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Simultaneous qualitative and quantitative analysis of triterpenic acids, saponins and flavonoids in the leaves of two *Ziziphus* species by HPLC–PDA–MS/ELSD

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ABSTRACT

The leaves of *Ziziphus jujuba* and *Z. jujuba* var. *spinosa* have been utilized as crude drugs for their health benefits in China for thousands of years. To control their quality, a reliable method based on high-performance liquid chromatography coupled with photo diode array and electrospray ionization tandem mass spectrometry detection (HPLC–PDA–ESI-MS/MS) was developed for exploration of the chemical profiles of these jujube leaves. As the results, fourteen constituents including three flavonoids, two saponins and nine triterpenic acids were identified or tentatively characterized. Then, twelve of them such as quercetin-3-*O*-rutinoside, zizyphus saponins I and II, ceanothic acid, alphitolic acid, maslinic acid, 2α-hydroxyursolic acid, zizyberanalic acid, epiceanothic acid, ceanothenic acid, betulinic acid, and oleanolic acid were selected as the chemical markers and were determined using an HPLC coupled with evaporative light scattering detection (ELSD) method. The separation was carried out on a Waters Sunfire C_{18} column with 0.2% acetic acid and acetonitrile as the mobile phase under gradient elution. The operating conditions of ELSD were set as 80 °C for drift tube temperature and 2.7 l/min for nitrogen flow rate. The developed method was fully validated in terms of linearity, sensitivity, precision, repeatability as well as recovery, and subsequently applied to evaluate the quality of eight batches of *Z. jujuba* and *Z. jujuba* var. *spinosa* leaves from different collections.

1. Introduction

The *Ziziphus* species (Rhamnaceae family) are considered to be multipurpose plants and have been used as foods, folklore medicines, the environmental protection plants, etc. [1]. The two species, *Ziziphus jujuba* Mill. and *Z. jujuba* var. *spinosa* (Bunge) Hu ex H.F. Chow, are indigenous to China and have been widely distributed in northern China with a history of over 4000 years. Their fruits and seeds are usually applied in traditional Chinese medicine (TCM) for the treatment of various diseases, such as anorexia, lassitude, insomnia, anxiety, etc. [2], and many studies about their chemical constituents [3–7] as well as pharmacology activities [8–10] have been reported. Besides of their fruits and seeds, the leaves of these two *Ziziphus* species are also used as a folk medicine to treat children suffering from typhoid fever, furuncle and ecthyma in China [2]. In addition, they are also utilized as tea for their benefits for human health. Phytochemical studies on the leaves of these two species have resulted in the isolation of several saponins [11,12], terterpenic acids [13] and flavonoids [14], which exhibited multiple activities, such as antimicrobial [15], anti-inflammatory [5,16], antitumor [6,17] and anti-sweet [11,12] effects. Therefore, these compounds could be tentatively assigned as the chemical markers for the quality control of these herb drugs. As for the quality control methods of these two plants, most studies focused on their seeds and fruits, and the analytical methods for the determinations of the triterpenic acids, saponins and flavonoids in their seeds and fruits have been established using high-performance liquid chromatography (HPLC), respectively [18–24]. However, the method for the simultaneous determination of these three classes of compounds in these plants on one HPLC separation procedure has not been found. According to our knowledge, up to now, there were few analytical methodologies available for the quality control of their leaves, which was insufficient for guarantee their quality consistency and restricted the further application of them. Besides, it is known that the therapeutic effect of TCM is due to the synergic activities of their multiple bioactive compounds and the multi-components analytical method has been widely used for quality control of TCM [25–27]. Therefore, it is imperative to establish an analytical method using multiple pharmacologically active compounds as standards for the quality control of these herb drugs.

In light of this, the objective of the present study was to develop a multiple markers analytical method for quality control of *Z. jujuba* and *Z. jujuba* var. *spinosa* leaves. For this purpose, a hyphened

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method of HPLC coupled with photo diode array and electrospray ionization tandem mass spectrometry detection (HPLC–PDA–ESI-MS/MS) method was established firstly to explore the chemical profiles of these two species leaves. Furthermore, several triterpenic acids, saponins and flavonoids were selected as the chemical markers for their quality control. Owing to the low UV absorption for the triterpenic acids and saponins, an HPLC coupled with evaporative light scattering detection (ELSD) method was proposed for the quantitative analysis purpose.

