ISSN 1061-9348, Journal of Analytical Chemistry, 2020, Vol. 75, No. 4, pp. 459–467. © Pleiades Publishing, Ltd., 2020.

ARTICLES

Extraction Optimization of Six Alkaloids and Four Lignans in *Zanthoxylum armatum* by Orthogonal Design and Ultra-Fast Liquid Chromatography–Tandem Quadrupole Mass Spectrometry

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Received August 17, 2017; revised October 6, 2019; accepted October 6, 2019

Abstract—Alkaloids and lignans in *Zanthoxylum armatum* display important biological activities, but the quanitification method of alkaloids has not been reported. In this study, an effective extraction method was developed through an orthogonal design, and ten compounds in roots, stems, branches and leaves of *Zanthoxylum armatum* were simultaneously quantified by ultra-fast liquid chromatography–tandem quadrupole mass spectrometry (**UFLC–MS/MS**). The roots, stems, branches and leaves of *Z. armatum* were ultrasonically extracted with methanol (solvent–to–sample ratio 100 : 1, v/w) for 10 min. An UFLC–MS/MS method was developed with a gradient UFLC mobile phase and triple quadruple tandem mass spectrometry with electrospray ionization in the positive ion mode. The method was validated for linearity, precision, repeatability and accuracy. The limits of detection and quantification were within 0.01–7.5 and 0.04–30 ng/mL, respectively. The root samples collected from Tian'e County were abundant in N-methylanhydrotetrahydroberberrubine A, escholidine perchlorate and pinoresinol monomethyl ether, eudesminthe.

Keywords: alkaloids, lignans, quality evaluation, UFLC–MS/MS, *Zanthoxylum armatum*

DOI: 10.1134/S1061934820040164

Zanthoxylum armatum DC (Rutacea), Zhuyejiao in Chinese, is an evergreen shrub or small tree of 3–4 m height grown at the slopes and roadside under 2300 m. It is widely distributed in the eastern, southern and southwest China, such as Shaanxi and Gansu provinces. In Chinese medicine, its roots and fruits are employed to remedy the stomach ache, cold, headache and rheumatoid arthralgia. The leaves are used for alleviating the pain or swelling caused by injuries or infection [1]. In previous studies, researchers confirmed that *Z. armatum* possesses many kinds of pharmacological activities, including antinociceptive and anti–inflammatory [2, 3], hepatoprotective [4, 5], antimicrobial [6], anticancer [7, 8], anthelmintic [9, 10] and antioxidant [11, 12] activities. The phytochemical studies showed that the chemical components of *Z. armatum* were mainly alkaloids, lignans, volatile oils and coumarins [10, 13–17].

The prominent bioactivities of *Z. armatum* are closely related to its alkaloids and furofuran lignans. Su [18] found that both of alkaloids and furofuran lignans have potential anti-diabetes activities through inhibition of α-glycosidase and α-amylase. Some of alkaloids, isodecaline and dictamine, have obvious anti-analgesic effects [18]. Other compounds, like planispine A and eudesmin, have potential antinociceptive and anti-inflammatory activities [2].

In previous research, Li [19] developed an HPLC method to determine the contents of four lignans in *Z. armatum* from Gansu Province. Vinod Bhatt et al. [20] developed an ultra performance liquid chromatography with diode array detection coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UPLC–DAD-ESI–QTOF–MS/MS) method to quantify and identify the flavonoids, lignans, and coumarin in leaves of *Z. armatum*. However, there is no report on the determination of alkaloids, which is closely related with its bioactivity.

In this paper, a rapid and effective UFLC–MS/MS method was validated, which was successfully employed to determine the content of six alkaloids and four lignan compounds in roots, stems, branches and leaves of *Z. armatum* from different harvest zones of China.

