Preliminary Investigation of the HPLC Fingerprint of *Lysimachia foenum-graecum* Hance

Guilin YANG, Qiji ZHOU, Chengtong LIU, Weimei HE, Lixiang LU, Xueping WEI, Lizhen LIN, Xinying MO*

Affiliated Hospital of Youjiang Medical University for Nationalities, Baise 533000, China; Guangxi Key Laboratory of Artificial Intelligence for Genetic Diseases of Long-dwelling Nationalities, Baise 533000, China; Key Laboratory of Research on Prevention and Control of High Incidence Diseases in Western Guangxi, Baise 533000, China

Abstract [**Objectives**] To develop an HPLC fingerprint analysis method for the medicinal material of *Lysimachia foenum-graecum* Hance, thereby providing a foundation for its quality control. [**Methods**] Samples of *L. foenum-graecum* collected from 10 distinct locations in Guangxi were analyzed using HPLC, and chromatographic fingerprints were established. The Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (2012 Edition) was employed for common peak calibration and similarity evaluation. Additionally, principal component analysis was performed on the common peak area data. [**Results**] An HPLC fingerprint of *L. foenum-graecum* was developed, identifying a total of 13 common peaks. Among these, four characteristic components were specifically identified: chlorogenic acid, myricetin, quercetin, and kaempferol. The kaempferol chromatographic peak, exhibiting good resolution and a stable peak shape, was selected as the reference peak. The similarity indices between the fingerprints of the 10 sample batches and the reference fingerprint ranged from 0.954 to 0.995, indicating a relatively high consistency in the chemical composition of *L. foenum-graecum* from different origins. Principal component analysis identified two principal components, which together accounted for 89.45% of the cumulative variance, effectively capturing the primary chemical differences among the samples. [**Conclusions**] The established HPLC fingerprint method is straightforward to implement, stable, reliable, and exhibits high specificity. When combined with similarity evaluation and principal component analysis, it offers a scientific basis for developing quality standards for *L. foenum-graecum* medicinal materials.

Key words *Lysimachia foenum-graecum* Hance, HPLC, Fingerprint, Similarity evaluation, Principal component analysis

1 Introduction

Lysimachia foenum-graecum Hance is a species within the genus *Lysimachia*, belonging to the Primulaceae family, predominantly found in regions such as Guangxi and Yunnan[1]. Both the stems and leaves of this plant are utilized for medicinal purposes. It is characterized by a pungent and sweet flavor and a warm nature, exhibiting pharmacological effects including stomach strengthening, induction of sweating, and pain relief. Consequently, it is commonly employed in the treatment of conditions such as colds, abdominal pain, and eczema[2-3]. *L. foenum-graecum* serves as a significant raw material for spices and is recognized as one of the "Three Spices of Guangxi", alongside cinnamon and star anise[4]. Contemporary studies have demonstrated that *L. foenum-graecum* contains a variety of bioactive compounds, including flavonoids, triterpenoids, and polyphenols[5]. Nevertheless, systematic research on the quality control of *L. foenum-graecum* remains inadequate, and standardized evaluation methods are lacking. Chromatographic fingerprinting technology offers a comprehensive characterization of the overall chemical profile of Chinese medicinal materials and represents an effective approach for assessing the consistency and stability of traditional Chinese medicine quality[6].

This study collected *L. foenum-graecum* samples from 10 distinct locations in Guangxi to develop a high-performance liquid chromatography (HPLC) fingerprint analysis method. By employing similarity evaluation and principal component analysis, the chemical composition similarities and differences among *L. foenum-graecum* samples from various origins were comprehensively assessed. The objective was to provide a scientific foundation for the rational development and utilization of this Zhuang medicinal resource, as well as for the establishment of quality standards.

2 Materials and methods

2.1 Materials

2.1.1 Experimental medicinal materials. The 10 batches of *L. foenum-graecum* medicinal materials were collected from various locations in Guangxi in 2024 (Table 1). These samples were authenticated as dried whole plants of *L. foenum-graecum*, belonging to the Primulaceae family, by Chief Pharmacist Zhong Wen of the Guangxi International Zhuang Medicine Hospital, Affiliated to Guangxi University of Chinese Medicine. Subsequently, the samples were dried at 55 ℃, ground, sieved through a No. 2 mesh, and stored in a desiccator for subsequent use.