2. Experimental

2.1. Chemicals, reagents and materials

Chemical standards of quercetin-3-*O*-rutinoside, zizyphus saponin II, zizyphus saponin I, ceanothic acid, alphitolic acid, maslinic acid, 2α-hydroxyursolic acid, zizyberanalic acid, epiceanothic acid, ceanothenic acid, betulinic acid, and oleanolic acid were isolated from the fruits of *Z. jujuba* or the fruits of *Z. jujuba* var. *spinosa* in our laboratory, and their structures were determined by NMR and MS. Their structures are presented in Fig. 1. The purity of each reference compound was determined to be over 98% by normalization of the peak area detected by HPLC–ELSD.

Acetonitrile was HPLC-grade from Merck (Darmstadt, Germany) and deionized water was purified by an EPED superpurification system (Eped, Nanjing, China). Other reagent solutions such as methanol and acetic acid were of analytical grade (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

The leaves of *Z. jujuba* and *Z. jujuba* var. *spinosa* were collected from Ningxia and Hebei provinces, China in September 2010. Their botanical origins were identified by the corresponding author, and the voucher specimens were deposited at the Herbarium in Nanjing University of Chinese Medicine, China. After collection, the leaves were dried at 45 °C.

2.2. Apparatus and chromatographic conditions

2.2.1. HPLC–PDA–MSD analysis

The analyses were performed on a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA), equipped with a quaternary pump solvent management system, an auto-sampler, and an on-line degasser. The separation was carried out on a Waters Sunfire C_{18} column (250 mm × 4.6 mm, 5 μm) at a column temperature of 25 °C. The mobile phase was composed of A (acetonitrile) and B (0.2% aqueous acetic acid (v/v)) with a gradient elution at the flow rate of 0.5 ml/min: 0–12 min, 22–30% A; 12–13 min, 30–33% A; 13–31 min, 33–48% A; 31–35 min, 48–63% A; 35–80 min, 63–100% A. Re-equilibration duration was 15 min between individual runs. A Waters 2996 photo diode array (PDA) was connected to the liquid chromatography for detection of the raw data. The MS analysis was performed on a Micromass Q/TOF Mass Spectrometer connected to the HPLC instrument via an electrospray ionization interface (ESI) source. All the data were acquired and analyzed by MassLynx Software Version 4.1. High purity nitrogen was used as the nebulizer and auxiliary gas; argon was utilized as the collision gas. The Q/TOF mass spectrometer was operated in negative ion mode with a capillary voltage of 3 kV, sampling cone voltage of 10 V, cone gas flow of 50 l/h, desolvation gas flow of 700 l/h, desolvation temperature of 350 °C, source temperature of 120 °C, collision energy of 45 V, and the full scan spectra from 100 to 1000 Da.

2.2.2. HPLC–ELSD analysis

The quantification analysis was performed on a Waters 2424 evaporative light scattering detector connected to the liquid chromatography for detection of the raw data which were further acquired and processed with Empower Software. The HPLC conditions were identical to those used for HPLC–PDA–MSD analyses mentioned above. The drift tube temperature for ELSD was set at 80 °C and the nitrogen flow rate was 2.7 l/min.

2.3. Preparation of standard solutions

A mixed standard stock solution containing quercetin-3-*O*-rutinoside (**1**), zizyphus saponin II (**4**), zizyphus saponin I (**5**), ceanothic acid (**6**), alphitolic acid (**7**), maslinic acid (**8**), 2α-hydroxyursolic acid (**9**), zizyberanalic acid (**10**), epiceanothic acid (**11**), ceanothenic acid (**12**), betulinic acid (**13**), and oleanolic acid (**14**), was prepared in methanol. The working standard solutions were prepared by diluting the mixed standard solution with methanol to a series of proper concentrations within the ranges: **1**, 22.55–902.00 μg/ml; **4**, 13.70–548.00 μg/ml; **5**, 5.40–216.00 μg/ml; **6**, 6.05–242.00 μg/ml; **7**, 6.75–270.00 μg/ml; **8**, 3.750–150.00 μg/ml; **9**, 6.00–240.00 μg/ml; **10**, 7.60–304.00 μg/ml; **11**, 6.60–264.00 μg/ml; **12**, 2.90–116.00 μg/ml; **13**, 10.70–428.00 μg/ml; and **14**, 2.75–110.00 μg/ml. The standard stock and working solutions were all stored at 4 °C until use and filtered through a 0.22 μm membrane prior to injection.

