Phyton-
International Journal of
Experimental Botany

Tech Science Press

http://doi.org/10.32604/phyton.2026.084444

ARTICLE

Survey and Comparative Analysis of Secondary Metabolites in Mulberry Leaves from Different Cultivars and Geographical Origins in Chongqing

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Received: 22 April 2026; Accepted: 25 June 2026; Published: 30 July 2026

ABSTRACT: Mulberry leaf is a widely used medicinal and edible homologous plant, and its quality is closely related to the contents of secondary metabolites. In this study, 17 batches of mulberry leaves from 3 cultivars and 7 geographical origins in Chongqing were used as materials. An HPLC method was established for the simultaneous determination of mulberroside A, chlorogenic acid, rutin, isoquercitrin, and astragalin, and the effects of cultivar and origin on the accumulation of these components were systematically compared. The established method exhibited satisfactory linearity ($R^2 \geq 0.9998$), together with good precision, stability, repeatability, and accuracy, which proved to be reliable for the quantitative analysis of multiple components in mulberry leaves. Using this validated approach, obvious variations in active component contents were observed among different cultivars. *Morus alba* L. showed the highest content of core active components, followed by *Morus australis* and *Morus cathayana*. Regarding different geographical origins, samples from Wanzhou District possessed the highest contents of chlorogenic acid, rutin, isoquercitrin, and astragalin. Samples from urban areas, including Nan'an Districts showed the lowest contents. The overall content presented a gradient distribution of outer suburban mountains higher than suburban hills and suburban hills higher than urban areas. This study provided preliminary insights into the variation patterns of secondary metabolites in mulberry leaves from different cultivars and origins in Chongqing, and offered a reference basis for germplasm evaluation, quality control, and high-value utilization of mulberry leaf resources.

KEYWORDS: *Morus alba*; mulberry leaves; flavonoids; phenolic acids; HPLC-DAD; geographical variation; quality evaluation

1 Introduction

Morus alba L. (Moraceae, *Morus* genus) is a perennial deciduous tree with a long cultivation history and extensive applications in China. As a traditional Chinese medicinal material, mulberry leaf has been used for thousands of years [1]. Widely distributed across the country, mulberry serves as a key economic tree species in the traditional sericulture industry and also represents a typical medicinal and edible homologous plant [2,3]. According to traditional Chinese medicine (TCM) theory, mulberry leaves are characterized as sweet, bitter, and cold in nature, and are associated with the lung and liver meridians. They exhibit traditional efficacies including dispelling wind and heat, clearing the lung and moistening dryness, and clearing the liver to improve eyesight, with a long-standing application record in traditional Chinese medicine [4,5]. Modern pharmacological studies have confirmed that mulberry leaves are rich in various

bioactive constituents such as flavonoids, phenolic acids, alkaloids, and polysaccharides. These components exert antioxidant, anti-inflammatory, hypoglycemic, hypolipidemic, and immunoregulatory activities, showing great development potential in health food and pharmaceutical products [6–8].

The therapeutic efficacy of mulberry leaves is closely related to the composition and content of their secondary metabolites, among which flavonoids and phenolic acids are the primary material basis and also serve as a crucial indicator group for evaluating the intrinsic quality of mulberry leaves [9–11]. Mulberroside A features high specificity and stable content, with significant anti-inflammatory, antioxidant, chondroprotective, and neuroprotective effects, making it a suitable marker component for quality assessment of mulberry leaves [12,13]. Chlorogenic acid, the most abundant phenolic acid in mulberry leaves, exhibits prominent antioxidant and anti-inflammatory activities, which can delay senescence, protect cell membranes, and inhibit oxidative damage, thus acting as a core index for quality evaluation [14,15]. Rutin, isoquercitrin, and astragalin are important flavonol glycosides in mulberry leaves. With similar structures and synergistic bioactivities, they possess multiple functions including free radical scavenging, vascular protection, glycolipid metabolism regulation, and microcirculation improvement, representing typical indicator components of mulberry flavonoids [16–19]. These five components are characterized by high contents, well-defined bioactivities, and strong representativeness, enabling assessment of the chemical quality differences among mulberry leaves.