2.1.2 Experimental reagents. The reference substances of chlorogenic acid (batch No.: 110753-202520), myricetin (batch No.: 112153-202501), quercetin (batch No.: 10008-202411), and kaempferol (batch No.: 110861-201911) were obtained from the National Institutes for Food and Drug Control of China. Acetonitrile and methanol, both of chromatographic grade, were procured from Fisher (USA). Phosphoric acid was of analytical

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Guilin YANG, master's degree, associate chief pharmacist. * Corresponding author. Xinying MO, master's degree, associate chief pharmacist.

grade, and ultrapure water was used throughout the experiments.

Table 1 Information regarding the samples of *Lysimachia foenum-graecum*

No.	Place of origin	Collection time
LXC1	Jingxi City, Guangxi	2024 – 10
LXC2	Napo County, Baise City	2024 – 08
LXC3	Lingyun County, Guangxi	2024 – 11
LXC4	Du'an County, Guangxi	2024 – 10
LXC5	Hezhou City, Guangxi	2024 – 07
LXC6	Tian'e County, Hechi City	2024 – 12
LXC7	Bama County, Guangxi	2024 – 09
LXC8	Fuchuan County, Guangxi	2024 – 10
LXC9	Gongcheng County, Guilin City	2024 – 05
LXC10	Binyang County, Guangxi	2024 – 08

2.1.3 Main instruments. The primary instruments utilized in this study comprised a high-performance liquid chromatograph (e2695, Waters Technologies, USA), an ultrasonic cleaner (KQ-500DE, Kunshan Ultrasonic Instrument Co., Ltd.), and an electronic balance (BSA224S, Sartorius Scientific Instruments Co., Ltd.).

2.2 Methods

2.2.1 Preparation of the test solution. Precisely 1.00 g of *L. foenum-graecum* powder, sieved through a No. 2 mesh, was weighed, soaked with 95% ethanol, and subjected to ultrasonic extraction twice, each extraction lasting 30 min. The residue was subsequently extracted twice more under the same conditions using 95% ethanol. The combined filtrates were evaporated to dryness in a water bath at 60 ℃ and reserved for further analysis. The dried extract was dissolved in methanol, diluted to a final volume of 5 mL in a volumetric flask, and centrifuged at 13 000 rpm for 10 min. The resulting supernatant was filtered through a 0.45 μm microporous membrane to obtain the test solution of *L. foenum-graecum*.

2.2.2 Preparation of the reference solution. Appropriate amounts of chlorogenic acid, myricetin, quercetin, and kaempferol reference substances were accurately weighed and individually dissolved in methanol to prepare single reference stock solutions with concentrations of 0.48, 0.42, 0.35, and 0.71 mg/mL, respectively. Subsequently, precise volumes of each stock solution were aspirated, diluted with methanol, and adjusted to constant volume to prepare a mixed reference solution containing chlorogenic acid (0.048 mg/mL), myricetin (0.042 mg/mL), quercetin (0.035 mg/mL), and kaempferol (0.071 mg/mL).

2.2.3 Chromatographic conditions. The chromatographic column used was a ZORBAX SB C_{18} (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of solvent A, acetonitrile, and solvent B, 0.1% phosphoric acid solution. Gradient elution was performed with the following program: 0 – 10 min, 8% – 92% A; 10 – 25 min, 18% – 82% A; 25 – 36 min, 45% – 55% A; 36 – 50 min, 62% – 38% A; and 50 – 60 min, 78% – 22% A. The detection wavelength was set at 320 nm, the flow rate was maintained at 1 mL/min, the column temperature was controlled at 30 ℃, and the injection volume was 10 μL.

2.2.4 Methodological investigation[6-7]. A single sample of the test solution of *L. foenum-graecum* (S1) was repeatedly injected six times under the chromatographic conditions specified in Section **2.2.3** to assess precision. Additionally, six separate powder samples of *L. foenum-graecum* from the same location were prepared in parallel according to the procedure in Section **2.2.1**, and repeatability was evaluated. For the stability test, aliquots of the same test solution were stored at room temperature for 0, 2, 4, 8, 12, and 24 h, respectively, before injection. All experiments utilized the kaempferol peak as a reference to calculate the relative retention time and relative peak area of each common peak, with their relative standard deviations (*RSD*) subsequently determined.

2.2.5 Fingerprint establishment and similarity evaluation. The HPLC chromatograms of 10 batches of *L. foenum-graecum* test solutions were imported into the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (2012 Edition). The reference fingerprint (R) was generated using the median method, with the time window width set at 0.5 min. Chromatographic peaks present in all sample batches were designated as common peaks. Each batch's fingerprint was then compared to the reference fingerprint to calculate similarity.