2.4. Preparation of sample solutions

The dried powders of *Z. jujuba* and *Z. jujuba* var. *spinosa* leaves (1.0 g, 40 mesh) were weighed accurately into a 50 ml conical flask with stopper, and 20 ml 80% methanol was added. After accurately weighing, ultrasonication (40 kHz) was performed at room temperature for 30 min, and then same solvent was added to compensate for the lost weight during the extraction. After centrifugation (13,000 r/min, 10 min), the supernatant was stored at 4 °C and filtered through a 0.22 μm membrane filter before injection into the HPLC system for analysis.

2.5. Validation of the HPLC method

2.5.1. Calibration curves, limits of detection and quantification

For calibration, the working standard solutions with at least six different concentrations were analyzed, and the calibration curves were calculated by linear regression of the double logarithmic plots of the peak area versus the concentration of the reference solution injected. The limits of detection (LODs) and quantification (LOQs) under the present chromatographic conditions were determined by diluting the standard solution when the signal-to-noise ratios (S/N) of analytes were about 3 and 10, respectively. The S/N was calculated as the peak height divided by the back ground noise value.

2.5.2. Precision, repeatability and accuracy

The intra-day and inter-day variations, which were chosen to determine the precision of the developed method, were investigated by determining the 12 analytes in six replicates during a single day and by duplicating the experiments on three consecutive days. Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (RSD).

Repeatability was confirmed with six independent analytical sample solutions prepared from the same batch of sample (the leaf of *Z. jujuba* collected from Linwu in Ningxia) and variations were expressed by RSD. One of the sample solutions mentioned above was stored at 25 °C, and injected into the apparatus at 0, 2, 4, 8, 12, and 24 h, respectively, to evaluate the stability of the solution.

Recovery test was used to evaluate the accuracy of this method. The test was performed by adding accurate amounts of the 12