EXPERIMENTAL

Chemicals and materials. The dried roots, stems, branches and leaves of *Z. armatum* were collected from different places of production (Tian'e county of Guangxi province, Wuyi Mountain of Fujian province, Fuzhou of Fujian province, Jiangxi province, Hunan province, China) and identified by vice Prof. Jian–ping Tian of Hainan Medical University. Reference standards of isodecaline (**1**), 6-acetonyldihydrochelerythrine (**2**), N-methylanhydrotetrahydroberberrubine A (**3**), escholidine perchlorate (**4**), allocryptopine (**5**), dictamine (**6**), planispine A (**7**), pinoresinol monomethyl ether (**8**), de-4'-*o*-methylyangambin (**9**) and eudesmin (**10**) were isolated and purified by Prof. Guo. The structures of reference standards were ensured by UV, NMR and MS analysis, and their purity detected using HPLC with diode array detector was over 98.0%. Their structures are listed in Fig. 1.

Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany), and deionized water was purified by a Cascada IV super purification system (Pall Corporation, NY, USA). Other reagent solutions were analytical grade (Shanghai Chemical Reagent Company, Shanghai, PR China).

Sample preparation. Eleven samples of *Z. armatum* were ground into fine powder. Each aliquot (0.5 g) was weighed precisely and extracted by ultrasonic with 50 mL of methanol for 10 min. The solution was filtered through 0.22 μm membrane and stored at −20°C before use. A 5 μL of supernatant centrifuged at 13000 rpm for 10 min was injected into the UFLC–MS/MS system for analysis.

Standard solutions. Ten isolated compounds isodecaline (**1**), 6-acetonyldihydrochelerythrine (**2**), N-methylanhydrotetrahydroberberrubine A (**3**), escholidine perchlorate (**4**), allocryptopine (**5**), dictamine (**6**), planispine A (**7**), pinoresinol monomethyl ether (**8**), de-4'-*o*-methylyangambin (**9**) and eudesmin (**10**) were precisely dissolved in methanol as a mixed standard stock solution. The working standard solutions were diluted from the mixed standard stock solution with methanol. Berberine hydrochloride was selected as the internal standard. All standard solutions were filtered through 0.22 μm membrane and stored at −20°C till use. The supernatant of the standard solution (5 μL), after being centrifuged at 13000 rpm for 10 min, was injected for analysis.

Ultra-fast liquid chromatography conditions. The determination was performed on a Shimadzu LC-20 AD UFLC system (Shimadzu, Tokyo, Japan) with a binary pump solvent management system, a SIL-20A HT autosampler, a CTO-20A column oven, and a model DGU-20A_{3R} online degasser. A Phenomenex Kinetex 2.6u X-C18 100A column (2.1 × 50 mm) was employed with the column temperature of 40°C. The mobile phase consisted of water containing 0.01% formic acid (A) and acetonitrile containing 0.01% formic acid (B) with a gradient programmed as follows: 0.00–0.50 min, 2% B; 0.51–5.00 min, 40–75% B; 5.01–6.00 min, 2% B. A 5 μL of supernatant was injected with a flow rate of 0.45 mL/min.

Mass spectrometry conditions. An AB–SCIEX API 4000^+ mass spectrometer (Toronto, Canada) was interfaced via a Turbo V ion source with a Shimadzu Prominence UFLC chromatographic system (Kyoto, Japan). The AB–SCIEX Analyst software packages were used to control the UFLC–MS/MS system, data acquisition and processing. The mass spectrometer was operated in the positive ion electrospray ionization (**ESI**) mode with optimized multiple reaction monitoring (**MRM**) mode for all the analytes. The pneumatically nebulized ESI was achieved by using inner coaxial nebulizer N_2 gas (GS1) at 35 psi through a Turbo V ion spray probe, a high voltage of +5.0 kV applied to the sprayer tip, and heated dry N_2 gas (GS2) of 45 psi at 500°C from two turbo heaters adjacent to the probe. A curtain N_2 gas (CUR) of 45 psi was applied between the curtain plate and the orifice to avoid solvent droplets from entering and contaminating the ion optics. The insource collision gas (CAD) flow was set at level 8, and the parameters for MRM were optimized for each analyte and the selected values are shown in Table 1.

