.73
A3 ^d	2.662	138705	0.886	0.917	0.839	0.845	0.818	0.804	1.219	1.179	1.145	1.159	1.092	1.096	16.11
A4 ^d	3.296	352654	1.063	1.123	0.926	0.991	0.935	0.970	1.082	1.072	0.996	0.982	0.927	0.931	6.88
A5 ^d	5.689	152146	1.047	0.952	0.935	0.974	0.983	0.970	1.024	1.037	0.981	0.992	1.049	1.057	4.11
A6 ^d	7.527	385975	1.009	1.049	1.029	0.991	1.163	1.130	0.930	0.882	0.880	0.881	1.009	1.045	9.35
A7 ^d	7.748	108146	1.575	1.672	0.550	0.653	0.660	0.694	1.715	1.600	0.571	0.543	0.880	0.889	48.67
A8 ^d	8.030	2457611	1.048	1.045	0.974	0.972	1.092	1.078	0.968	0.961	0.984	0.987	0.952	0.940	5.14
A9 ^d	8.431	220362	0.922	0.974	0.948	0.961	0.959	0.931	1.012	0.990	1.086	1.119	1.038	1.060	6.35
A10 ^d	10.248	168950	1.039	0.869	1.011	0.993	0.979	0.971	1.001	0.982	1.023	1.063	1.027	1.039	4.97
A11 ^d	10.485	211302	0.963	1.025	0.931	0.953	1.001	1.012	0.909	0.900	1.202	1.226	0.951	0.926	10.74
A12 ^d	12.592	217763	0.983	0.962	0.868	0.927	1.029	1.011	0.936	0.903	1.191	1.195	0.987	1.007	10.17
B1 ^e	1.340	446235	1.013	1.006	0.833	0.823	1.191	1.178	1.014	0.898	0.906	1.044	1.066	1.028	11.84
B2 ^e	9.279	408437	1.198	1.069	0.911	0.920	0.960	0.922	1.037	0.895	0.923	1.063	1.059	1.044	9.25
B3 ^e	9.423	7728449	0.969	0.955	1.015	0.995	1.016	0.979	1.080	0.897	0.906	1.052	1.057	1.079	6.17
B4 ^e	9.565	1135465	1.018	0.984	1.040	1.078	1.084	1.071	1.009	0.782	0.769	1.060	1.060	1.044	10.89
B5 ^e	9.716	7405738	0.982	0.972	1.050	1.045	1.014	1.004	1.092	0.782	0.809	1.072	1.079	1.099	10.43
B6 ^e	11.580	372710	0.942	0.921	1.018	1.024	0.982	0.965	1.076	0.905	0.907	1.097	1.101	1.062	7.31
C1 ^f	3.646	429846	0.910	0.817	1.061	1.078	1.186	1.153	0.547	0.660	1.209	1.258	1.075	1.045	22.31
C2 ^f	9.435	168667	0.994	0.984	0.986	0.987	0.925	0.915	1.140	1.133	0.938	0.938	1.026	1.033	7.39
C3 ^f	9.538	138705	1.271	1.258	1.424	1.368	1.336	1.372	0.791	0.748	0.570	0.576	0.642	0.643	36.10
C4 ^f	9.977	352654	0.907	0.885	0.902	0.894	0.862	1.138	1.307	1.034	0.861	0.877	1.164	1.169	15.57
C5 ^f	14.846	152146	0.936	0.898	0.905	0.909	0.831	0.849	1.248	1.232	0.997	1.015	1.071	1.110	14.01
C6 ^f	15.173	385975	1.097	1.091	1.076	1.112	1.011	1.012	1.123	1.122	0.821	0.833	0.848	0.855	12.44
C7 ^f	15.394	108146	0.991	0.990	1.002	1.004	0.964	0.951	1.127	1.120	0.909	0.926	1.003	1.015	6.63
C8 ^f	16.281	2457611	1.035	1.040	0.731	0.723	1.277	1.282	0.969	0.969	0.993	1.017	0.971	0.994	16.83
C9 ^f	16.691	220362	1.243	1.235	1.312	1.379	1.584	1.574	0.763	0.763	0.444	0.598	0.545	0.561	42.74
C10 ^f	17.015	168950	0.878	0.875	0.605	0.702	1.136	1.113	1.126	1.120	1.146	1.152	0.997	1.149	19.17
C11 ^f	17.713	211302	0.783	0.837	0.782	0.803	1.109	1.100	1.062	1.062	1.111	1.079	1.015	1.256	15.78
C12 ^f	18.017	217763	0.859	0.812	0.819	0.899	1.021	1.013	1.079	1.061	1.105	1.071	0.996	1.265	13.32

d represented the peak recorded at 254 nm; e represented the peak recorded at 280 nm; f represented the peak recorded at 440 nm.

