

Development of a rapid resolution liquid chromatography-diode array detector method for the determination of three compounds in *Ziziphora clinopodioides* Lam from different origins of Xinjiang

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ABSTRACT

Context: As a traditional Uygur medicinal plant, *Z. clinopodioides* Lam has various uses in Xinjiang. **Aims:** A reversed-phase rapid resolution liquid chromatography (RP-RRLC) method with diode array detector (DAD) was developed for simultaneous determination of diosmin, linarin, and pulegone from *Ziziphora clinopodioides* Lam, a widely used in traditional Uygur medicine for treating heart disease, high blood pressure, and other diseases. **Settings and Design:** Compounds were separated on a XDB-C18 reversed-phase analytical column (50 mm × 4.6 mm, 1.8 μm) with gradient elution using methanol and 1% aqueous acetic acid (v/v) at 0.9 mL/min. The detection wavelength was set at 270 nm. **Materials and Methods:** *Ziziphora clinopodioides* Lam. were collected from ten different origins in Xinjiang, including the Ban fang ditch, Tuoli, the Altay mountains, Terks, Xiata Road, Zhaosu Highway, Guozigou, Fukang, Jimsar, Wulabo. **Statistical Analysis Used:** The intra-day and inter-day precisions of all three compounds were less than 0.89% and the average recoveries ranged from 97.4 to 104.1%. There were highly significant linear correlations between component concentrations and specific chromatographic peak areas ($R^2 > 0.999$). **Results:** The proposed method was successfully applied to determine the levels of three active components in *Z. clinopodioides* Lam. samples from different locations in Xinjiang. **Conclusions:** The proposed method is simple, consistent, accurate, and could be utilized as a quality control method for *Z. clinopodioides* Lam.

Key words: Diosmin, linarin, pulegone, reversed-phase-rapid resolution liquid chromatography-diode array detector, *Ziziphora clinopodioides* Lam

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INTRODUCTION

Herbal medicines have been used over many centuries in Asia and have become more popular worldwide in recent decades. Medicinal herbs may contain hundreds of complex active components, and it is often impractical to identify all these substances by quantitative analysis^[1] Therefore, we can only determine the composition is relatively high, and have bioactive ingredients in order to quality control. We decided to develop a Rapid Resolution LC method suitable for determination of different compounds in crude extracts of selected medicinal herbs, on a short C_{18} analytical column

packed with 1.8 μm silica-based particles, using methanol as an organic solvent in a binary mobile phase system.

Traditional Uygur medicines are natural therapeutic agents used in accordance with the guiding theory of traditional Uygur medical science. They have been widely used in China since antiquity for prevention and treatment of diseases. *Ziziphora clinopodioides* Lam of the family Lamiaceae is indigenous to China, Mongolia, Turkey, Kazakhstan, and Kyrgyzstan. It is a semi-perennial shrub-like plant that grows on low hills, grasslands, and arid slopes.^[2] As a traditional Uygur medicinal plant, *Z. clinopodioides* Lam has various uses in Xinjiang, including the treatment of heart disease, high blood pressure, asthma hyperhidrosis, palpitation insomnia, edema, cough, bronchitis, and lung abscess.^[3] Several studies have revealed that it has a wide range of antimicrobial^[4,5]