Method validation. To establish the calibration curves, a series of concentrations of mixed standard solution were prepared from the stock solution. The graph was plotted by the peak areas against the corresponding concentrations. UFLC–MS/MS analysis was using internal standard methods with available reference standards. The limits of detection and quantification (**LOD** and **LOQ**) were determined at the signal to noise ratio (S/N) about 3 and 10, respectively. The S/N of analytes was calculated as the peak height divided by the background noise value. To determine the precision of the developed method, the intra- and inter-day variations were tested by determining all analytes in six replicates during a single day and by repeating the experiments on three consecutive days. Variations of the concentrations were taken as the measure of precision and expressed as percentage relative standard deviations (**RSD**). Six independent analytical sample solutions, extracted of the roots from Tian'e County, were investigated to evaluate the repeatability of solution. Stability was confirmed by analyzing one sample solution stored at the room temperature (25°C), at 0, 2, 4, 8, 12 and 24 h, respectively. Recovery test was used to check the accuracy of the method. Adding

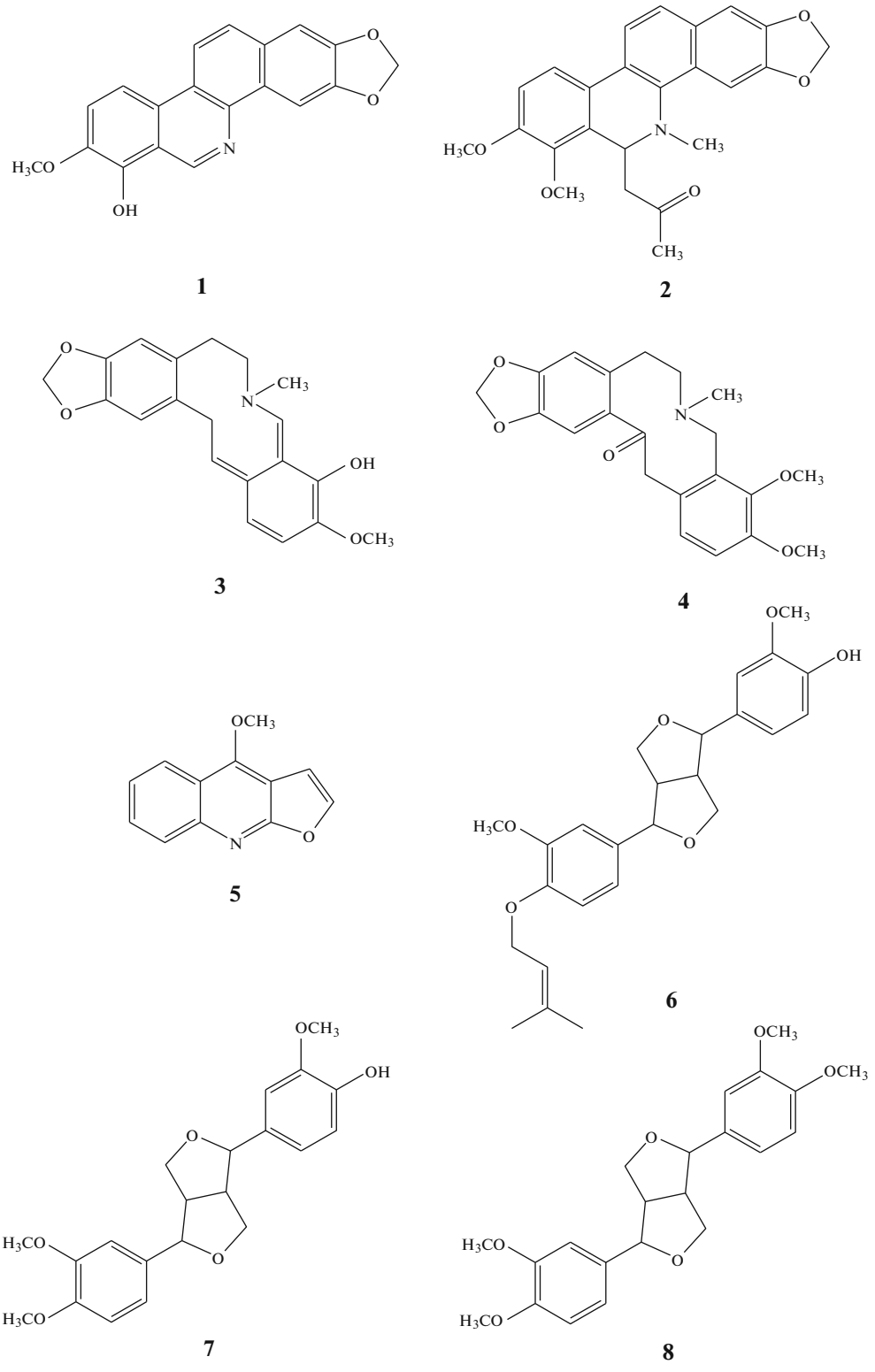

Fig. 1. Structures of 10 compounds in *Z. armatum*. **1**—isodecaline, **2**—6-acetonyldihydrochelerythrine, **3**—N-methylanhydrotetrahydroberberrubine A, **4**—escholidine perchlorate, **5**—allocryptopine, **6**—dictamine, **7**—planispine A, **8**—pinoresinol monomethyl ether, **9**—de-4'-*o*-methylyangambin, **10**—eudesmin.

Table 1. Retention times (t_R) and parameters for multiple reaction monitoring mode of analytes and internal standard (IS)

Compound	t_R, min	$[M + H]^+$, m/z	Quantitation ion, m/z	DP, V	EP, V	CE, V	CXP, V