The accumulation of secondary metabolites in medicinal plants is jointly affected by multiple environmental and genetic factors, including regional climate, soil conditions, altitude, and cultivated varieties, leading to significant geographical variation and cultivar specificity [20,21]. Previous studies have demonstrated that the contents of flavonoids and phenolic acids in mulberry leaves from different producing areas can differ by several times. Even the same cultivar grown under distinct ecological conditions shows obvious differences in the accumulation patterns of major secondary metabolites [22–24]. As a typical medicinal and edible plant, the chemical composition and content of mulberry leaves are also susceptible to growth environment, resulting in remarkable discrepancies in quality and efficacy among samples from different origins and varieties. Reliance on samples from a single producing area or cultivar is insufficient to reflect the genuine quality profile of mulberry leaves.

Chongqing is located in the subtropical humid monsoon climate zone of southwestern China, characterized by complex terrain, diverse climates, and abundant soil types, providing superior natural conditions for mulberry growth. It is an important sericultural production base and a rich region of mulberry germplasm resources in southwestern China [25]. Given the considerable influence of producing area on mulberry leaf quality, current studies on simultaneous quantitative determination of multiple components and systematic comparison of geographical and cultivar variations of mulberry leaves in Chongqing remain insufficient, which can hardly meet the demands for resource evaluation and high-value utilization. Therefore, it is of great practical significance to conduct component analysis of mulberry leaves from different producing areas and cultivars in Chongqing.

In this study, an HPLC method was established for the simultaneous determination of mulberroside A, chlorogenic acid, rutin, isoquercitrin, and astragalin in mulberry leaves. Furthermore, a comparison of the secondary metabolite contents among different cultivars and geographical origins was conducted, and the association patterns between these two factors and component accumulation were documented. This study aims to provide a scientific basis for germplasm evaluation, quality control and high-value utilization of mulberry resources in Chongqing.

2 Experimental

2.1 Materials and Reagents

Sixteen batches of mulberry leaves were collected during the mature stage in October from various districts and counties of Chongqing, China. The samples were authenticated by Professor Nong Zhou from the College of Biology and Food Engineering, Chongqing Three Gorges University, as fresh leaves of *Morus australis* Poir., *Morus cathayana* Hemsl., and *Morus alba* L. (Moraceae). Voucher specimens of *Morus alba* leaves were deposited at Room 223, Chongqing Engineering Research Center for Green Cultivation and Deep Processing of Genuine Medicinal Materials in the Three Gorges Reservoir Area, with detailed sample information and corresponding accession numbers listed in Table 1. The leaves were washed, dried to constant weight at 45°C, ground into powder, passed through an 80-mesh sieve, and stored in sealed containers at 4°C until analysis.

Reference substances of mulberroside A, chlorogenic acid, rutin, isoquercitrin, and astragalin were purchased from Chengdu Dest Biotechnology Co., Ltd., with batch numbers DST190408-068, DST180504-021, DST181221-156, DST180108-068, and DST190312-001, and purities of 98.6%, 95.6%, 98.5%, 98.4%, and 98.8%, respectively. Acetonitrile of chromatographic grade was obtained from Chengdu Nuoshike Technology Co., Ltd., and phosphoric acid of chromatographic grade was purchased from Chengdu Kelong Chemical Reagent Factory.

Table 1: Sample information of mulberry leaves collected from Chongqing.