3.3.3 Evaluation of similarity

Similarity evaluation software was utilized to calculate the similarity based on the “vector cosine” method[32] between different samples of ZZDHD and the control fingerprint. The control fingerprint was generated from 12 batches of ZZDHD samples (Table 6). The results showed that the similarity of single- and co-boiled ZZDHD was higher than 0.997 at 254 nm, 280 nm, and 440 nm, indicating that there was no significant change in chemical substances in the two different preparation methods.

Table 6
Similarity analysis of Zhi-Zi-Da-Huang decoction samples.

Sample No.	Similarity			Sample No.	Similarity		
	254 nm	280 nm	440 nm		254 nm	280 nm	440 nm
S1	1.000	1.000	1.000	S7	0.999	0.997	0.999
S2	0.999	1.000	1.000	S8	0.999	0.998	0.999
S3	0.999	1.000	0.999	S9	0.999	1.000	0.999
S4	0.999	1.000	0.999	S10	0.999	1.000	0.999
S5	0.999	1.000	0.999	S11	1.000	1.000	0.998
S6	0.999	1.000	0.999	S12	1.000	1.000	0.998
S1-S6 were samples of the co-boiled Zhi-Zi-Da-Huang decoction; S7-S12 were samples of the single-boiled Zhi-Zi-Da-Huang decoction.							

3.3.4 Chemometric analysis

Further quality assessment of the samples was performed using a PCA on 12 samples with 30 common peaks. The results showed that there was a separation between CB and SB (Fig. 6A). These samples were clearly clustered into two groups: Group 1 including six samples (S1–S6) and Group 2 including six samples (S7–S12), which indicated that the different preparation methods resulted in changes to the ZZDHD. PCA was also employed to map the RPA of 30 common peaks onto the samples in the loading plot (Fig. 6B), which was utilized to find the compounds accountable for the separation of the groups.

Before subjecting different samples to HCA, 30 common peaks were selected. To visualize the classification trends in ZZDHD samples, HCA was conducted based on the RPA of 30 common peaks, which could divide tested samples into different categories. As the dendrogram showed in Fig. 6C, 12 batches of ZZDHD samples were sorted into two clusters. All CB samples (S1-S6) were grouped as group I, and SB samples (S7-S12) were grouped as group II. This corresponded to the PCA plot shown in Fig. 6A, although the similarity values of these samples were much closer. It was concluded that the classification of the sample quality might be mostly relevant to the boiling method of ZZDHD.

Based on the results of the HCA, PLS-DA was further established to obtain important variables with CB and SB samples. The VIP plot displays the importance of variables both to explain X correlated to Y. Based on the VIP values (i.e., larger than 1.0), 13 compounds played key roles in the clustering (Fig. 7). These 13 compounds, including C3, A3, crocetin, daidzin, C5, phycion, G2, G1, chrysophanol, emodin, A2, A5, and C6, corresponded to those points far away from zero in the loading plot of 30 common peaks (Fig. 6B).

4 Discussion

Currently, Traditional Chinese Medicine decoctions and formula granules have been widely studied and applied around the world. The study of chemical compounds is the key factor for the development of traditional Chinese medicine decoctions into formula granules. Unambiguous chemical substances guarantees the quality and therapeutic efficacy of Traditional Chinese Medicine decoctions, so investigations of Traditional Chinese Medicine is essential. However, the chemical composition of Traditional Chinese Medicines is rather complicated, which poses great challenge for the separation and identification of their chemical compositions. UPLC-qTOF-MS/MS has allowed for the perfect combination of ultra-high performance liquid chromatography and high-quality mass spectrometry with high resolution, which have improved performances compared with LC-MS. UPLC uses small particle packing (1.7 μm) as the stationary phase to achieve faster, more sensitive, and higher resolution chromatographic separation of samples, which has advantages over traditional HPLC. QTOF-MS/MS with high quality resolution and high sensitivity can accurately determine molecular weight and predict the molecular composition[33]. UPLC-qTOF-MS/MS can be used for qualitative or quantitative analysis of trace samples with extremely complex components, especially for the study of metabolomics and drug metabolism. Therefore, UPLC-qTOF-MS/MS was used to separate and analyze the chemical components in ZZDHD. A total of 147 chemical compounds were preliminarily determined, and 11 compounds found in CB were distinguishing components of the single- and co-boiled ZZDHD. Our results indicated that chemical compounds changed after the combination of four herbs. Through comparing the mass spectrometry information of the single-boiled herbs, affiliations of 11 compounds were clarified. Gallic acid-4-O-β-D-glucopyranoside, glucosylsyringic acid, Unknown 4, gallate-O-diglucoside, and glycerinmonogallate were derived from DH. Unknown 15 originated from ZZ. In addition, 3', 4', 5, 5', 6, and 7-Hexamethoxyflavone was present in ZS. Importantly, Unknown 59, Unknown 61, Unknown 66, and Unknown 69 were not found in the four herbs or SB. We speculated that the co-boiling of the four herbs together might result in detection of the former six compounds and the generation of the other four compounds. However, there are no further study on the specific mechanism.