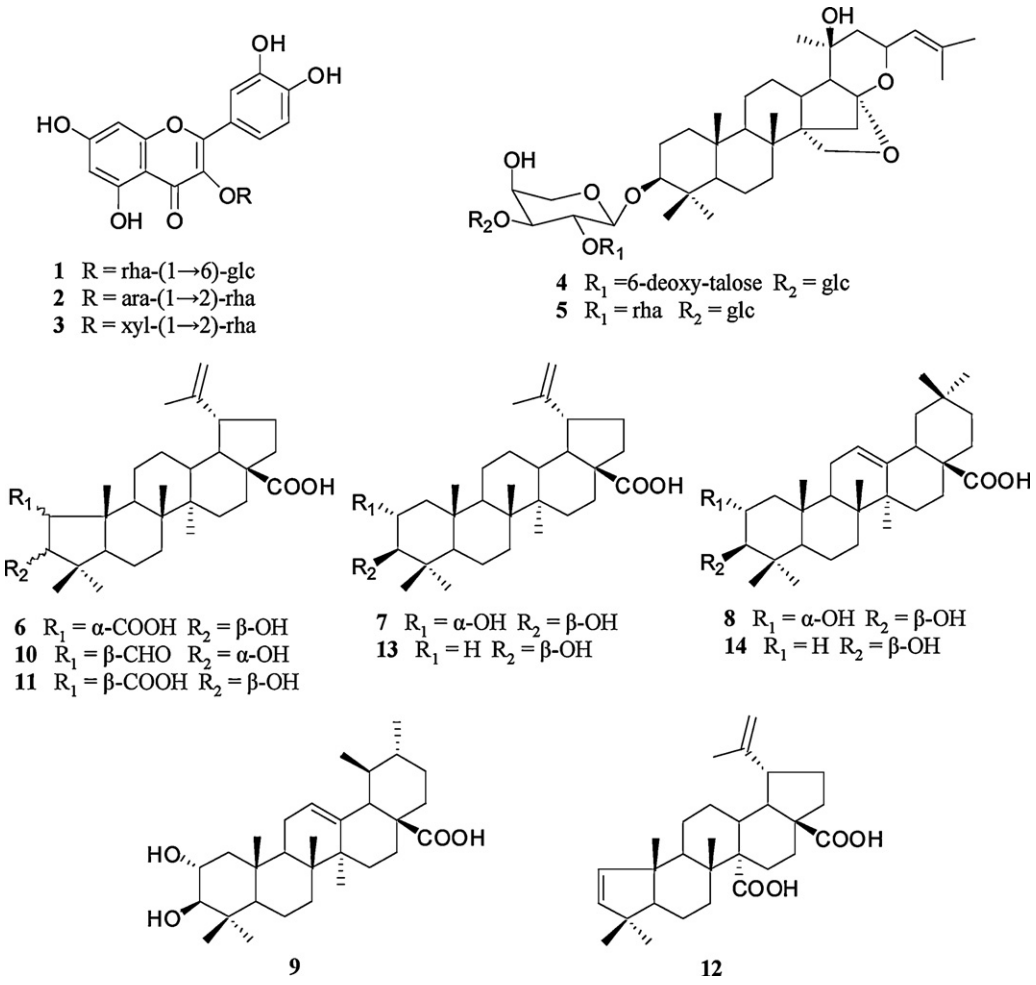

Fig. 1. Chemical structures of the identified compounds in the leaves of *Ziziphus jujuba* and *Z. jujuba* var. *spinosa*.

standards into a certain amounts (0.5 g) of *Z. jujuba* leaves (collected from Linwu in Ningxia) separately. The spiked samples were then extracted, processed, and quantified in accordance with the methods mentioned above. Six replicates were performed for the test. The average recovery percentage was calculated by the formula: recovery (%) = (observed amount − original amount)/spiked amount × 100%.

2.6. Identification and quantification

Identification of the target peaks was performed by comparing their HPLC retention times, UV data and mass/charge ratios with those of the standards or those of the published compounds in *Ziziphus* species. In order to further confirm the structures of the constituents, standards and samples were analyzed by LC/ESI-MS/MS in negative ion modes. Quantification was performed on the basis of linear calibration plots of the logarithm of peak areas versus the logarithm of concentration.

3. Results and discussion

3.1. Optimization of sample preparation procedure

To achieve the optimal extraction conditions, three important factors, namely, extraction solvents, extraction methods and extraction time which might influence the extraction efficiency of the target constituents, were optimized. The different levels of each factor including extraction solvent (50% methanol, 80% methanol and 100% methanol), extraction method (ultrasonic extraction versus heat reflux extraction), extraction time (15, 30 and 60 min) were investigated individually by using univariate approach. The results revealed that for the triterpenic acids, 80% methanol showed similar extraction efficiencies with 100% methanol, which were all higher than those of 50% methanol. For flavonoids and the saponins, the extraction efficiencies were similar for 50% and 80% methanol, which were higher than those of 100% methanol. Thus, 80% methanol was chosen as the suitable extraction solvent. In addition, ultrasonic extraction for 30 min was found to be adequate and appropriate for the analysis.

3.2. Optimization of the HPLC chromatographic conditions

Our previous study showed that Hypersil C_{18} column (250 mm × 4.6 mm, 5 μm) was suitable for the separation of the triterpenic acids. However, it showed a poor separation for the saponins analyzed in this experiment. Therefore, other analytical columns, such as Waters SunFire C_{18} (250 mm × 4.6 mm, 5 μm) and Apollo C_{18} (250 mm × 4.6 mm, 5 μm), were compared to separate the target compounds, and the former exhibited a better resolution for these three types of constituents. As for the mobile phase, different kinds of solvent systems, such as acetonitrile and methanol with a variety of modifiers, were tested. It was found that the mixture of acetonitrile and 0.2% acetic acid solution was a suitable solution, which not only can simultaneously separate the three dif-