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and antioxidant^[6] effects. Senejoux and others also explored the vasodilating effects of *Z. clinopodioides* Lam and the underlying mechanisms.^[7] Till date, research on *Z. clinopodioides* Lam has focused mainly on the chemical constituents of its essential oils and their bioactive constituents, of which pulegone is considered the main ingredient.^[8-10] In preliminary studies, our research group studied the stability of the plant's essential oils,^[11] inhibit bacterial activity screening, and volatile oil chemical composition analysis of *Z. clinopodioides* Lam,^[12] determined its oleanolic acid and ursolic acid content by HPLC^[9] and simultaneous determination of caffeic acid and rosmarinic acid in *Z. clinopodioides* Lam from different sources in Xinjiang.^[13] Moreover, we investigated the total polyphenolic and flavonoid content as well as the antioxidant activity of *Z. clinopodioides* Lam extracts of different polarity^[6] and determination of ten metal elements in *Z. clinopodioides* Lam by microwave digestion-FAAS.^[14] Other components in *Z. clinopodioides* Lam are caffeic acid, rosmarinic acid and oleanolic acid, ursolic acid, salicylic acid, and flavonoids.^[15] Flavonoids are known to possess potent anti-inflammatory activity in both humans and animals, and recently their topical application has met with considerable interest.^[16,17] Diosmin and linarin are common naturally occurring flavonoids with a number of interesting biological activities. As a flavonoid, diosmin [Figure 1a] also exhibits anti-inflammatory, free-radical scavenging, and antimutagenic properties,^[18] linarin [Figure 1b] is believed to possess parasiticide, anti-microbial, analgesic, anti-viral, anti-proliferative, anti-hypertensive, anti-oxidant, and anti-inflammatory properties.^[19] These pharmacological effects are consistent with the herbs of *Z. clinopodioides* Lam. Pulegone [Figure 1c], a monoterpene hydrocarbon reported to be one of the major active components of *Z. clinopodioides* Lam, can act against Gram-positive and Gram-negative bacteria. It also has a wide range of antimicrobial and antioxidant

effects.^[20] We develop an RRLC method that allows for the simultaneous determination of at least three of these putative bioactive ingredients, diosmin, linarin, and pulegone. This method may form the basis for a more efficient analytic procedure to assess the medicinal quality of *Z. clinopodioides* Lam samples and as a preparative aid for future studies on therapeutic mechanisms.

MATERIALS AND METHODS

Materials and reagents

Whole plant samples were collected from ten different origins in Xinjiang. Samples from the Ban fang ditch, Tuoli, the Altay mountains, Terks, Xiata Road, Zhaosu Highway, and Guozigou were obtained in July, 2010 (NO:ZYMZY20100701-07). Samples from Fukang and Jimsar were collected in August, 2009 (NO:ZYMZY20090801) and the samples from Wulabo were collected in July, 2008 (NO:ZYMZY20080701-02). All specimens were stored in the Traditional Chinese Medicine Ethnical Herbs Specimen Museum of Xinjiang Medical University. The plant materials were identified by Yonghe Li, a chief apothecary of the Chinese Medicine Hospital of Xinjiang. The standards of pulegone were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The standards of diosmin and linarin were purchased from Sigma (USA). Acetic acid of analytical grade was obtained from Tianjin, Fuyu Chemical Reagents Company. Reverse phase RRLC grade methanol was supplied by Fisher Scientific (USA) and water was obtained from a Millipore Q3 ultra-pure water system (Millipore, USA).

Rapid resolution liquid chromatography analysis

Analyses were carried out on an Agilent 1200 system with diode array detector (DAD). The detection wavelength was set at 270 nm. An Agilent XDB-C₁₈ column (50 mm × 4.6 mm, 1.8 μm) was used with a flow rate of 0.9 mL/min. The injection volume was 5 μL and the column temperature was maintained at 30°C. The mobile phase consisted of solvent A (1% acetic acid in water) and solvent B (methanol), using gradient elution as follows: 0–10 min, 62–52% A; 10–20 min, 52–40% A.

Preparation of standard solutions

Each standard of diosmin, linarin, and pulegone were accurately weighed, dissolved in methanol:DMSO (3:1 v/v) and diluted to the appropriate concentration for analysis. All stock solutions were stored at 4°C.