Previous studies showed that iridoids, anthraquinones, flavonoids, isoflavonoids, coumarins, and crocetin glycosides were the major bioactive compounds in ZZDHD[28]. Based on this, we determined 15 representative compounds by UPLC-DAD in order to further evaluate the quality of samples and comprehensively compare the differences of chemical substances in ZZDHD. ZZDHD has been extensively used for clearing heat and eliminating dampness and jaundice for many years in China, and it is further thought to have liver-protective, choleric, and anti-inflammatory effects. A previous study has reported that geniposide could prevent hepatocyte injury[34]. Geniposide is hydrolyzed into genipin under the action of beta-D-glucosidases in intestinal bacteria, which is required for its choleric action[35]. Genipin could also influence the induction of the inflammatory mediators inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)- α through the inhibition of nuclear transcription factor- κ B (NF- κ B) activation in hepatocytes; therefore, it might have therapeutic potential for liver injury[36]. Moreover, as one of the major pharmacodynamic substances, G1 generated geniposide and genipin via deglycosylation involving intestinal bacteria, and then distributed in the target organ for hepatinica and cholagogue[37]. As one of the primary constituents of crocin, Crocin-I could reduce oxidative damage in liver tissue in diabetic rats[38]. Crocetin exhibited inhibitory potential against cyclooxygenase-2 (COX-2), iNOS, proinflammatory factors (interleukin [IL]-1 β , IL-6, IL-10, and TNF- α) in *in vitro* assays, and it significantly decreased hepatic parameters, such as serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and alkaline phosphatase (ALP), which suggested protective and therapeutic effects on inflammatory tissue[39]. DH and its anthraquinone constituents, including rhein, emodin, and chrysophanol, could exert a protective effect against liver injury[18, 40–42]. Emerging studies suggested that physcion suppressed hepatocellular carcinoma cell metastasis[43]. Narirutin, naringin, hesperidin, and neohesperidin, the active constituents of ZS, could inhibit pro-inflammatory factors, attenuate liver damage, modulate the state of antioxidants, and have hypoglycemic and hypolipidemic effects[44–47]. Some studies have revealed that the major isoflavonoids of DDC, such as daidzein, have a significant hepatoprotective effect against oxidative damage induced by tert-butylhydroperoxide (TBHP) in rat hepatocyte BRL-3A cell line, which is closely related to the efficacy of ZZDHD[15, 16, 18]. Some pharmacokinetics indicated that isoflavone glycosides of DDC, including daidzein and genistin, could be hydrolyzed in the intestinal wall by bacterial β -glucosidases after oral administration, and further transformed into corresponding bioactive aglycones, and all of them could be excreted into bile[48]. As a glucoside form of isoflavones, daidzin markedly reduced the elevated serum aminotransferase activity and attenuated the apoptosis of hepatocytes[49]. Another study reported that the complex interactions among four herbs may result in a synergistic or antagonistic effect on the solubility of active compounds[50]. Therefore, it was reasonable to select these quantitative compounds, and all of 15 quantitative compounds were representative compounds in four herbs as well.

Then we used fingerprinting to evaluate the similarity of 30 common peaks in 12 batches of samples. The number and retention time of peaks in CB were similar to that of SB, but the peak area of many peaks increased. We therefore conclude that co-boiling was beneficial to the dissolution of effective herbal compounds, providing scientific support for traditional decoction preparation. Determination and chemometric analysis of the fingerprinting were integrated. In 30 common peaks, we found that crocetin, daidzin, physcion, G2, chrysophanol, and emodin with contents changed were included in 15 quantitative compounds. The change in content might be one of the reasons responsible for the discrimination of CB and SB; therefore, these compounds may be effective quality control markers to differentiate the two methods of preparation of ZZDHD. To our knowledge, the quality standard of ZZDHD has not yet been described by the Pharmacopoeia of the People's Republic of China. The quantification, fingerprinting methods, and results of the 15 compounds presented in this study could provide a reference for the establishment of quality control of ZZDHD.

