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Original Research Article

Quality evaluation of cortex berberidis from different geographical origins by simultaneous high performance liquid chromatography combined with statistical methods

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Abstract

Purpose: *To develop an effective method for evaluating the quality of Cortex berberidis from different geographical origins.*
Methods: *A simple, precise and accurate high performance liquid chromatography (HPLC) method was first developed for simultaneous quantification of four active alkaloids (magnoflorine, jatrorrhizine, palmatine, and berberine) in Cortex berberidis obtained from Qinghai, Tibet and Sichuan Provinces of China. Method validation was performed in terms of precision, repeatability, stability, accuracy, and linearity. Besides, partial least squares discriminant analysis (PLS-DA) and one-way analysis of variance (ANOVA) were applied to study the quality variations of Cortex berberidis from various geographical origins.*
Results: *The proposed HPLC method showed good linearity, precision, repeatability, and accuracy. The four alkaloids were detected in all samples of Cortex berberidis. Among them, magnoflorine (36.46 - 87.30 mg/g) consistently showed the highest amounts in all the samples, followed by berberine (16.00 - 37.50 mg/g). The content varied in the range of 0.66 - 4.57 mg/g for palmatine and 1.53 - 16.26 mg/g for jatrorrhizine, respectively. The total content of the four alkaloids ranged from 67.62 to 114.79 mg/g. Moreover, the results obtained by the PLS-DA and ANOVA showed that magnoflorine level and the total content of these four alkaloids in Qinghai and Tibet samples were significantly higher ($p < 0.01$) than those in Sichuan samples.*
Conclusion: *Quantification of multi-ingredients by HPLC combined with statistical methods provide an effective approach for achieving origin discrimination and quality evaluation of Cortex berberidis. The quality of Cortex berberidis closely correlates to the geographical origin of the samples, with Cortex berberidis samples from Qinghai and Tibet exhibiting superior qualities to those from Sichuan.*

Keywords: *Tibetan medicine, Cortex berberidis, Origin discrimination, Quality evaluation, Magnoflorine, Palmatine, Berberine, Jatrorrhizine*

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INTRODUCTION

Cortex berberidis is an important and frequently used crude drug in traditional Tibetan medicinal system. It has been recorded in the "Drug Standards of Tibetan Medicines" since 1995 [1]. Several classic Tibetan medicine books [2-4] including "Jing Zhu Materia Medica" describe its traditional uses and botanical species. *Cortex berberidis* has been used for the treatment of diarrhea, urinary frequency, dysuria, fever, red eyes, trachoma, and nephritis for a long time [1-4], and its relevant medicinal preparations such as Bawei Xiaobopi powder and Xiaobo

ophthalmic ointment are frequently used to treat urinary tract and eye infections, respectively. Modern investigations have shown that *Cortex berberidis* possesses significant biological properties, such as antibacterial, anti-inflammatory and anti-diabetic activities [5-7].

In China, *Cortex berberidis* is widely distributed in various locations of Tibetan people inhabited areas, such as Qingha, Tibet and Sichuan provinces. As is well known, the quality of herbal medicines is somewhat jagged according to different geographical origins because of the disparity of growing environments such assoil and climate. Therefore, it is very necessary and important to evaluate the quality of *Cortex berberidis* from various geographical origins for its safe use, quality control and clinical popularization.

The major active compounds isolated from Berberis medicinal plants are alkaloids such as berberine, magnoflorine and palmatine. At present, several analytical approaches including high performance liquid chromatography (HPLC) [8], dual wavelength thin-layer scanning chromatography [9], and non-aqueous capillary electrophoresis (NACE) [10] have been used to quantitatively analyze one or several alkaloids in *Cortex berberidis*. However, to our knowledge, there is no report about combination of quantification of multi-ingredients and statistical analysis for discrimination of *Cortex berberidis* from different geographical origins. In this paper, a simple, rapid and accurate HPLC method was firstly developed for simultaneous quantitative determination of four bioactive alkaloids (magnoflorine, jateorrhizine, palmatine, and berberine) in *Cortex berberidis*. Moreover, we have systematically analyzed the quality variations of *Cortex berberidis* from three geographical origins (Qingha, Tibet and Sichuan) with the help of the statistical methods. The present study will provide important information for the geographical characterization, rational use and quality control of *Cortex berberidis*.