Isodecaline	3.07	320.1	277.0	120	10	49	16
6-Acetonyldihydrochelerythrine	4.28	406.0	348.1	60	8	22	22
N-Methylanhydrotetrahydroberberrubine A	3.00	339.9	135.1	85	9.5	35.7	7
Escholidine perchlorate	2.86	341.1	135.1	88	10	36	7
Allocryptopine	2.69	370.1	195.9	80	5	22	12
Dictamine	2.59	200.1	129.1	87	8	44	6
Planispine A	3.53	427.3	235.1	70	8	11	15
Pinoresinol monomethyl ether	3.66	373.2	189.0	69	10	23	10
De-4'-*o*-methylyangambin	2.47	433.2	218.9	70	10	26	15
Eudesmin	2.74	387.4	369.0	63	4	8	10
IS	2.39	337.1	321.1	89	10	41	7

known amounts of the 10 standards at low (80% of the known amounts), medium (the same as the known amounts) and high (120% of the known amounts) levels, the spiked samples were then extracted and analyzed in triplicate. The average recovery percentage was calculated by the formula:

$$\text{Recovery}\,(\%) = (\text{Observed amounts} - \text{Original amounts})/\text{Spiked amounts} \times 100\%.$$

Optimization of extraction. To optimize the conditions of ultrasonic extraction, the root from Tian'e (0.5 g) was extracted. The conditions of solvent (A: methanol, ethanol, 50% methanol, v/v), extraction time (B: 10, 20, 30 min) and solvent-to-sample ratio (C: 25 : 1, 50 : 1, 100 : 1, v/w) were optimized. All factors were tested through the orthogonal design ($L_9 3^3$) and each of extraction was investigated in triplicate. Then, the rest samples of roots, stems, branches and leaves from different harvest zones were extracted under the optimal extraction conditions. Each sample (0.5 g) was weighed precisely and extracted by ultrasonic with 50 mL of methanol for 10 min. The extraction solution was filtered through 0.22 μm membrane and stored at −20°C before use. A 5 μL of supernatant centrifuged at 13000 rpm 10 min was injected into the UFLC–MS/MS system for quantification.