No.	Variety	Variety Code	Origin	Origin Code		Regional Classification	Altitude (m)
S1	*M. alba*	S	Wanzhou District	WZ	S-WZ-001	outer suburban mountainous area	595.2
S2	*M. alba*	S	Yunyang County	YY	S-YY-001	outer suburban mountainous area	456.7
S3	*M. alba*	S	Fengjie County	FJ	S-FJ-001	outer suburban mountainous area	848.2
S4	*M. cathayana*	HS	Yunyang County	YY	HS-YY-001	outer suburban mountainous area	474.8
S5	*M. cathayana*	HS	Fengjie County	FJ	HS-FJ-001	outer suburban mountainous area	648.2
S6	*M. cathayana*	HS	Kaizhou District	KZ	HS-KZ–001	outer suburban hilly area	325.4
S7	*M. cathayana*	HS	Banan District	BN	HS-BN-001	Suburban hilly area	320.1
S8	*M. cathayana*	HS	Nan'an District	NA	HS-NA-001	urban area	154.2
S9	*M. cathayana*	HS	Kaizhou District	KZ	HS-KZ-002	outer suburban hilly area	321.3
S10	*M. australis*	JS	Kaizhou District	KZ	JS-KZ–001	outer suburban hilly area	347.1
S11	*M. australis*	JS	Yunyang County	YY	JS-YY-001	outer suburban mountainous area	415.3
S12	*M. australis*	JS	Wanzhou District	WZ	JS-WZ-001	outer suburban mountainous area	514.3
S13	*M. australis*	JS	Fengjie County	FJ	JS-FJ-001	outer suburban mountainous area	756.3
S14	*M. australis*	JS	Nan'an District	NA	JS-NA-001	urban area	141.8
S15	*M. australis*	JS	Banan District	BN	JS-BN-001	Suburban hilly area	357.6
S16	*M. australis*	JS	Kaizhou District	KZ	JS-KZ-002	outer suburban hilly area	304.7

Note: Regional classification is defined according to local topography and geographical location. Mountainous areas refer to low mountain terrain in remote suburbs, hilly areas represent gentle rolling land, and urban areas cover built-up urban zones.

2.2 Reference Standard Preparation

An appropriate amount of mulberroside A, chlorogenic acid, rutin, isoquercitrin and astragalin reference substances was accurately weighed and transferred into the same volumetric flask. The mixture was dissolved with 70% methanol to prepare a mixed reference solution with mass concentrations of 0.184, 1.342, 1.262, 0.914 and 0.791 mg/mL for the above components, respectively, and stored for further use.

2.3 Test Sample Preparation

An accurately weighed 0.500 g sample of mulberry leaf powder was placed in a stoppered conical flask, and precisely added 10 mL of 70% methanol solution. The total weight was recorded, and the mixture was sonicated for 30 min at a power of 240 W and frequency of 40 kHz. After cooling to room temperature, 70% methanol was added to compensate for the weight loss. The solution was shaken well and filtered through a 0.45 μm microporous membrane.

2.4 Chromatographic Conditions

A Shimadzu LC-20AT HPLC system (Shimadzu, Kyoto, Japan) equipped with an InertSustain C18 chromatographic column (250 mm × 4.6 mm, 5 μm) was employed for HPLC analysis. The separation was carried out using gradient elution with a mobile phase consisting of acetonitrile (A) and 0.1% phosphoric acid aqueous solution (B) at a flow rate of 1.0 mL/min. The gradient program was as follows: 0–10 min, 8% A; 10–30 min, 8% A → 35% A; 30–31 min, 35% A → 90% A; 31–36 min, 90% A; 36–37 min, 90% A → 8% A; 37–45 min, 8% A. The column temperature was set at 35°C, the detection wavelength at 350 nm, and the injection volume at 10 μL.

2.5 Methodological Validation

2.5.1 Specificity

An appropriate amount of mixed reference solution, test solution (Sample No. S2), and blank solution were accurately pipetted and injected for analysis. Each sample was tested in triplicate, and the chromatograms were recorded. The specificity of the method was evaluated by comparing the chromatograms of the mixed reference solution, test solution, and blank solution to check whether interfering peaks appeared at the retention times corresponding to each compound in the blank solution.

2.5.2 Linearity

A certain volume of mixed reference stock solution was accurately pipetted and serially diluted with 70% methanol to prepare a series of reference solutions at 5–6 different concentration gradients, ensuring that the concentration range covered the corresponding content in the test sample. All solutions were injected in triplicate, and the average peak area was recorded. Standard curves were constructed by the least-squares method with the concentration (x, μg/mL) as the abscissa and the peak area (y) as the ordinate. The correlation coefficients (R^2) were calculated to verify the linearity within the corresponding concentration range.

2.5.3 Precision

The same batch of mixed reference solution was injected continuously for 6 times, and the peak areas of each compound were recorded. The relative standard deviations (RSD) of the peak areas of each compound were calculated.

2.5.4 Stability

The same test solution (Sample No. S2) was stored protected from light at room temperature (25 ± 2°C) and analyzed at 0, 2, 4, 8, 12, 16, and 24 h. The peak areas of each compound were recorded, and the RSD was calculated to evaluate the stability.