EXPERIMENTAL

Reagents and materials

Acetonitrile purchased from Fisher Chemicals (Pittsburgh, PA, USA) was of chromatographic grade. Analytic grade hydrochloric acid and methanol were purchased from Chengdu Kelong Reagent Co., Ltd (Sichuan, China). Deionized water was purified by a Hi-tech water purification system (Henan, China). Standard compounds of berberine, jatrorrhizine and palmatine were purchased from National Institutes for Food and Drug Control (Beijing, China) while magnoflorine was purchased from Chengdu Must Biotechnology Co., Ltd (Sichuan, China). The purity of each standard compound was over 98 %.

A total of 17 batches of *Cortex berberidis* were collected from various geographical locations in China. The materials were identified by Professor Gang Fan, and their voucher specimens deposited in the herbarium of College of Ethnic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, China. Detailed information of the samples are shown in Table 4(a) and (b).

Instrumentation and chromatographic condition

Chromatographic analysis was performed on an Agilent 1200 Series HPLC system equipped with a diode array detector (DAD), an auto-sampler and a column temperature controller. All the separations were carried out at a column temperature of 30 °C on an InertSustain C18 column (4.6 mm × 250 mm, 5 µm). The mobile phase was consisted of 0.2 % phosphoric acid (A) and acetonitrile (B) in a gradient elution mode as follows: 17 - 19 % B at 0 - 10 min, 19 - 28 % B at 10 - 11 min, and 28 - 34 % B at 11 - 18 min. The flow rate was 1.0 mL/min and the injection volume was 10 µL. The detection wavelength was monitored at 270 nm.

Sample preparation

The four reference compounds were accurately weighed and dissolved in methanol in a 50 mL volumetric flask to make a mixed standard stock solution (1.560, 0.138, 0.072, and 0.432 mg/mL for magnoflorine, jatrorrhizine, palmatine, and berberine, respectively). Working standard solutions were freshly prepared from the stock solution by further dilution with the appropriate volume of methanol.

All the tested samples were crushed into powder with a mean particle size of 700 µm (24-mesh); 0.5 g powder of each dried sample was accurately weighed into a Erlenmeyer flask and extracted with 50 mL hydrochloric acid/70 % aqueous methanol (1:100, v/v) by ultrasonication for 30 min. The extract was adjusted to the original weight with the extraction solvent and filtered through a 0.45 µm filter membrane. An aliquot of 10 µL of the successive filtrate was injected into the HPLC system for analysis.

HPLC analysis

Samples were analyzed using Agilent 1200 Series HPLC system. Diode array detector was used for the detection, and the wavelength was 270 nm. All chromatographic parameters were optimized for better separation, including mobile phase composition, gradient elution mode, flow rate, and column temperature. All chromatographic data were recorded and analyzed with Agilent Chromatographic Work Station software. The concentrations of magnoflorine, jatrorrhizine, palmatine, and berberine in each sample were calculated based on the established calibration curves.

Validation of HPLC method

The developed HPLC method was validated in terms of linearity, precision, repeatability, stability, and accuracy. The stock solution containing the four alkaloids was prepared and diluted to appropriate concentrations for establishment of calibration curves. The calibration graphs were plotted after linear regression of the peak areas versus the corresponding concentrations. In addition, limit of detection (LOD) and of quantification (LOQ) were determined at signal-to-noise (S/N) ratios of 3 and 10, respectively. The precision of the HPLC method was assessed by six replicate measurements of the same sample solution in one day. For the stability test, the same sample solution was stored at room temperature, and analyzed at 0, 1, 2, 4, 8, 12, and 24 h, respectively. The method repeatability was evaluated by analyzing six different sample solutions independently prepared from the same sample. Recovery tests were carried out to evaluate the accuracy by standard addition method. Low, medium and large amounts of the four standards were added to a previously analyzed sample for which the concentrations of the compounds were known. The mixtures were then extracted and analyzed according to the above-established procedures.

Data analysis

Partial least squares discriminant analysis (PLS-DA) was done to sort samples into groups and obtain an overview of variation among groups using SIMCA-P software (version 11.5, Umetrics, Umeå, Sweden). Moreover, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were performed with GraphPad Prism software (version 5.0, GraphPad Software Inc, San Diego, CA, USA) to reveal paired differences between groups. Probability values (p) < 0.05 were considered to be significant in all experiments.