Preparation of samples

The herbal samples of *Z. clinopodioides* Lam. were first crushed into coarse powder. The pulverized powder (0.2000 g) was added to methanol-DMSO in a 30 ml Erlenmeyer flask. For chemical extraction, the mixture

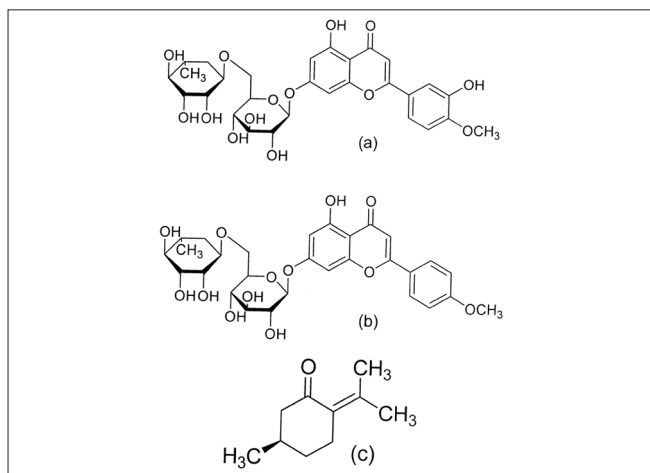


Figure 1: Chemical structures of the three bioactive components in *Z. clinopodioides* Lam

was first left at room temperature for 1 h and then ultrasonicated for 30 min. The solution was then filtered through the 0.22 μm membrane filter.

RESULTS

Method development and optimization

To obtain chromatograms with well-resolved peaks and minimal analysis time per run, chromatographic conditions were optimized, including the mobile phase, column temperature, and flow rate. Various mixing ratios of water to methanol as the mobile phase were used but no satisfactory separation was achieved. It was found that the presence of acids in the mobile phase enhanced the resolution. The results showed that 1% acetic acid buffer in the mobile phase significantly improved the retention behavior and peak shape of the different components in *Z. clinopodioides* Lam. However, using isocratic elution, the three compounds could not be separated effectively and so gradient elution was used throughout the study. Other chromatographic variables were also optimized, including column temperatures (25, 30, or 35°C) and flow rate (0.6, 0.8, 0.9, and 1.0 mL/min). Eventually, the optimal separation was achieved at a column temperature of 30°C and flow rate of 0.9 mL/min.

To determine the appropriate wavelength for simultaneous determination of diosmin, linarin, and pulegone solutions, standards were injected into the RRLC system and the UV spectra measured over the range 190 to 400 nm by DAD. The UV spectra of all three compounds showed the same absorption maximum at 270 nm.

Extraction efficiency of different solvents, including methanol-DMSO (75:25, v/v), methanol, methanol-water (50:50, v/v), ethanol, and ethanol-water (50:50, v/v), were compared. Diosmin and linarin were difficult to dissolve in

methanol, but DMSO as a co-solvent helped in the separation.

A typical RRLC chromatogram is shown in Figure 2. Retention times for the three compounds were 3.66 min (diosmin), 7.98 min (linarin), and 14.23 min (pulegone).

System suitability

System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Resolution (R), retention time (RT), number of theoretical plates (N), and tailing factor (T) were evaluated in five replicate injections of the drugs at 5 μL . As shown in Table 1, all parameters were within acceptable limits.

Method validation

Linearity, limits of detection, and quantification

Regression analyses were performed using GraphPad Prism 4.00. The correlation coefficient r^2 and linear regression equations were computed by the partial least square method and the quality of the curve fit obtained was assessed. The linear calibration curves were constructed from five concentration assays performed in triplicate. The linear regression equation was $Y = aX + b$, where Y and X are the values of peak area and concentration of the reference compound, respectively. The results of the regression analyses and the calculated correlation coefficients (r^2) are listed in Table 2. The high correlation coefficient values ($r^2 > 0.999$) indicated good linearity between peak areas (Y) and compound concentrations (X , mg/mL) over relatively wide concentration ranges.