2.2.6 Principal component analysis. The absolute peak area data corresponding to 13 common peaks from 10 batches of samples were imported into SPSS 21.0. Following data standardization, principal component analysis was performed to extract principal components with eigenvalues greater than 1, in order to assess the overall chemical differences among samples originating from different locations.

3 Results and analysis

3.1 Methodological verification results In the precision, repeatability, and stability assessments, the *RSD* of the relative retention time for the 13 common peaks was consistently below 0.5%, while the *RSD* of the relative peak area was below 3.0% ($n=6$). These results demonstrate that the method exhibits excellent precision and high repeatability. Stability testing indicated that the test solution remained stable for up to 12 h; however, a significant decrease in the area of certain chromatographic peaks was observed after 24 h. Consequently, it is recommended that samples must be analyzed within 12 h of preparation.

3.2 Establishment of reference fingerprint Based on the common chromatographic patterns of *L. foenum-graecum* samples from 10 distinct locations, a representative control fingerprint was established and designated as the reference fingerprint for *L. foenum-graecum* (Fig. 1). Subsequently, the superimposed HPLC chromatograms of *L. foenum-graecum* samples from various locations were obtained (Fig. 2).

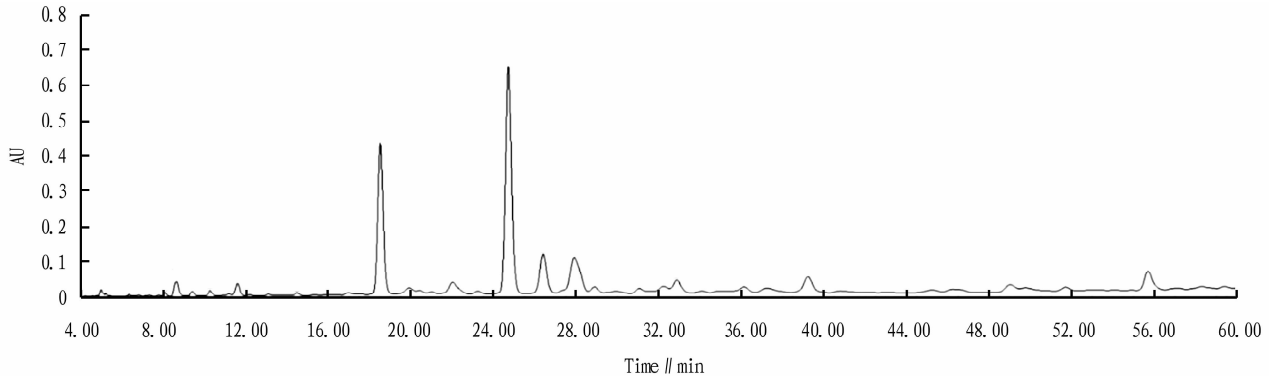


Fig. 1 Reference fingerprint of *Lysimachia foenum-graecum*

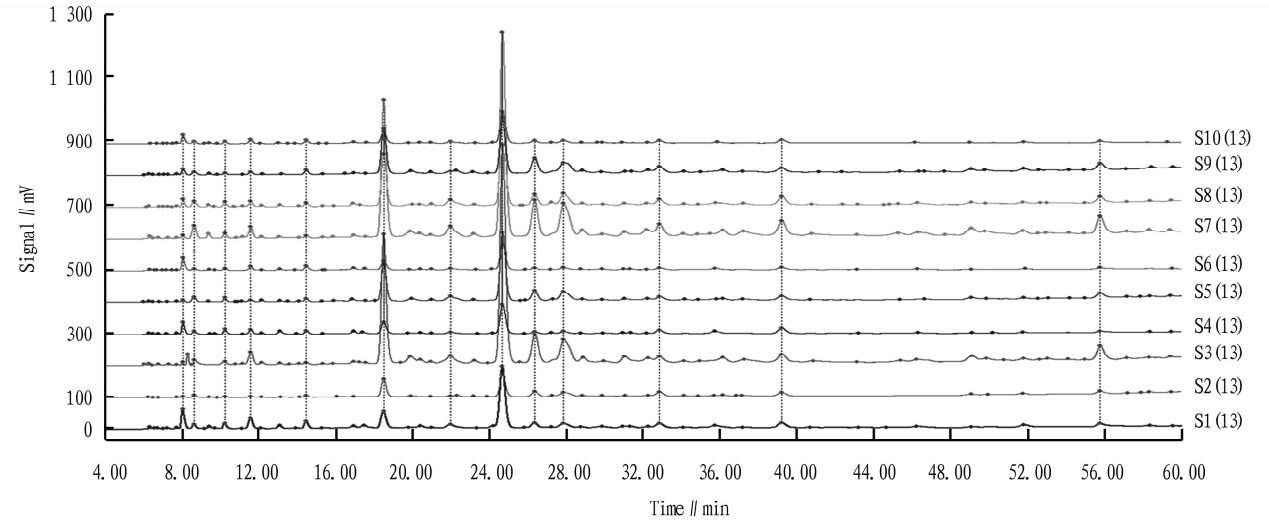