RESULTS

Optimized chromatographic conditions

In order to obtain the best separation for all the studied analytes, different types of columns including InertSustain C18 column (4.6 mm × 250 mm, 5 μm), Inerstil ODS-3 column (4.6 mm × 250 mm, 5 μm) and WondaSil C18-WR column (4.6 mm × 250 mm, 5 μm) were tested in different elution modes by using acetonitrile - water with different concentrations of phosphoric acid as the mobile phase. Comparing the separation and elution time of characteristic peaks, the InertSustain C18 column gave the best results. 0.2 % phosphoric acid (A) and acetonitrile (B) system were selected as an appropriate mobile phase, and the gradient elution mode was decided as follows: 17 - 19 % B at 0 - 10 min, 19 - 28 % B at 10 - 11 min, and 28 - 34 % B at 11 - 18 min. The detection wavelength was set at 270 nm, where all the targeted compounds could be detected and had adequate absorption. Moreover, the flow rates (0.8, 1.0 and 1.2 mL/min) and column temperatures (25, 30 and 35 °C) were also examined and optimized. Finally, 1.0 mL/min and 30 °C were selected as the best conditions. Under these selected chromatographic conditions, good baseline separation and peak resolution for all the studied compounds were achieved (Figure 1).

Optimized extraction conditions

In order to obtain satisfactory extraction efficiency, several experimental factors including extraction method, extraction solvent, number of times of extraction, extraction time, and solvent volume were tested and optimized. First, three extraction methods (ultrasonic, refluxing, and standing extraction) were compared. It was found that ultrasonic extraction was simpler and more effective for extraction of the four alkaloids. Various solvent systems were evaluated for their extraction efficiency. The best solvent was found to be hydrochloric acid - 70 % methanol (1:100, v/v) solution. In addition, other factors including number of times of extraction (1, 2 and 3 times), solvent volume (30, 40, 50, and 60 mL) and extraction time (20, 30, 40, and 60 min) were also tested and optimized. Finally, the optimal extraction condition was determined as follows: samples were extracted with 50 mL of hydrochloric acid - 70 % methanol solution (1:100, v/v) by ultrasonic method for 30 min.

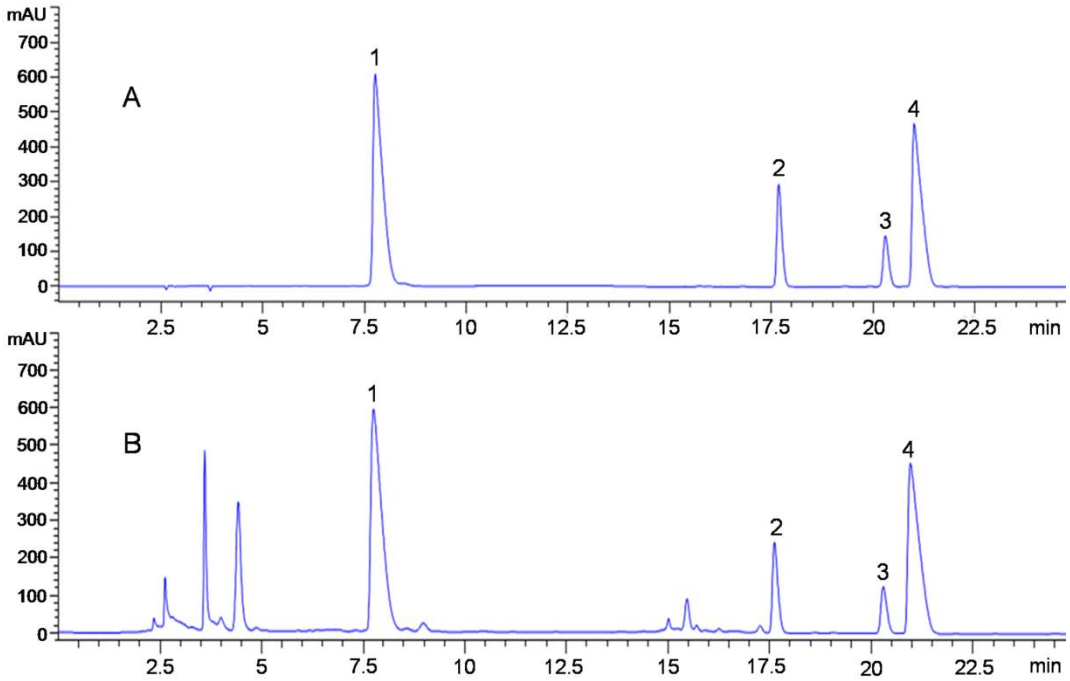

Figure 1: Representative HPLC chromatograms of mixed standard compounds (A) and *Cortex berberidis* extract (B). Peaks: 1. Magnoflorine; 2. Jatrorrhizine; 3. Palmatine; 4. Berberine