2.5.5 Repeatability

To evaluate the repeatability of the method, six parallel samples (Sample No. S2, 0.500 g each) were accurately weighed, extracted, and analyzed by HPLC. Based on the standard regression equations, the contents of the five components were calculated, and the repeatability was assessed by the RSD of the six parallel results.

2.5.6 Recovery

Six parallel samples (0.500 g each, Sample No. S2) with known component contents were accurately weighed. Each sample was spiked with 10 mL of 70% methanol solution containing 5 reference standards at given concentrations, including mulberroside A (0.300 mg), chlorogenic acid (9.000 mg), rutin (0.700 mg), isoquercitrin (2.500 mg), and astragalin (1.500 mg). The spiked sample solutions were prepared following the test solution preparation procedure. The samples were then injected, and peak areas were measured to calculate the recoveries and RSD values.

2.5.7 Robustness

An accurately weighed 0.500 g of S2 sample was prepared into the test solution according to the established procedure. The determination was carried out under varying experimental conditions, including different HPLC systems (Shimadzu LC-20AT, Agilent 1260), C18 chromatographic columns (Zhongpu Red C18, Agilent Eclipse C18, InertSustain C18; all 250 mm × 4.6 mm, 5 μm), flow rates (0.8, 1.0, and 1.2 mL/min), and column temperatures (25, 30, and 35°C). All tests were performed in triplicate under each condition. The peak areas were recorded, and the RSD values of the content results under different conditions were calculated.

2.6 Analysis

HPLC-DAD data acquisition and processing were conducted on a Shimadzu Prominence LC-20AT system. Radar charts, box plots and column charts were generated using Origin 2024. Heatmap visualization and hierarchical cluster analysis were performed using TBtools V2.225. ANOVA was used for the comparison of different cultivars via SPSS Statistics 26.0, and $p < 0.05$ was regarded as statistically significant.

3 Results and Discussion

3.1 HPLC Method Validations

The specificity was evaluated by comparing the chromatograms of blank solvent, mixed reference solution, and test solution to assess selectivity, as shown in Fig. 1. All target analytes were effectively separated on the chromatographic column. The retention times of mulberroside A, chlorogenic acid, rutin, isoquercitrin, and astragalin were 20.75, 21.45, 27.90, 29.09, and 37.89 min, respectively. No interfering peaks were observed near the corresponding retention times of each target peak, indicating that the method possessed satisfactory specificity.

The results of methodological validation for the five active components are presented in Tables 2 and 3. All analytes showed good linearity within their corresponding concentration ranges, with $R^2 \geq 0.9998$. For precision and repeatability, the RSD values ranged from 1.01% to 1.89% and from 1.41% to 2.18%, respectively, demonstrating favorable precision and repeatability of the method. In the stability test, the standard solution remained stable after storage at room temperature for 24 h (RSD ≤ 2.56%). Meanwhile, the spiked recoveries of the five active components ranged from 94.3% to 98.5%, with RSD ≤ 1.84%, indicating that the method was accurate for the quantification of the five active components. Robustness results showed that the RSD values measured under different conditions were all less than 2.73%, suggesting that the method exhibited good adaptability for the quantitative analysis of multiple components.