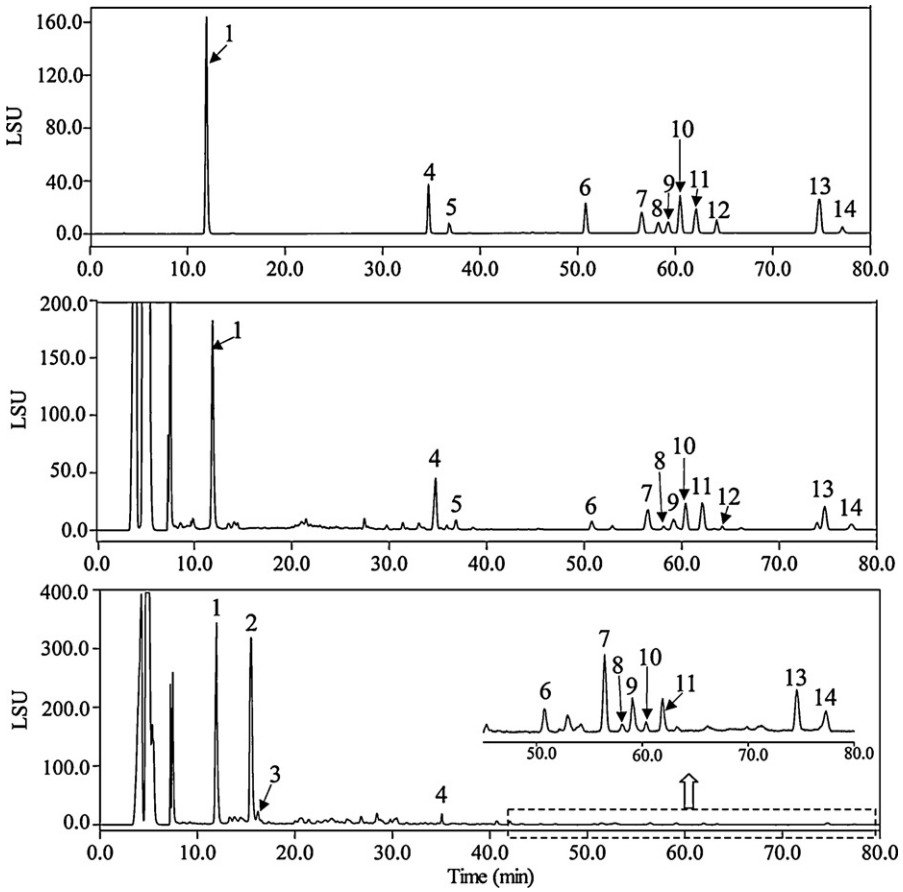

Fig. 2. Typical HPLC–ELSD chromatograms of mixed standards and samples. (A) Mixed standards, (B) the leaves of *Ziziphus jujuba*, (C) the leaves of *Z. jujuba* var. *spinosa*. **1**, Quercetin-3-*O*-rutinoside; **2**, quercetin-3-*O*-α-L-arabinosyl-(1 → 2)-α-L-rhamnoside; **3**, quercetin-3-*O*-β-D-xylosyl-(1 → 2)-α-L-rhamnoside; **4**, zizyphus saponin II; **5**, zizyphus saponin I; **6**, ceanothic acid; **7**, alphitolic acid; **8**, maslinic acid; **9**, 2α-hydroxyursolic acid; **10**, zizyberanalic acid; **11**, epiceanothic acid; **12**, ceanothenic acid; **13**, betulinic acid; **14**, oleanolic acid.

ferent types of compounds in the samples, but also be compatible to ELSD and MS detectors. The operating conditions for ELSD such as the nebulizing gas flow rate and the drift tube temperature were referred to our previous report, and the maximum S/N values for these three types of compounds were obtained by using a drift tube temperature of 80 °C and nitrogen flow of 2.7 l/min. Representative chromatograms for the standard analytes and the samples are shown in Fig. 2.