RESULTS AND DISCUSSION

Optimization of the chromatographic and mass spectrometric conditions. To develop an effective analysis method, the separation conditions, mobile phase, column temperature, flow rate, and gradient program were optimized respectively. A shiseido capcell core C_{18} column (2.1 × 50 mm, 2.7 μm) was used for 10 compounds analytics, but the shape of peaks tended to front extended. Waters XBridge HILIC column (2.1 × 100 mm, 3.5 μm) and XBridge C_{18} column (2.1 × 100 mm, 3.5 μm) were also tested, and the retention times of 10 compounds on former one were all in 2 min and become longer on the another one. So we finally selected the Phenomenex Kinetex 2.6u XB–C18 100A column (2.1 × 50 mm), with the optimal performance of retention time and peak shape. The chromatograms of different combinations of the mobile phase, such as methanol (containing 0.01% formic acid)–water (containing 0.01% formic acid), acetonitrile (containing 0.01% formic acid and 1 mM ammonium acetate) – water (containing 0.01% formic acid and 1 mM ammonium acetate) and acetonitrile–water (containing 0.01% formic acid and 1 mM ammonium acetate), were obtained. The first combination had a low performance in peak shape, and the others were not good at peak height. Finally, the mobile phase consisting of water (containing 0.01% formic acid) and acetonitrile (containing 0.01% formic acid) was selected. To maintain fine column pressure, the column temperature was set at 40°C, rather than 30 or 35°C, and the flow rate was optimized at 0.45 mL/min, rather than 0.35 or 0.4 mL/min. The UFLC–MS/MS chromatograms of 10 analytes are shown in Fig. 2.

The conditions of mass spectrometry were set both in positive and negative ion modes. However, some of compounds cannot be determined in negative ion mode, so positive ion mode was selected for detection. Retention times and MS parameters for MRM of compounds, including $[M + H]^+$, quantitation ions, scan time, declustering potential (**DP**), entrance