Validation results of quantitative analysis

Detailed information about the calibration curves, linear ranges, LOD, and LOQ is listed in Table 1. All calibration curves showed good linear regression (r^2 > 0.9997) within test ranges. The range of LOD for the four alkaloids was from 0.05 to 0.07 µg/mL, and LOQ was from 0.18 to 0.24 µg/mL. For the precision test, relative standard deviation (RSD) values for the contents of four alkaloids were in the range of 0.14 - 0.56 %. Moreover, the analytes were stable within 24 h, and RSD values of the contents of four alkaloids were less than 1.65 %. For the repeatability evaluation, The RSD values of the contents of four alkaloids ranged from 0.55 to 1.55 %, which showed high repeatability. Detailed data about the precision, stability and repeatability are listed in Table 2. In addition, the average recoveries of the four alkaloids were between 98.77 and 101.81 % with RSD < 2.86 % (Table 3). Therefore, the developed HPLC method was precise and accurate enough for simultaneous quantitative analysis of the four alkaloids in *Cortex berberidis*.

Table 1: Linear regression data, LOD and LOQ of investigated compounds

Alkaloid	Calibration curve [a]	r^2	Linear range (mg/mL)	LOD [b] (µg/mL)	LOQ [c] (µg/mL)
Magnoflorine	y = 14632 x + 45.114	0.9997	0.0780～1.2480	0.05	0.18
Jatrorrhizine	y = 35640 x + 26.526	0.9997	0.0069～0.1104	0.06	0.20
Palmatine	y = 38575 x + 0.8934	1.0000	0.0036～0.0576	0.07	0.20
Berberine	y = 34466 x + 26.506	0.9999	0.0216～0.3456	0.06	0.24

[a] y and x refer to the peak area and the concentration of the analyte (mg/mL), respectively; [b] LOD refers to the limit of detection, S/N = 3; [c] LOQ refers to the limit of quantification, S/N = 10

Table 2: Stability, repeatability and precision of the HPLC method for determination of the four alkaloids

Alkaloid	Stability (24h, n = 7)		Repeatability (n = 6)		Precision (n = 6)	
	Content (mg/g) [a]	RSD (%) [b]	Content (mg/g) [a]	RSD (%) [b]	Content (mg/g) [a]	RSD (%) [b]
Magnoflorine	80.01 ± 0.52	0.65	79.83 ± 0.58	0.73	80.09 ± 0.24	0.30
Jatrorrhizine	6.86 ± 0.07	1.07	6.84 ± 0.11	1.55	6.82 ± 0.04	0.56
Palmatine	3.55 ± 0.06	1.65	3.53 ± 0.05	1.52	3.54 ± 0.01	0.15
Berberine	26.86 ± 0.19	0.71	26.82 ± 0.15	0.55	26.62 ± 0.04	0.14

[a] Content = mean ± SD; [b] RSD (%) = (SD/mean) × 100

Table 3: Recovery of the four alkaloids determined by standard addition method

Alkaloid	Original (mg)	Spiked (mg)	Found (mg)	Recovery [a] (%)	Mean recovery (%)	RSD (%)
	20.037	15.5	35.272	98.29		
Magnoflorine	20.024	19.5	39.679	100.79	99.70	1.29
	19.987	23.5	43.492	100.02		
	1.717	1.5	3.169	96.80		
Jatrorrhizine	1.713	1.7	3.447	102.00	98.77	2.86
	1.717	2.0	3.667	97.50		
	0.886	0.5	1.387	100.20		
Palmatine	0.884	0.9	1.804	102.22	101.81	1.42
	0.886	1.0	1.916	103.00		
	6.682	5.5	12.309	102.31		
Berberine	6.668	6.5	13.197	100.45	100.56	1.68
	6.682	8.0	14.597	98.94		