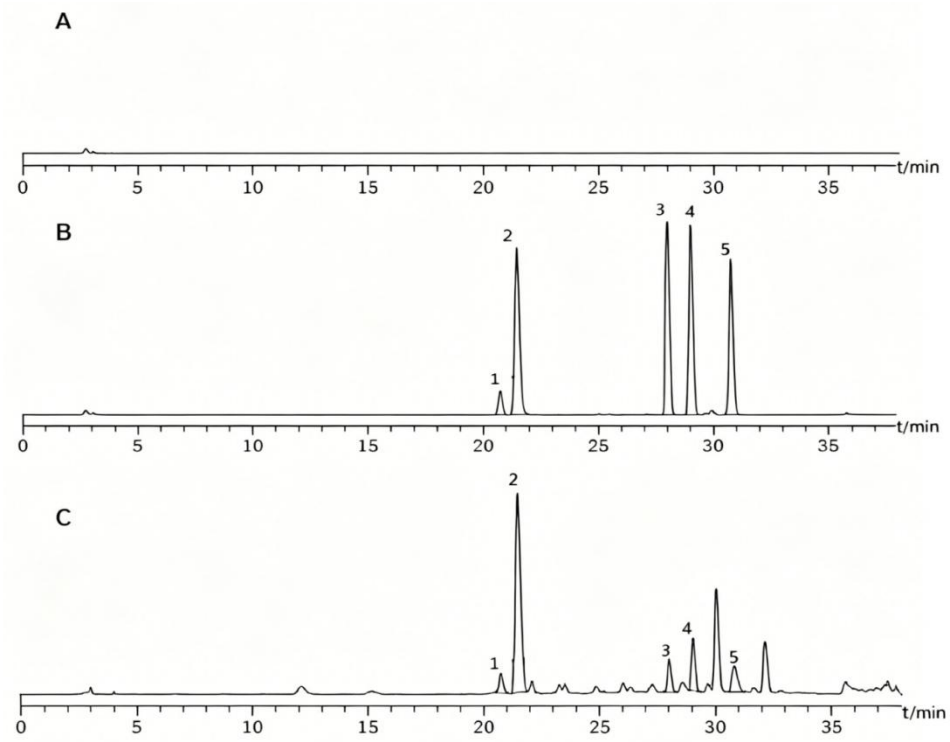

Figure 1: The result of Selectivity. (**A**) Methanol solution. (**B**) Mixed standard solution. (**C**) Sample solution. 1. Mulberroside A; 2. Chlorogenic acid; 3. Rutin; 4. Isoquercitrin; 5. Astragalin.

Table 2: Linearity, LOD and LOQ, precision, stability, repeatability and accuracy of the assay (n = 6).

Compounds	Linearity			Precision	Stability	Repeatability	Accuracy	
	Calibration Curve	Range (μg/mL)	R^2	RSD (%)	RSD (%)	RSD (%)	Recovery (%)	RSD (%)
Mulberroside A	y = 14,134.0x + 927.8	9.19~91.88	0.9999	1.54	1.42	1.41	94.3	1.35
Chlorogenic acid	y = 16,772.3x + 168,560	63.10~1262.08	0.9998	1.77	1.35	1.61	98.4	0.60
Rutin	y = 14,784.4x + 18,169.1	67.11~1342.22	0.9999	1.89	2.56	2.18	96.2	1.84
Isoquercitrin	y = 22,655.4x + 12,479.5	45.70~914.08	0.9999	1.01	1.71	1.75	98.5	0.87
Astragalin	y = 22,400.7x + 8378.9	35.56~791.14	0.9999	1.12	1.22	1.46	97.5	1.66

Table 3: Robustness investigation results (n = 3).

Compounds	RSD (%)			
	HPLC Instrument	Chromatographic Columns	Column Temperature	Flow Rate
Mulberroside A	1.32	2.17	1.02	2.01
Chlorogenic acid	1.42	1.81	2.73	1.85
Rutin	0.89	2.51	1.81	1.75
Isoquercitrin	0.93	1.73	1.70	1.21
Astragalin	1.75	1.27	2.50	0.86

3.2 Quantitative Determination of Five Secondary Metabolites in 16 Mulberry Leaf Samples

Based on the validated HPLC-DAD analytical method established above, the contents of mulberroside A, chlorogenic acid, rutin, isoquercitrin and astragalin in 16 batches of mulberry leaf samples collected from seven geographical regions and three cultivars in Chongqing were quantitatively determined, and the overall content distribution characteristics of the five target secondary metabolites were statistically analyzed. The quantitative results of all samples are summarized in Table 4.

As shown in the statistical results, the five detected components exhibited distinct content differences across all test samples. Among the five active substances, chlorogenic acid had the highest average content in mulberry leaves, reaching 5.137 ± 2.896 mg/g, accompanied by the largest standard deviation, which indicated that this component was greatly affected by cultivar and growth environment, and presented extremely significant inter-sample variation. Followed by isoquercitrin with an average content of 1.997 ± 1.204 mg/g, rutin and astragalin showed moderate average contents of 1.076 ± 0.965 mg/g and 1.044 ± 0.547 mg/g respectively. Mulberroside A had the lowest average content at 0.247 ± 0.183 mg/g, and its content fluctuation among samples was relatively mild compared with phenolic acids and flavonoids.