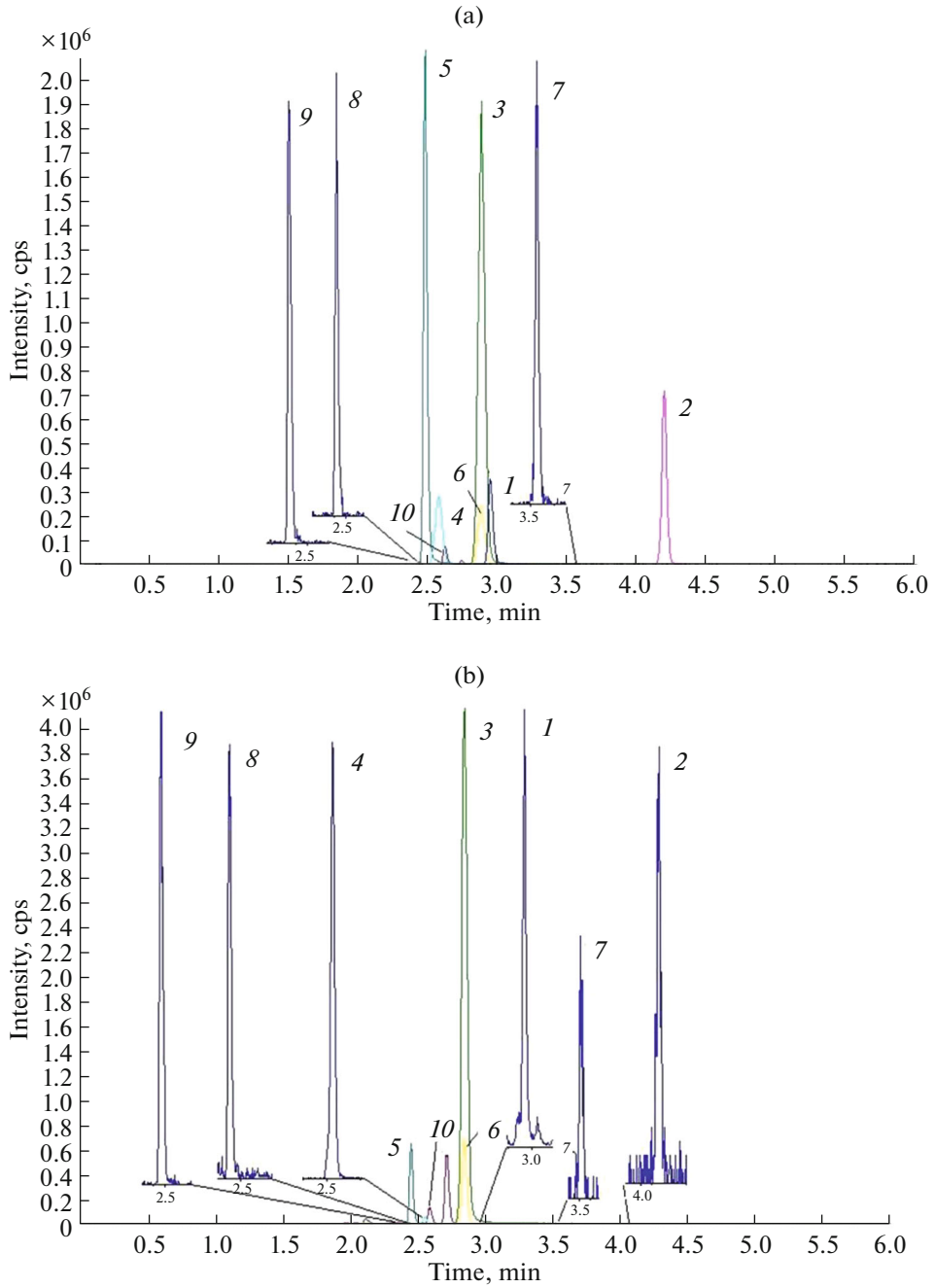

Fig. 2. LC–MS/MS chromatograms of standard (a) and the roots of *Z. armatum* from Tian'e (b). Names of compounds corresponding to numbers *1–10* see above in the experimental part.

potential (**EP**), collision energy (**CE**) and collision cell exit potential (**CXP**), are listed in Table 1.

Optimization of the extraction conditions. To select the optimal extraction conditions, the yields of two types of extraction were weighed and quantitatively analyzed using evaluation indices *k* and *R* through the orthogonal ($L_9 3^3$) test. The parameters are shown in Tables 2 and 3. The value of *R* (range) showed that factor A (solvent) was the most significant for the yields of two types of compounds, and factor B (extraction time) was the least important among three factors. The rank of importance for the overall two types of ingredients was as follows: solvent > solvent-to-sample ratio > extraction time. According to the yields of two types of components, methanol gave a higher yield than other solvents, and extraction time of 10 min also provided a great performance. The solvent-to-sample ratio shows different effect of the yield for alkaloids and lignans. To extract compounds completely, sol-

Table 2. Orthogonal ($L_9 3^3$) extraction results

No.	A (solvent)	B (extraction time)	C (solvent-to-sample ratio)	Yield, mg/g	
				alkaloids	lignans
1	A2 (EtOH)	B1 (10 min)	C2 (50 : 1)	0.744	0.944
2	A3 (50% MeOH)	B1 (10 min)	C3 (100 : 1)	1.123	1.585
3	A1 (MeOH)	B3 (30 min)	C2 (50 : 1)	1.194	1.644
4	A2 (EtOH)	B3 (30 min)	C3 (100 : 1)	0.854	1.037
5	A1 (MeOH)	B1 (10 min)	C1 (25 : 1)	1.055	1.529
6	A2 (EtOH)	B2 (20 min)	C1 (25 : 1)	0.849	1.066
7	A1 (MeOH)	B2 (20 min)	C3 (100 : 1)	0.981	1.274
8	A3 (50% MeOH)	B3 (30 min)	C1 (25 : 1)	0.856	1.165
9	A3 (50% MeOH)	B2 (20 min)	C2 (50 : 1)	0.955	1.457