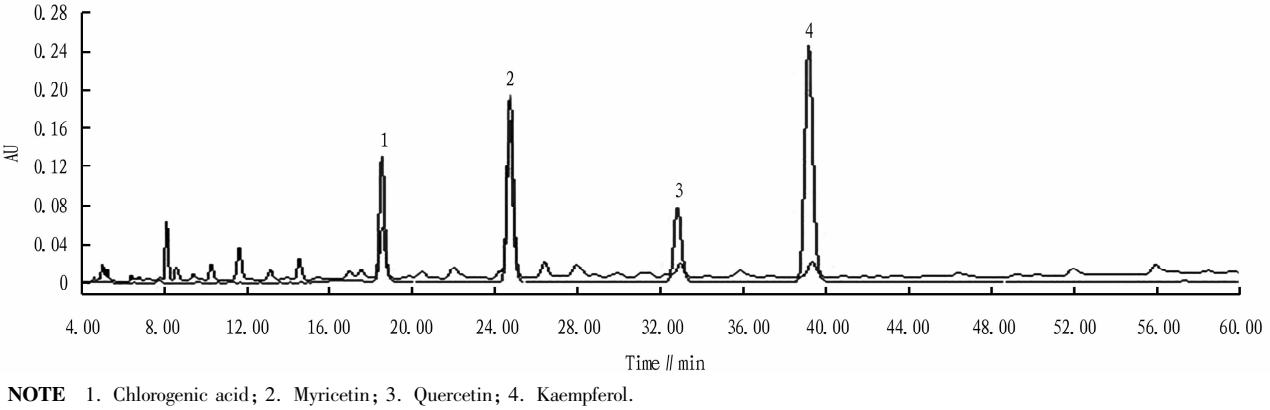


Fig. 2 Superimposed HPLC chromatograms of *Lysimachia foenum-graecum* samples from 10 distinct locations

3.3 Reference peak identification The research group previously identified that *L. foenum-graecum* contains chlorogenic acid, myricetin, quercetin, and kaempferol. These four compounds are considered characteristic components of *L. foenum-graecum*. Comparison with reference standards using HPLC chromatography revealed that the retention time of *L. foenum-graecum* matched those observed in the ultraviolet absorption spectra, confirming the presence of these four components in *L. foenum-graecum*. In the HPLC chromatogram of *L. foenum-graecum*, the four common components exhibited well-resolved chromatographic peaks, with peak symmetries ranging from 0.95 to 1.05 and relatively stable peak areas. Due to its favorable separation, relative stability, and distinctive characteristics, kaempferol was selected as the reference peak. The chromatogram of the mixed reference is presented in Fig. 3.



NOTE 1. Chlorogenic acid; 2. Myricetin; 3. Quercetin; 4. Kaempferol.

Fig. 3 Superimposed UPLC chromatograms of ethyl acetate and mixed reference substances

3.4 Determination of common peaks and reference peaks

The No. 4 peak in the fingerprint was designated as the reference peak. The retention time and peak area of this reference peak were used as the baseline value to calculate the relative retention time and relative peak area of the common peaks across 10 batches of *L. foenum-graecum* (Tables 2 – 3).