3.3. Identification of constituents in the samples by HPLC–PDA–ESI-MS

Under the present chromatographic and MS conditions, nearly 20 peaks were detected from these two *Ziziphus* species leaves. Twelve of these major peaks were identified as quercetin-3-*O*-rutinoside (peak 1), zizyphus saponin II (peak 4), zizyphus saponin I (peak 5), ceanothic acid (peak 6), alphitolic acid (peak 7), maslinic acid (peak 8), 2α-hydroxyursolic acid (peak 9), zizyberanalic acid (peak 10), epiceanothic acid (peak 11), ceanothenic acid (peak 12), betulinic acid (peak 13), and oleanolic acid (peak 14) by comparison of their HPLC retention times and ESI-MS/MS spectrometric data with those of reference compounds. Owing to unavailability of authentic standards, peaks 2 and 3 were tentatively assigned as quercetin-3-*O*-α-L-arabinosyl-(1 → 2)-α-L-rhamnoside and quercetin-3-*O*-β-D-xylosyl-(1 → 2)-α-L-rhamnoside, respectively, by comparing the UV and ESI-MS/MS spectrometric data as well as the elution order with those of the published compounds in *Ziziphus* species [23]. The results are summarized in Table 1 and Fig. 1. In the MS spectra, the most prominent mass-to-charge ratios corresponded to deprotonated molecular ions for all 14 compounds. Furthermore, dimer ions $[2M-H]^-$ were found for compounds **6**–**9**, **11**, **13** and **14**, which further confirming their molecular formulas. In addition, the fragments of losing the sugar moieties for compounds **1**–**5** were found in the tandem mass spectrometry (MS/MS). As for the triterpenic acids (compounds **6**–**14**), the fragments in the MS/MS spectrometry were mainly correspondent with the losses of hydroxyl and carboxyl moieties. All the fragment ions mentioned above were consistent with those of reference standards, which further confirmed the identification of the constituents in these two species leaves.

3.4. HPLC method validation

The proposed HPLC–ELSD method for quantitation analysis was validated to determine the linearity, LOD, LOQ, intra-day and inter-day precisions, stability, and accuracy. As shown in Table 2, all calibration curves showed good linearity ($r^2 > 0.9906$) within the test ranges, and the overall LODs and LOQs were in the range of 2.90–6.85 μg/ml and 5.80–13.70 μg/ml, respectively. The RSD values of intra- and inter-day variations, repeatability and stability of the 12 analytes were all less than 5% (Table 3). The overall recoveries lay between 94.74% and 102.54% with RSD less than 3.5%. In addition, peak purity was investigated by studying the PDA and MS data of all peaks analyzed in this study, and the results showed that no indications for impurities could be found. All the results mentioned above indicated that the established method was accurate

Table 1
Chromatographic and spectrometric data of compounds found in the leaves of *Z. jujuba* and *Z. jujuba* var. *spinosa*.

Peak	Compound	t_R (min)	$[M-H]^-$ (m/z)	MS/MS (m/z)	UV (λ_{max}, nm)
1	Quercetin-3-*O*-rutinoside	11.93	609	301	255, 355
2	Quercetin-3-*O*-α-L-arabinosyl-(1 → 2)-α-L-rhamnoside	15.47	579	301	255, 345
3	Quercetin-3-*O*-β-D-xylosyl-(1 → 2)-α-L-rhamnoside	16.21	579	301	255, 345
4	Zizyphus saponin II	34.57	911	749, 603	
5	Zizyphus saponin I	36.70	911	749, 603	
6	Ceanothic acid	50.72	485	423	
7	Alphitolic acid	56.44	471	393	
8	Maslinic acid	58.15	471	393	
9	2α-Hydroxyursolic acid	59.14	471	393	
10	Zizyberanalic acid	60.37	469	423	
11	Epiceanothic acid	61.97	485	423	
12	Ceanothenic acid	64.14	453	409	
13	Betulinic acid	74.65	455		
14	Oleanolic acid	77.01	455		

Table 2
Calibration curves, LOD and LOQ data of investigated compounds by HPLC–ELSD.

Analytes	Calibration curves[a]	r^2	Linear range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
1 Quercetin-3-*O*-rutinoside	$y = 1.6107x + 2.4973$	0.9906	22.55–902.00	5.64	11.28
4 Zizyphus saponin II	$y = 1.6212x + 2.2258$	0.9989	13.70–548.00	6.85	13.70
5 Zizyphus jujuba I	$y = 1.5695x + 2.3802$	0.9974	10.80–216.00	5.40	10.80
6 Ceanothic acid	$y = 1.4185x + 3.0524$	0.9991	6.05–242.00	3.03	6.05
7 Alphitolic acid	$y = 1.3955x + 2.9641$	0.9980	6.75–270.00	3.38	6.75
8 Maslinic acid	$y = 1.6267x + 2.6553$	0.9988	7.50–150.00	3.75	7.50
9 2α-Hydroxyursolic acid	$y = 1.6568x + 2.3315$	0.9996	12.00–240.00	6.00	12.00
10 Zizyberanalic acid	$y = 1.427x + 3.0468$	0.9988	7.60–304.00	3.80	7.60
11 Epiceanothic acid	$y = 1.4247x + 2.9792$	0.9989	6.60–264.00	3.30	6.60
12 Ceanothenic acid	$y = 1.4792x + 3.0025$	0.9943	5.80–116.00	2.90	5.80
13 Betulinic acid	$y = 1.6502x + 2.4368$	0.9995	10.70–428.00	5.35	10.70
14 Oleanolic acid	$y = 1.8411x + 1.6357$	0.9987	11.00–110.00	5.50	11.00