Combined with the data of single samples, extreme content differences were observed for individual components. For chlorogenic acid, the maximum content was 12.283 mg/g in sample S-WZ-001, while the minimum value was only 0.029 mg/g in sample HS-KZ-001, with a content gap of nearly 424 times. A similar large variation was found in rutin: the highest content was 3.758 mg/g in S-WZ-001, whereas rutin was not detected in HS-KZ-001. For isoquercitrin and astragalin, the highest contents were 4.737 mg/g and 2.176 mg/g (both in S-WZ-001), while the lowest contents were 0.286 mg/g and 0.194 mg/g (both in HS-KZ-001). Differently, mulberroside A could be detected in all 16 samples, with the highest content of 0.619 mg/g in HS-BN-001 and the lowest of 0.073 mg/g in JS-NA-001, and the overall content range was relatively narrow.

The heatmap (Fig. 2) visually reflected the accumulation level of five secondary metabolites in each sample. The color gradient intuitively presented the content difference of target components among different specimens: samples collected from Wanzhou District (S-WZ-001) showed the darkest color for chlorogenic acid, rutin, isoquercitrin and astragalin, demonstrating the highest accumulation of these four core active ingredients. Samples from urban areas represented by Nan'an District (HS-NA-001, JS-NA-001) displayed the lightest color for all five components, meaning their secondary metabolite contents were at the lowest level. In addition, partial samples from Kaizhou District, Banan District, Yunyang County and Fengjie County showed medium component contents, with uneven accumulation of different metabolites.

In general, the quantitative results preliminarily proved that the contents of flavonoids and phenolic acids in mulberry leaves from Chongqing were unevenly distributed. The huge content differences among samples suggested that various cultivars and origins were the key factors leading to the variation of

secondary metabolites in mulberry leaves. This study will further analyze compositional variations among different cultivars and producing areas, and explore the effects of such variations on mulberry leaf quality.

Table 4: Determination results of five active components in 16 batches of mulberry leaf samples.

Specimen Numbers	Mulberroside A	Chlorogenic Acid	Rutin	Isoquercitrin	Astragalin
S-WZ-001	0.082	12.283	3.758	4.737	2.176
S-YY-001	0.297	9.020	0.650	2.673	1.322
S-FJ-001	0.529	6.39	0.548	1.561	1.028
HS-YY-001	0.464	4.496	0.733	1.981	1.090
HS-FJ-001	0.159	4.352	0.800	1.987	1.080
HS-KZ-001	0.519	0.029	-	0.286	0.194
HS-BN-001	0.619	6.241	0.879	0.956	0.734
HS-NA-001	0.150	1.071	0.176	0.497	0.200
HS-KZ-002	0.099	6.69	1.686	2.486	1.245
JS-KZ-001	0.106	6.001	2.26	3.239	1.290
JS-YY-001	0.082	4.108	0.794	3.216	1.595
JS-WZ-001	0.360	5.152	0.547	1.942	1.192
JS-FJ-001	0.196	5.238	0.907	1.783	1.082
JS-NA-001	0.073	1.087	0.120	0.534	0.265
JS-BN-001	0.158	5.149	0.767	2.073	1.236
JS-KZ-002	0.080	5.692	2.297	3.289	1.647
Mean ± SD	0.247 ± 0.183	5.137 ± 2.896	1.076 ± 0.965	1.997 ± 1.204	1.044 ± 0.547