Table 2 Relative retention time of common peaks in the fingerprint of *Lysimachia foenum-graecum* min

Sample	1	2	3	4R	5	6	7	8	9	10
LXC1	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC2	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC3	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC4	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC5	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC6	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC7	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC8	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC9	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505
LXC10	0.436	0.468	0.554	1	0.627	0.783	1.187	1.334	1.424	1.505

Table 3 Relative peak area of common peaks in the fingerprint of *Lysimachia foenum-graecum*

Sample	1	2	3	4R	5	6	7	8	9	10
LXC1	0.617	0.201	0.238	1	0.528	0.326	0.294	3.88	0.411	0.466
LXC2	0.034	0.112	0.038	1	0.047	0.078	0.048	1.395	0.346	0.443
LXC3	0.011	0.027	0.018	1	0.088	0.015	0.119	1.885	0.313	0.387
LXC4	0.535	0.175	0.277	1	0.311	0.245	0.243	2.875	0.198	0.317
LXC5	0.267	1.011	0.679	1	0.392	0.638	2.356	19.476	3.27	4.524
LXC6	0.755	0.13	0.154	1	0.344	0.383	0.248	3.219	0.288	0.429
LXC7	0.015	0.083	0.028	1	0.07	0.015	0.148	1.669	0.347	0.474
LXC8	0.077	0.094	0.069	1	0.084	0.058	0.228	1.85	0.264	0.381
LXC9	0.075	0.051	0.028	1	0.049	0.076	0.043	1.326	0.433	0.567
LXC10	0.368	0.12	0.136	1	0.277	0.233	0.27	2.888	0.303	0.387

3.5 Proportions of common peak area and non-common peak area

Among the HPLC fingerprints of the 10 batches of *L. foenum-graecum* medicinal materials, the proportion of non-common peak areas exceeded 10% in all cases (Table 4). This variation may be attributed to differences in the collection regions, diverse growth environments, and varying harvest periods, which collectively led to significant disparities in the chemical composition of the medicinal materials and consequently a relatively large area of non-common peaks.

Table 4 Proportions of common peak area and non-common peak area %

Sample	Proportion of common peak area	Proportion of non-common peak area
LXC1	73.57	26.45
LXC2	74.77	25.23
LXC3	79.33	20.67
LXC4	76.26	23.74
LXC5	80.20	19.80
LXC6	74.07	25.93
LXC7	78.72	21.28
LXC8	76.07	23.93
LXC9	74.29	25.71
LXC10	76.87	22.13

3.6 Similarity analysis

According to the similarity evaluation results presented in Table 5, the similarity indices for all 10 batches of *L. foenum-graecum* exceeded 0.9. Specifically, the similarity values for samples from 10 distinct locations, including Jingxi City, Napo County, Lingyun County, Du'an County, Hezhou City, and Tian'e County, ranged from 0.954 to 0.995, all above 0.90. These findings indicate a high consistency in the chemical composition of *L. foenum-graecum* across different origins. However, notable variations were observed in the contents of individual chemical components.

Table 5 Similarity evaluation results of *Lysimachia foenum-graecum* from various locations

Sample	Place of origin	Similarity
LXC1	Jingxi City, Guangxi	0.961
LXC2	Napo County, Baise City	0.985
LXC3	Lingyun County, Guangxi	0.993
LXC4	Du'an County, Guangxi	0.962
LXC5	Hezhou City, Guangxi	0.989
LXC6	Tian'e County, Hechi City	0.963
LXC7	Bama County, Guangxi	0.995
LXC8	Fuchuan County, Guangxi	0.994
LXC9	Gongcheng County, Guilin City	0.971
LXC10	Binyang County, Guangxi	0.954

3.7 Principal component analysis

Principal component analysis was performed on the peak area data of 13 common peaks.

The eigenvalues of the first two principal components exceeded 1, and the cumulative variance contribution rate reached 89.45%, indicating that these components represent the majority of the chemical information (Table 6).

Table 6 Eigenvalues of principal components and their contribution rates

Component	Initial eigenvalue			Extracted eigenvalue and contribution rate		
	Total	Variance contribution rate//%	Accumulation//%	Total	Variance contribution rate//%	Accumulation//%
1	9.124	70.187	70.187	9.124	70.187	70.187
2	2.504	19.256	89.453	2.504	19.265	89.453
3	0.689	5.301	94.753			
4	0.415	3.192	97.945			
5	0.162	1.247	99.192			
6	0.059	0.454	99.646			
7	0.032	0.246	99.893			
8	0.013	0.096	99.989			
9	0.001	0.011	100			
11	1.01E-13	1.06E-13	100			
12	1.00E-13	1.02E-13	100			
13	-1.00E-13	-1.01E-13	100			
14	-1.01E-13	-1.09E-13	100			

The PCA scree plot (Fig. 4) demonstrated that the slopes of the first three factors were relatively steep, indicating that the eigenvalues in this region were large and contained substantial information. Beyond the component 3, the slope of the scree plot became more gradual, corresponding to smaller eigenvalues and less informational content. Using an eigenvalue threshold greater than 2 as the extraction criterion, the initial eigenvalue contribution analysis revealed that the cumulative variance explained by the first two factors was 89.453%, signifying their considerable influence on the components. In conjunction with the PCA scree plot, the first two factors were therefore selected as the primary components for subsequent analysis.