5 Conclusion

In the present research, a rapid and efficient method to integrate UPLC with UPLC-qTOF-MS/MS analysis was successfully established to comprehensively discriminate and assess the quality of ZZDHD samples. Simultaneously, combining chemometric analysis with chromatographic fingerprints served as a powerful tool to apply to the quality control of the co- and single-boiled ZZDHD. We identified a total of 147 chemical compounds, 15 of which were quantified and 12 batches of samples were qualitatively fingerprinted for a systematic analysis of the chemical substances of the two preparation methods of ZZDHD. This study shows the difference of chemical substances between two preparation methods of ZZDHD, which provided an experimental basis for explaining the rationality of the preparation methods. However, research remains limited on the pharmacodynamics, clinical verification, and correlation between changes in chemical composition, pharmacodynamics, and clinical efficacy of ZZDHD prepared via different methods; therefore, further research is required.

Abbreviations

ZZDHD: Zhi-Zi-Da-Huang decoction; ZZ: Gardeniae Fructus; DH: Rhei Radix et Rhizoma; ZS: Fructus Aurantii Immaturus; DDC: Sojae Semen Praeparatum; CB: co-boiled Zhi-Zi-Da-Huang decoction; SB: single-boiled Zhi-Zi-Da-Huang decoction; MS: mass spectrometry; UHPLC: ultra-high-performance liquid chromatography; UPLC-qTOF-MS/MS: ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry; PCA: principal component analysis; PLS-DA: partial least-squares discrimination analysis; HCA: hierarchical cluster analysis; G1: [genipin-1-O- \$\beta\$ -D-gentiobioside](#); G2: geniposide; RPA: relative peak area; VIP: variable importance in projection; RSD: relative standard deviation; RRT: relative retention time; TNF: tumor necrosis factor; iNOS: inducible nitric oxide synthase; TNF: tumor necrosis factor; NF- κ B: nuclear transcription factor- κ B; COX-2: cyclooxygenase-2; SGOT: serum glutamic-oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase; ALP: alkaline phosphatase; TBHP: tert-butylhydroperoxide.

Declarations

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Not applicable.

Authors' contributions

CZ, XZ and YW were responsible for the design of this study. XZ and HW collected and detected samples. MX and HW analyzed data. MX, XZ and CZ wrote and revised the article. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Chemical structures of 15 reference standards in Zhi-Zi-Da-Huang decoction.

Figure 2

Total ion chromatogram of Zhi-Zi-Da-Huang decoction obtained by UPLC-qTOF-MS/MS. (a) the co-boiled Zhi-Zi-Da-Huang decoction in negative ion mode; (b) the single-boiled Zhi-Zi-Da-Huang decoction in negative ion mode; (c) the co-boiled Zhi-Zi-Da-Huang decoction in positive ion mode; (d) the single-boiled Zhi-Zi-Da-Huang decoction in positive ion mode.

Figure 3

Representative UPLC chromatograms under 254 nm, 283 nm and 440 nm. (a) the reference standards; (b) the sample. Peaks 1 to 15 were identified as genipin-1-O- β -D-gentiobioside (G1), geniposide (G2), daidzin, daidzein, genistein, narirutin, naringin, hesperidin, neohesperidin, crocin-I, rhein, crocetin, emodin, chrysophanol and physcion, respectively. (X-axi: running time; Y-axi: peak intensity).

Figure 4

Comparison the content of 15 compounds of the co-boiled Zhi-Zi-Da-Huang decoction (CB) and the single-boiled Zhi-Zi-Da-Huang decoction (SB).

Figure 5

UPLC overlap of the co-boiled Zhi-Zi-Da-Huang decoction (S1-S6), the single-boiled Zhi-Zi-Da-Huang decoction (S7-S12) and the representative fingerprint (R). The peaks marked with A1–A12, B1-B6 and C1-C12 represent the 30 characteristic common peaks.

Figure 6

a, Principal component analysis plot of 12 samples of the Zhi-Zi-Da-Huang decoction. b, Loading plot of 30 common peaks. c, Hierarchical cluster analysis dendrogram of different samples of the co-boiled Zhi-Zi-Da-Huang decoction (S1-S6) and the single-boiled Zhi-Zi-Da-Huang decoction (S7-S12).

Figure 7

Variable importance in projection (VIP) plot of the co-boiled Zhi-Zi-Da-Huang decoction (CB) and the single-boiled Zhi-Zi-Da-Huang decoction (SB) with confidence intervals.

Figure 8

Fig. A1 The proposed fragmentation pathways of geniposide(a) and crocin-I(b) in the negative ion mode. 3.1.2 Identification of compounds in Fructus Aurantii Immaturus

Figure 9

Fig. A2 The MS/MS spectra and proposed fragmentation pathways of narirutin(a), naringin(b) and hesperidin(c) in the negative ion mode.