[a] Recovery (%) = [(found − original)/spiked] × 100

Sample analysis

The developed analytical method was subsequently applied to simultaneous determination of magnoflorine, jatrorrhizine, palmatine, and berberine in 16 batches of samples collected from different regions of China. Each sample was analyzed in triplicate to determine the mean content, and the results are shown in Tables 4(a) and 4(b). The four alkaloids were detected in all samples of *Cortex berberidis*. Among them, magnoflorine (36.46 - 87.30 mg/g) was found to be the main component of *Cortex berberidis*, followed by berberine (16.00 - 37.50 mg/g). The compounds with low concentrations included jatrorrhizine (1.53 - 16.26 mg/g) and palmatine (0.66 - 4.57 mg/g). Moreover, the total content of the four alkaloids in all samples ranged from 67.62 to 114.79 mg/g. Interestingly, it was found that samples from Qinghai and Tibet had the total content over 90 mg/g, while the total content of the four compounds in Sichuan samples was not more than 81 mg/g. These data suggest that there may be some notable differences in the contents of the four alkaloids among *Cortex berberidis* samples from different geographical origins.

PLS-DA was performed to obtain an overview of variation among different *Cortex berberidis* samples. The PLS-DA score plot (Figure 2A) clearly showed that samples from Qinghai and Tibet were clustered together and could not be differentiated by PLS1 and PLS2. However, the six Sichuan samples could be clearly separated from those of Qinghai and Tibet by PLS1. In addition, the corresponding loading plot (Figure 2B) of PLS-DA revealed the compounds having significant contributions to the intergroup differences. The loading plot of PLS1 versus PLS2 showed that all of the identified alkaloids were more abundant in Qinghai and Tibet samples than those in Sichuan samples, and that magnoflorine and the total content of the four alkaloids had greater positive loading values on PLS1 than other alkaloids.

Moreover, in order to observe whether these differences are statistically significant, one-way ANOVA followed by Tukey's multiple comparison tests were performed. As shown in Figure 3, no significant differences in the levels of magnoflorine, jatrorrhizine, palmatine, and berberine and the total content of the four alkaloids were observed between Qinghai and Tibet samples. However, the magnoflorine level and the total content of the four compounds in Qinghai and Tibet samples were obviously higher than those in Sichuan samples. In addition, the Qinghai samples contained significantly more jatrorrhizine compared with the Sichuan samples. These results indicated that geographical origins imposed a significant effect on phytochemical levels of *Cortex berberidis*.

Table 4: Content (mg/g) of four alkaloids in *Cortex berberidis* from different origins determined with the HPLC method (n = 3)

No.	Geographical origins	Detailed sources	Acquisition date	Magnoflorine	Jatrorrhizine	Palmatine	Berberine	Total
1	Qinghai	Xining herbal markets (Booth No. 503), Qinghai, China	August 12, 2014	68.48	4.95	3.73	37.50	114.66
2	Qinghai	Huangnan Tibetan Hospital, Qinghai, China	August 17, 2014	55.65	16.26	4.57	26.17	102.65
3	Qinghai	Yalang wholesale department of Tibetan Medicine, Xining, Qinghai, China	August 12, 2014	55.34	14.64	3.37	24.19	97.54
4	Qinghai	Xining herbal markets (Booth No. 403), Qinghai, China	August 12, 2014	60.36	8.96	3.18	24.80	97.30
5	Qinghai	Xining herbal markets (Booth No. 307), Qinghai, China	August 12, 2014	64.01	4.23	2.78	30.10	101.12
6	Qinghai	Qinghai Tibetan Hospital, China	August 10, 2014	87.30	6.91	1.60	16.00	111.81
7	Sichuan	Hongyuan County Tibetan Hospital, Sichuan, China	July 20, 2015	50.23	3.21	3.45	23.46	80.35
8	Sichuan	Dege County Tibetan Hospital, Sichuan, China	October 12, 2014	48.17	2.31	2.50	24.48	77.46
9	Sichuan	Derong County Tibetan Hospital, Sichuan, China	November 12, 2012	54.33	2.75	0.66	21.27	79.01
10	Sichuan	Baiyu County Tibetan Hospital, Sichuan, China	November 8, 2012	46.10	1.53	2.76	29.93	80.32
11	Sichuan	Dege County Tibetan Hospital, Sichuan, China	July 26, 2013	48.49	2.55	2.18	22.77	75.99
12	Sichuan	Zoige County Tibetan Hospital, Sichuan, China	October 6, 2015	36.46	4.04	2.88	24.24	67.62
13	Tibet	Xiongbalaqu Shenshui pharmaceutical factory of Tibetan medicine, Tibet, China	June 11, 2014	67.78	7.71	3.44	24.12	103.05
14	Tibet	Pharmaceutical factory of Tibetan medicine of Tibet, China	August 22, 2014	71.53	6.10	3.17	23.88	104.68
15	Tibet	Yongbulakang pharmaceutical factory of Tibetan medicine, Shannan, Tibet, China	July 15, 2014	77.30	4.05	3.60	26.39	111.34
16	Tibet	Yongbulakang pharmaceutical factory of Tibetan medicine, Shannan, Tibet, China	October 21, 2013	80.50	4.15	3.69	26.45	114.79
17	Tibet	Ganlu pharmaceutical factory of Tibetan medicine, Tibet, China	August 21, 2014	63.68	6.43	3.48	21.12	94.71