[a] y is the logarithmic value of peak area and x is the logarithmic value of the reference compound's concentration (μg/ml).

enough for the determination of the 12 chemical markers in the samples of jujube leaves.

3.5. Quantitative determination of constituents in jujube leaves by HPLC–ELSD

The proposed HPLC–ELSD method was then subsequently applied to simultaneous determination of the chemical markers including quercetin-3-*O*-rutinoside, zizyphus saponinin II, ziziphus saponin I, ceanothic acid, alphitolic acid, maslinic acid, 2α-hydroxyursolic acid, zizyberanalic acid, epiceanothic acid, ceanothenic acid, betulinic acid, and oleanolic acid in *Z. jujuba* and *Z. jujuba* var. *spinosa* leaves from different regions in China. The results (Table 4) showed there were remarkable differences among the contents of the chemical markers analyzed in different samples. For example, quercetin-3-*O*-rutinoside (**1**) was found to be a predominant constituent in both of *Z. jujuba* and *Z. jujuba* var. *spinosa* leaves, its content varied from 15.78 to 32.72 mg/g. The same variation could also be found in other constituents. Besides, although the chemical compositions in the leaves of *Z. jujuba* were similar to those of *Z. jujuba* var. *spinosa*, the total contents of the nine triterpenic acids analyzed in the leaves of *Z. jujuba* except for the sample 3 were higher than those of *Z. jujuba* var. *spinosa*. These results might be ascribed to multiple factors such as environmental conditions, genetic variation, etc. Thus, the establishment of a quality control method so as to ensure its efficacy and safety is essential.

In addition, it was found that the contents of ziziphus saponin II (**4**) were higher than those of ziziphus saponin I (**5**) in all the samples tested, and the average content of ziziphus saponin II was more than three times of ziziphus saponin I. Among the triterpenic acids

Table 3
Precision, repeatability, stability and recovery of 12 analytes.

Analytes	Precision (RSD, %)		Repeatability (RSD, %, $n = 6$)	Stability (RSD, %, $n = 6$)	Recovery (%, $n = 3$)	
	Intraday ($n = 6$)	Interday ($n = 6$)			Mean	RSD (%)
Quercetin-3-*O*-rutinoside	2.16	2.91	3.05	2.02	98.46	3.34
Zizyphus saponin II	1.61	2.90	2.97	3.48	102.54	2.46
Zizyphus saponin I	0.53	1.81	3.34	2.22	97.35	3.42
Ceanothic acid	1.45	2.72	2.61	2.11	98.63	2.26
Alphitolic acid	2.57	2.51	1.96	2.37	97.96	2.72
Maslinic acid	1.61	1.51	2.54	1.02	98.46	2.61
2α-Hydroxyursolic acid	1.74	3.21	2.12	1.61	97.91	2.30
Zizyberanalic acid	2.62	1.98	1.48	1.40	94.74	2.95
Epiceanothic acid	1.90	2.52	3.09	2.46	98.64	1.52
Ceanothenic acid	1.88	1.15	2.10	3.08	98.31	2.38
Betulinic acid	2.22	2.04	2.63	1.49	96.62	0.78
Oleanolic acid	1.04	3.01	3.22	2.21	97.27	1.56

Table 4
Contents (mg/g) of 12 investigated compounds in *Z. jujuba* and *Z. jujuba* var. *spinosa* leaves.