The limit of quantification (LOQ) was defined as the lowest concentration with peak height 10 times the baseline noise ($S/N = 10$). The minimum detectable concentration, as defined by a signal-to-noise ratio (S/N) of 3, was considered to be the limit of detection (LOD). The LOQ

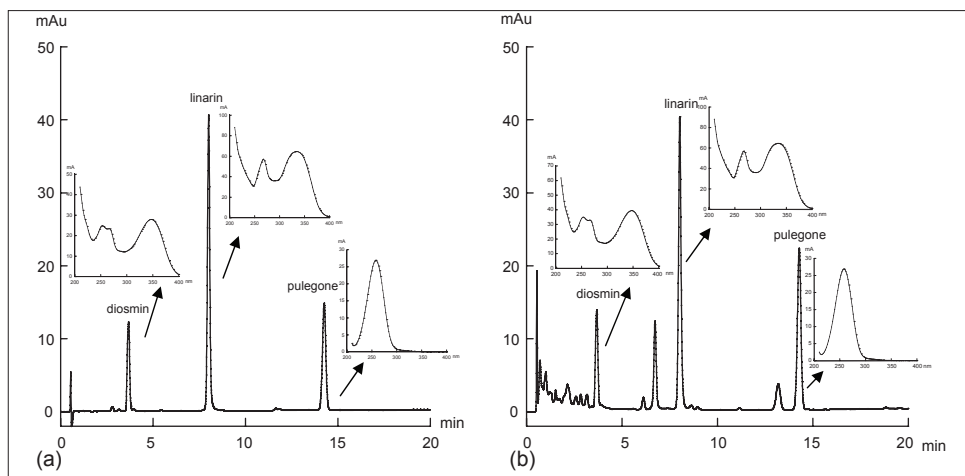


Figure 2: A typical rapid resolution liquid chromatography chromatogram and UV spectra of *Z. clinopodioides* Lam. (a) Standard chromatogram; (b) sample chromatogram

Table 1: System-suitability data. Mean \pm SD, ($n = 5$)

Property	Compounds	Value	RSD (%)	Required limits
Resolution (R)	Diosmin	22.46 \pm 0.15	0.65	R > 1.5
	Linarin	20.71 \pm 0.07	0.33	
	Pulegone	2.96 \pm 0.01	0.44	
Retention time (RT)	Diosmin	3.66 \pm 0.008	0.22	RSD \leq 1%
	Linarin	7.98 \pm 0.010	0.13	
	Pulegone	14.23 \pm 0.007	0.05	
Theoretical plates (N)	Diosmin	5439 \pm 87.6	1.61	N > 2000
	Linarin	21429 \pm 55.2	1.26	
	Pulegone	28813 \pm 321	1.11	
Tailing factor (T)	Diosmin	0.95 \pm 0.005	0.58	1.05 > T > 0.95
	Linarin	0.99 \pm 0.004	0.45	
	Pulegone	1.04 \pm 0.004	0.43	

Table 2: Statistical results of linear regression equation analysis in the determination of the three investigated compounds

Compounds	Regression equation	r ²	Linear range (mg/mL)	LOD (μ g/mL)	LOQ (μ g/mL)
Diosmin	Y = 890.4115X - 0.3198	0.999	0.0122-0.1708	0.313	0.607
Linarin	Y = 1196.5880X - 1.4305	0.999	0.0276-0.3864	0.177	0.504
Pulegone	Y = 3948.4880X - 1.4843	0.999	0.0063-0.0879	0.068	0.171

and LOD values for the three chemical components are also listed in Table 2.

Stability and precision

Intra-day and inter-day precision and accuracy were evaluated by analyzing quality-control samples. The intra-day variation was examined by analyzing five individual sample solutions from the same crude sample of *Z. clinopodioides* Lam on the same day. Inter-day precision and accuracy were determined by once daily trials for three consecutive days. Variations were expressed as relative standard deviation (RSD). The values of the intra-and inter-day variations were less than 2.0%. The instrumental precision was evaluated by five replicate injections of the ban fang ditch sample solution, and RSD value was below 0.89%.