The principal component loading matrix primarily indicates the contribution and direction of each variable to the principal components, with larger absolute values signifying stronger correlations. As presented in Table 7, common factor 1 exhibited positive correlations with the other 13 components, whereas common factor 2 showed positive correlations with components 3, 8, 9, 11, 12, and 13, and negative correlations with the remaining components.

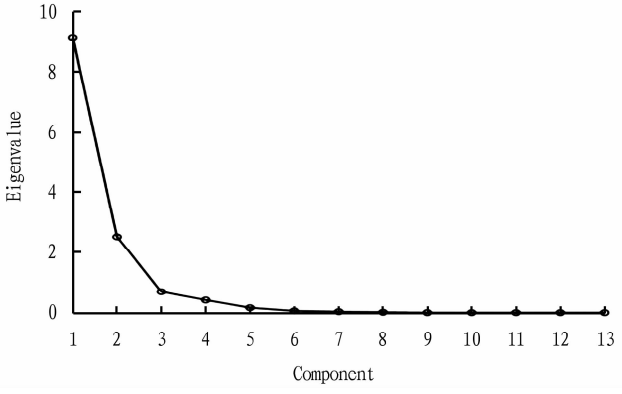


Fig. 4 PCA scree plot of *Lysimachia foenum-graecum*

Table 7 Principal component loading matrix

Component	Principal component	
	1	2
1	0.900	-0.394
2	0.605	-0.636
3	0.332	0.803
4	0.972	-0.205
5	0.925	-0.358
6	0.988	-0.032
7	0.991	-0.078
8	0.764	0.400
9	0.972	0.066
10	0.898	-0.123
11	0.922	0.243
12	0.225	0.844
13	0.932	0.292
14	0.582	0.794
15	0.912	-0.323

4 Conclusions and discussion

The results of the similarity analysis conducted in this study indicated that the chemical profiles of *L. foenum-graecum* samples from various locations in Guangxi were largely consistent. This similarity is attributable to their classification as the same species and the comparable growth environments and climatic conditions across these regions. Nevertheless, the proportion of non-common peak areas relative to the total peak area ranged from 19.80% to 26.45%, indicating the presence of certain variations in the types or contents of secondary chemical components among samples from different locations. These differences may be influenced by factors such as local microenvironmental conditions and harvest periods.

By comparison with reference substances, four flavonoids and phenolic acid compounds, including chlorogenic acid, myricetin, quercetin, and kaempferol, were successfully identified. These compounds possess well-established biological activities, including anti-inflammatory and antioxidant effects, which may un-

derlie the traditional heat-clearing and analgesic properties attributed to *L. foenum-graecum*. Furthermore, these findings provide a foundational basis for subsequent investigations into the plant's bioactivity.

Principal component analysis demonstrated that the first two principal components, accounting for a cumulative contribution rate of 89.45%, effectively differentiated subtle variations in the chemical characteristics of samples from diverse locations. Furthermore, in conjunction with load matrix analysis, future studies should prioritize chromatographic peaks that contribute significantly to the principal components. These peaks may serve as key markers for characterizing quality differences in *L. foenum-graecum*.

The primary limitation of this study is the relatively small sample size, which is confined to the Guangxi production area. Future research should consider expanding the sample collection scope and integrating content determination, chemometric analysis, and pharmacodynamic correlation analysis. Such an approach would facilitate a more comprehensive exploration of the intrinsic relationship between fingerprint profiles and the quality of medicinal materials, thereby contributing to the enhancement of quality standards for *L. foenum-graecum*.

This study successfully developed a stable, reliable, and specific HPLC fingerprint analysis method for Zhuang medicine *L. foenum-graecum*. Similarity evaluation confirmed that the overall chemical composition of *L. foenum-graecum* from different locations was consistent. Principal component analysis further elucidated the chemical variations present within the samples. The integration of fingerprinting with multimodal chemical analysis offers a more comprehensive and scientific approach to evaluating the quality of *L. foenum-graecum* medicinal materials, thereby providing essential technical support and data references for the future standardization and industrial development of this distinctive Zhuang medicinal resource.

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study are expected to provide an experimental basis for further investigation into the active components and pharmacological effects of *P. peltata*, and may offer valuable insights for the research and development of ethnomedicines.

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