Sample[a]	Contents of analyst (mean ± SD, $n = 3$)											
	1[b]	4	5	6	7	8	9	10	11	12	13	14
1	19.17 ± 0.62	6.62 ± 0.21	1.39 ± 0.03	2.06 ± 0.04	2.62 ± 0.04	0.49 ± 0.01	2.00 ± 0.06	1.83 ± 0.04	4.79 ± 0.09	0.32 ± 0.01	2.72 ± 0.06	1.05 ± 0.03
2	24.09 ± 0.58	5.12 ± 0.14	1.01 ± 0.02	2.75 ± 0.06	3.41 ± 0.03	nd[c]	1.06 ± 0.03	2.09 ± 0.03	7.63 ± 0.22	0.26 ± 0.01	4.35 ± 0.12	nd
3	30.82 ± 0.66	7.24 ± 0.16	2.20 ± 0.08	0.48 ± 0.01	0.89 ± 0.03	tr[d]	0.69 ± 0.03	tr	1.06 ± 0.02	tr	1.19 ± 0.03	0.59 ± 0.02
4	17.05 ± 0.29	2.59 ± 0.09	1.13 ± 0.02	0.76 ± 0.02	3.05 ± 0.06	0.59 ± 0.01	2.27 ± 0.05	1.13 ± 0.02	2.72 ± 0.07	nd	2.31 ± 0.02	0.84 ± 0.01
5	15.78 ± 0.41	2.90 ± 0.05	1.10 ± 0.02	0.85 ± 0.03	3.28 ± 0.11	0.55 ± 0.02	2.21 ± 0.03	1.44 ± 0.03	2.21 ± 0.04	nd	2.10 ± 0.04	0.71 ± 0.02
6	32.72 ± 0.68	2.00 ± 0.04	tr	5.77 ± 0.12	3.99 ± 0.11	1.66 ± 0.03	4.02 ± 0.07	2.37 ± 0.06	3.33 ± 0.05	nd	2.07 ± 0.06	1.47 ± 0.02
7	30.26 ± 0.54	2.73 ± 0.06	nd	0.71 ± 0.03	1.80 ± 0.04	0.44 ± 0.01	2.18 ± 0.05	tr	1.24 ± 0.03	nd	2.06 ± 0.04	0.99 ± 0.03
8	24.09 ± 0.61	6.98 ± 0.25	3.05 ± 0.06	0.45 ± 0.01	1.19 ± 0.04	tr	1.01 ± 0.03	tr	0.57 ± 0.02	tr	1.13 ± 0.02	0.93 ± 0.02
Mean	24.25	4.52	1.41	1.73	2.53	0.47	1.93	1.11	2.94	0.15	2.24	0.82

[a] Samples 1–3: the leaves of *Z. jujuba* collected from Linwu, Zhongning and Yingchuan in Ningxia, respectively; samples 4 and 5: the leaves of *Z. jujuba* collected from Jinzhou and Luquan in Hebei, respectively; sample 6: the leaves of *Ziziphus jujuba* collected from Jurong in Jiangsu; samples 7 and 8: the leaves of *Z. jujuba* var. *spinosa* collected from Yingchuan in Ningxia and Luquan in Hebei.
[b] The analysts are the same as in Table 2 and Fig. 1.
[c] Not detected.
[d] Under the LOQ.

tested in this study, alphitolic acid (**7**), epiceanothic acid (**11**) and betulinic acid (**13**) showed higher average contents than the others. Furthermore, our previous report showed that the jujube fruits contained many of the triterpenic acids with 3-carbonyl moieties such as betulonic acid, oleanolic acid and ursonic acid [19], while these compounds could not be detected in the jujube leaves. This result may due to the fact that the relevant enzyme activities in different organs are various and the detailed mechanism should be further study.

4. Conclusion

This study provided an HPLC–PDA–MS/ELSD method for simultaneous qualification and quantification of three classes of chemical markers, such as triterpenic acids, saponins and flavonoids, in two *Ziziphus* species leaves. Fourteen constituents including three flavonoids, two saponins and nine triterpenic acids were identified or tentatively characterized, and twelve of them were selected as the chemical markers and were determined. Based on the validation results of good accuracy, repeatability and precision, the proposed method could be used as a prerequisite for quality control of *Z. jujuba* and *Z. jujuba* var. *spinosa* leaves. On the basis of the analytical results that these jujube leaves are rich in quercetin-3-*O*-rutinoside and triterpenic acids, they could be the promising natural sources for future industrial research of quercetin-3-*O*-rutinoside and triterpenic acids with potential benefits for human health.

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