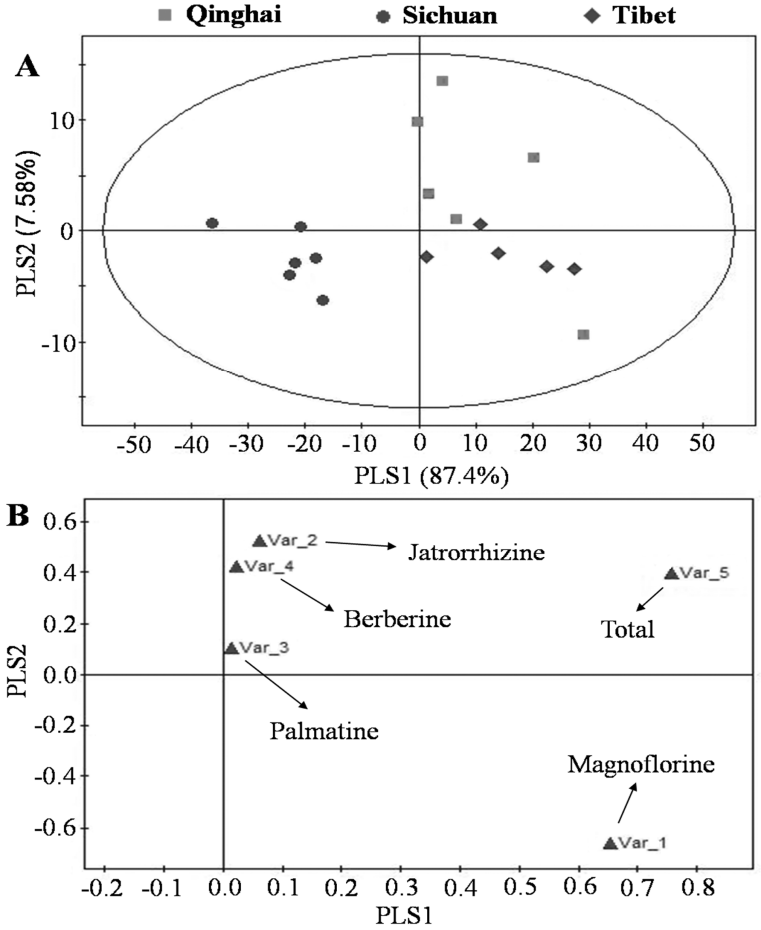

Figure 2: Score (A) and loading (B) plots of PLS-DA of the 17 tested samples using PLS1 (87.4 %) vs. PLS2 (7.58 %) derived from HPLC quantitative data