Table 3. Analysis of orthogonal results

Index	Yields of alkaloid, mg/g			Yields of lignanoid, mg/g		
*k*1	1.077	0.974	0.92	1.482	1.353	1.253
*k*2	0.816	0.928	0.964	1.016	1.266	1.348
*k*3	0.978	0.968	0.986	1.402	1.282	1.299
*R*b	0.261	0.046	0.066	0.466	0.087	0.095

Table 4. Regression data of compounds

Compound	Range, ng/mL	Linear regression equation	Correlation coefficient	LOD, ng/mL	LOQ, ng/mL
Isodecaline	1.6–400	$y = 0.00438x + 0.00315$	0.9949	0.5	1.6
6-Acetonyldihydrochelerythrine	0.04–20	$y = 0.083x + 0.00241$	0.9985	0.01	0.04
N-Methylanhydrotetrahydroberberrubine A	4–400	$y = 0.0235x + 0.0747$	0.9980	1	4
Escholidine perchlorate	1.6–400	$y = 0.00379x + 0.00442$	0.9989	0.5	1.6
Allocryptopine	4–800	$y = 0.00443x + 0.013$	0.9985	1	4
Dictamine	4–400	$y = 0.018x + 0.0659$	0.9976	1	4
Planispine A	15–1500	$y = 7.13 \times 10^{-5}x + 0.000225$	0.9953	5.00	15.0
Pinoresinol monomethyl ether	30–3000	$y = 3.54 \times 10^{-5}x + 0.000302$	0.9921	7.5	30
De-4'-*o*-methylyangambin	4–800	$y = 0.000296x + 0.000493$	0.9988	1	4
Eudesmin	8–800	$y = 0.00171x + 0.00311$	0.9958	2.5	8

Table 5. The results of precision and recovery tests

Compound	Precision (RSD, %)		Repeatability (RSD, %; $n = 6$)	Stability (RSD, %; $n = 6$)	Recovery, % ($n = 3$)	
	intra-day ($n = 6$)	inter-day ($n = 18$)			mean	RSD
Isodecaline	1.5	2.1	3.8	2.8	102.06	2.36
6-A-cetonyldihydrochelerythrine	3.6	4.8	5.1	2.6	99.20	1.06
N-Methylanhydrotetrahydroberberrubine A	3.0	2.3	4.8	1.7	102.55	1.27
Escholidine perchlorate	2.1	2.2	2.7	4.5	100.34	2.64
Allocryptopine	2.8	3.8	4.5	4.8	101.06	3.13
Dictamine	3.2	4.8	3.9	2.2	101.59	3.75
Planispine A	3.7	4.6	3.8	3.8	100.36	2.63
Pinoresinol monomethyl ether	2.4	4.7	4.4	3.2	101.53	2.72
De-4'-*o*-methylyangambin	3.1	4.0	3.0	1.8	100.22	2.26
Eudesmin	2.7	3.7	2.8	4.5	106.83	4.36

vent-to-sample ratio of 100 : 1 was selected. Finally, 100 : 1 of methanol for 10 min was chosen as the optimal extraction condition of compounds from *Z. armatum*.

Analytical method validation. The developed UFLC–MS/MS quantification method was validated by linearity, LOD, LOQ, intra- and inter-day precision, stability, repeatability and accuracy. As shown in Table 4, all compounds showed a good linearity ($r >$ 0.9921). LODs and LOQs for all compounds were between 0.01–7.5 and 0.04–30 ng/mL, respectively. The RSD values of 10 compounds in intra-day ($n = 6$) and inter-day ($n = 18$) variations, repeatability and stability were less than 5% (Table 5). The mean of recoveries were in range of 99.20–106.83% with RSDs of 1.06–4.36%. All the results mentioned above indicate that the established method is accurate and all the values are within acceptable range.