Reproducibility

The reproducibility of extraction was also investigated for the three components by comparing six samples from six independent extractions. Six 0.2000 g samples of *Z. clinopodioides* Lam powder from Ban fang ditch were accurately weighed, prepared, and analyzed by RRLC. The RSD values of the six replicates were less than 2.0% for all compounds, demonstrating the high reproducibility of the sample preparation procedure.

Recovery

Recovery tests were performed to further investigate the reproducibility and efficiency of the extraction and analysis method. Recoveries of the three compounds were determined by the method of standard addition. Three concentrations of the compound standard solutions were

used to spike *Z. clinopodioides* Lam samples containing known amounts of each compound (namely 50% of the compound). The mixture was extracted and analyzed as described. The mean recoveries of the three compounds were 104.1% for diosmin, 102.3% for linarin, and 97.4% for pulegone, with RSD values of 1.6, 1.2, and 2.1%, respectively.

The obtained results indicate that the developed analytical method was reproducible with high accuracy. It is therefore satisfactory for quantitative analysis.

Application to the analysis of *Ziziphora clinopodioides* Lam samples

The developed analytical method was successfully applied for the simultaneous determination of the three components in ten different samples of *Z. clinopodioides* Lam. All three compounds were detected from every sample. Each sample was determined in triplicate and the peaks in the chromatograms were identified by comparing the retention times and UV spectra with the authentic standards. The three compounds were quantified in the ten samples [Table 3].

DISCUSSION

We describe a new method of RRLC separation, using 1.8 μ m particle size of stationary phase instead of the usual 5 μ m columns, is proven to be efficient, precise, accurate, sensitive and time saving, and enabled determination of diosmin, linarin, and pulegone from *Z. clinopodioides* Lam using reversed-phase chromatography with gradient elution and a DAD detector. Simultaneous detection could allow

Table 3: Contents of the three compounds in ten different origin samples of *Ziziphora clinopodioides* Lam. (n = 3)

Sources	Diosmin contents (mg/g)	Linarin contents (mg/g)	Polegone contents (mg/g)
Tex	3.1186 ± 0.1156	9.1380 ± 0.1804	0.6102 ± 0.0012
Ban fang ditch	7.0749 ± 0.0501	15.7627 ± 0.1195	3.6721 ± 0.0088
Altay	7.6636 ± 0.1997	7.9919 ± 0.0599	4.0976 ± 0.0063
Tuoli	5.4379 ± 0.0831	24.2941 ± 0.0556	0.5174 ± 0.0047
Xiata Road	4.3156 ± 0.0568	14.1554 ± 0.0545	0.6751 ± 0.0008
Zhaosu Highway	5.7606 ± 0.1123	13.8769 ± 0.0104	1.8499 ± 0.0461
Guozigou	6.1576 ± 0.0338	18.8340 ± 0.0713	2.6950 ± 0.0118
Wulabo	4.9090 ± 0.0499	11.8823 ± 0.0405	0.3493 ± 0.0021
Fukang	7.3029 ± 0.1353	6.1507 ± 0.0564	0.8760 ± 0.0108
Jimsar	4.2613 ± 0.0852	4.7203 ± 0.0284	0.3941 ± 0.0025

for a large number of herbal samples to be analyzed in a relatively short period of time. Further studies are ongoing in our laboratory to further characterize activate compounds of *Z. clinopodioides* Lam. It is clear that there was a significant variation in the contents of the three compounds between the ten samples obtained from different regions. Similar variations have been found for other components and may be attributed to the different growing conditions at the sampling sites. These variations in the bioactive components influence the medicinal quality, so it was necessary to develop an effective qualitative and quantitative method to evaluate the overall quality of *Z. clinopodioides* Lam. The simultaneous analysis of several chemical components is a promising tool for the quality control of medicinal herbs.

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