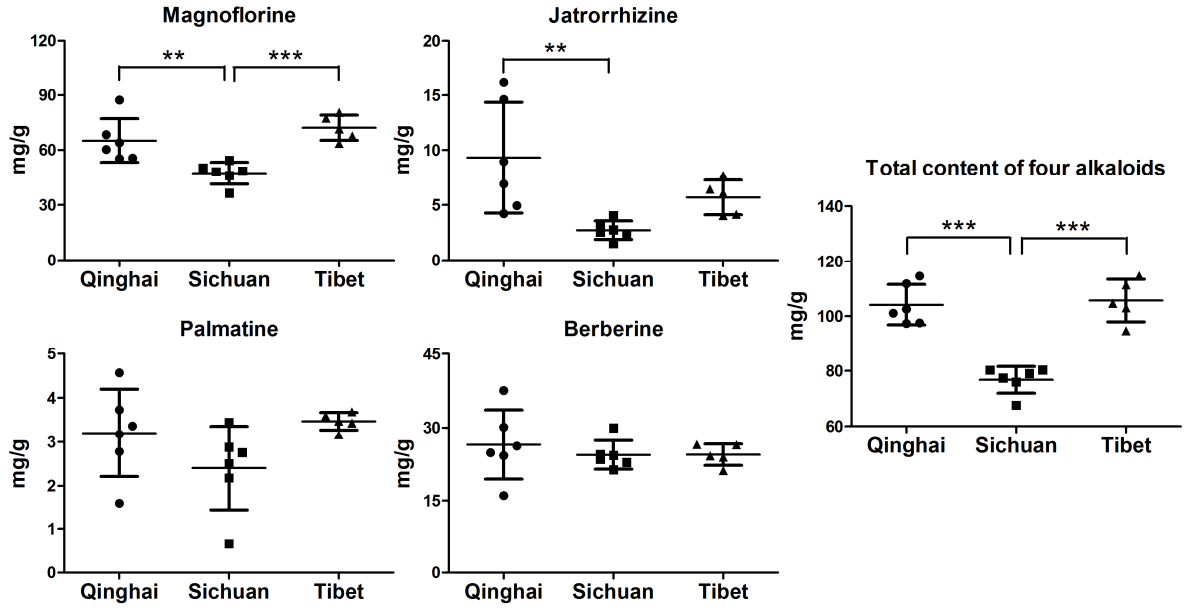

Figure 3: Contents (mg/g) of four alkaloids in *Cortex berberidis* from Qinghai, Sichuan and Tibet determined by the HPLC method. Each point represents an individual sample. Error bars indicate the mean ± standard error (n = 6 for Qinghai, n = 6 for Sichuan and n = 5 for Tibet). ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively, based on Tukey's multiple comparison tests

DISCUSSION

In the present study, a simple, accurate and reliable HPLC method was successfully developed for simultaneous determination of four alkaloids, namely magnoflorine, jatrorrhizine, palmatine, and berberine. It is the first time that these four active ingredients were analyzed simultaneously in *Cortex berberidis* with acceptable performance of linearity, precision, repeatability, and accuracy.

PLS-DA, an supervised pattern recognition method, was performed to obtain an overview of variation among the three geographical origins as well as to further observe how much the variable contributed to the separation between groups. The PLS-DA score plot (Figure 2A) indicated that the Qinghai samples had a close relationship with those from Tibet, but the samples of these two regions were quite different from those collected from Sichuan. Moreover, based on the loading plot of PLS-DA, we found that magnoflorine and the total content of the four alkaloids were the main chemical markers for discrimination of different *Cortex berberidis* samples. Among them, the total content of the four alkaloids had the largest positive loading value on PLS1, indicating that this indicator is mostly responsible for the discrimination between Qinghai and Tibet samples and Sichuan ones. Moreover, magnoflorine was found to have more influence than the other three alkaloids on the discrimination of *Cortex berberidis* from different origins because it showed a greater loading value on PLS1 than other alkaloids.

At present, alkaloids such as berberine and magnoflorine have been considered the primary active constituents of *Cortex berberidis*, because they have been proved to possess various pharmacological activities, such as antibacterial, anti-inflammatory and anti-diabetic effects [11–13]. It is well known that the quality of herbal medicines is closely related to the levels of their active ingredients.

Therefore, based on these results obtained by the PLS-DA and ANOVA, we can conclude that the quality of *Cortex berberidis* vary with the geographic origins, and that *Cortex berberidis* samples from Qinghai and Tibet have better quality than those from Sichuan. This may be due to some conceivable reasons such as the different growth environments (e.g., soil, climate and altitude) among them. Further studies are necessary to reveal the correlation between the phytochemical levels of *Cortex berberidis* and environmental factors.

CONCLUSION

The developed HPLC quantitative technique, combined with statistical methods, provide an efficient tool for the quality evaluation of *Cortex berberidis* from different geographical origins. The quality of Qinghai and Tibet samples is higher than that of Sichuan. Moreover, the results of this study confirm that magnoflorine, and especially the total concentration of the four alkaloids, play an important role in controlling the quality of *Cortex berberidis* because of their strong discriminating power. Thus, they can be used as chemical markers to assess the quality of *Cortex berberidis* in the future.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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