Quantitative analysis of samples. The contents of 10 compounds in eleven samples of *Z. armatum* were determined. The results are shown in Table 6. Alkaloids were the main bioactive component in the root samples, such as compounds 3 and 4. Compounds 1 and 2 were present in high concentration in stem samples. All the leaves samples had high content of lignans, especially compounds 8, 9 and 10. In branches, two types of compounds were both at low concentration. Compared of different harvest zone, samples from Tian'e County obviously contained higher contents of both alkaloids and lignans. The root from Jiangxi province had highest content of compound 1 and 7. Compound 10 was the major component in sample from Wuyi Mountain. In recent study, alkaloids were confirmed as the active ingredient for analgesia [19], lignans were responsible for anti-inflammatory activity [2, 6]. This result may explain the traditional usage of *Z. armatum* root rather than stem and leaf for alleviating pain and inflammatory disorders.

The results revealed that the concentrations of alkaloids and lignans varied with different parts of plant and different production region of *Z. armatum*. It maybe related to their therapeutic effects. Therefore, this UFLC–MS/MS method was necessary for quantification of multi-components in *Z. armatum* for quality control.

CONCLUSIONS

In this study, a rapid and effective ultrasonic extraction method was developed. Ten compounds in *Z. armatum* were simultaneously quantified by a validated UFLC–MS/MS method within 10 min. The quantification results indicated the samples collected from Tian'e County were abundant in alkaloids and lignans. Lignans in leaves of *Z. armatum* from Tian'e were the highest in all samples, particularly of compounds 8 and 10 that may be the constituents responsible of anti-inflammatory activity. Alkaloids, compounds 3 and 4, could be the active ingredients of analgesia. This study could offer a simple analytical method for the quality control and chemical information of *Z. armatum*.

Table 6. Concentrations of 10 analytes in eleven samples

Harvest zone	Sample	Part of plant	Compound, μg/g										Alkaloids, mg/mL	Lignans, mg/mL	Total, mg/mL
			1	2	3	4	5	6	7	8	9	10			
Tian'e	ZA–1	Root	1.50	0.0675	570.27	514.77	29.24	70.92	33.39	954.57	47.15	438.01	1.186	1.473	2.659
	ZA–2	Stem	2.03	0.0815	128.96	115.87	2.05	141.06	44.32	1053.39	36.42	952.99	0.390	2.087	2.477
	ZA–3	Leave	0.52	0.0057	7.42	8.94	55.12	0.71	138.01	4131.96	12.05	3396.47	0.072	7.678	7.751
Jiangxi	ZA–4	Root	6.82	0.0329	86.08	76.23	–	42.46	204.77	47.68	34.35	143.28	0.211	0.430	0.641
	ZA–5	Stem	2.45	–	33.94	33.37	15.98	12.11	116.78	18.05	11.56	276.68	0.097	0.423	0.520
	ZA–6	Branch	0.63	–	11.18	9.69	6.93	3.31	56.58	14.22	7.81	164.37	0.031	0.243	0.274
	ZA–7	Leave	0.80	0.0149	13.50	12.44	0.77	–	117.94	43.96	3.41	97.81	0.027	0.263	0.290
Hunan	ZA–8	Root	2.37	0.0136	116.87	106.12	8.63	17.58	45.15	10.56	18.28	105.96	0.251	0.180	0.431
	ZA–9	Leave	0.71	0.0084	16.32	14.57	1.00	–	59.17	39.06	3.19	26.40	0.032	0.127	0.160
Wuyi Mountain	ZA–10	Stem	–	–	15.60	14.06	24.96	–	24.50	53.82	20.42	357.69	0.054	0.456	0.5110
Fuzhou	ZA–11	Branch	1.23	–	22.93	21.10	22.51	1.38	131.09	53.48	5.76	65.92	0.069	0.256	0.325

FUNDING

This study was financially supported by the National Natural Science Foundation of China (81360476), Gansu Province Natural Science Foundation (1112RJ2A016), and the instrument supported of Hainan Provincial Key Lab of R&D on Tropic Herbs, Hainan Medical University.

CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

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