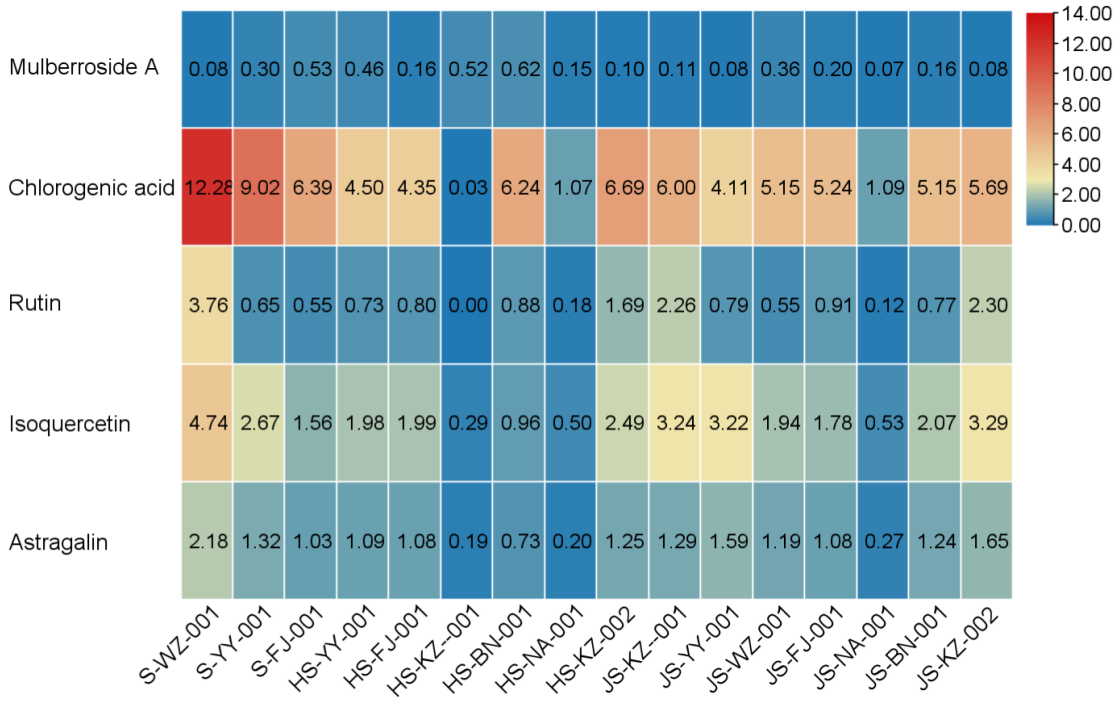

Figure 2: Heatmap of five secondary metabolites in 16 batches of mulberry leaf samples. Note: Origin code refers to Table 1.

3.3 Comparison among Different Mulberry Varieties

The contents of the five active components differed noticeably among three mulberry cultivars when comparing the mean values across all geographical origins (Figs. 3 and 4). Among them, the accumulation

characteristics of components in *Morus alba* L., *M. cathayana*, and *M. australis* showed obvious differentiation. As the official medicinal source of mulberry leaves specified in the Chinese Pharmacopoeia, *M. alba* showed a relatively higher overall accumulation level of active components, and the radar chart showed that its component profile was the outermost. The contents of four core components (chlorogenic acid, rutin, isoquercitrin and astragalin) in *M. alba* were markedly higher than those in *M. cathayana* and *M. australis*. Specifically, chlorogenic acid in *M. alba* was significantly higher than that in *M. cathayana* ($p < 0.05$). This is consistent with the conclusion clearly proposed by Jan et al. [24] that *M. alba* is the optimal variety for mulberry leaf medicinal use and has a higher level of active component accumulation than other related Morus species, and further confirmed the impact of varieties on component accumulation in combination with the study by Lee et al. [26]. According to Figs. 3 and 4, *M. australis* had higher contents of all five tested components than *M. cathayana*. In particular, the mean isoquercitrin content was 2.2966 mg/g for *M. australis* and 1.3655 mg/g for *M. cathayana*, showing a significant difference ($p < 0.05$). This indicates that *M. australis* had intermediate levels of active substances, while *M. cathayana* ranked the lowest among the three varieties. Notably, no obvious content variation of mulberroside A was observed across the three varieties, with average contents ranging from 0.15 to 0.34 mg/g. This range is consistent with the findings of Chan et al. [27], implying that varietal genetics exert a limited influence on the accumulation of this constituent.

In conclusion, the advantage of *M. alba* in core components provides experimental support for the selection of high-quality raw material varieties, and it is necessary to strictly distinguish varieties in actual production to avoid *M. cathayana* and *M. australis* affecting the quality of medicinal materials. It must be emphasized that the overall data is limited by unbalanced sampling. *M. alba* was only collected from three regions and no samples of *M. cathayana* and *M. australis* were obtained in Wanzhou District. Therefore, the overall mean can only reflect the general trend. Further expansion of sample size will be carried out to resolve this limitation.

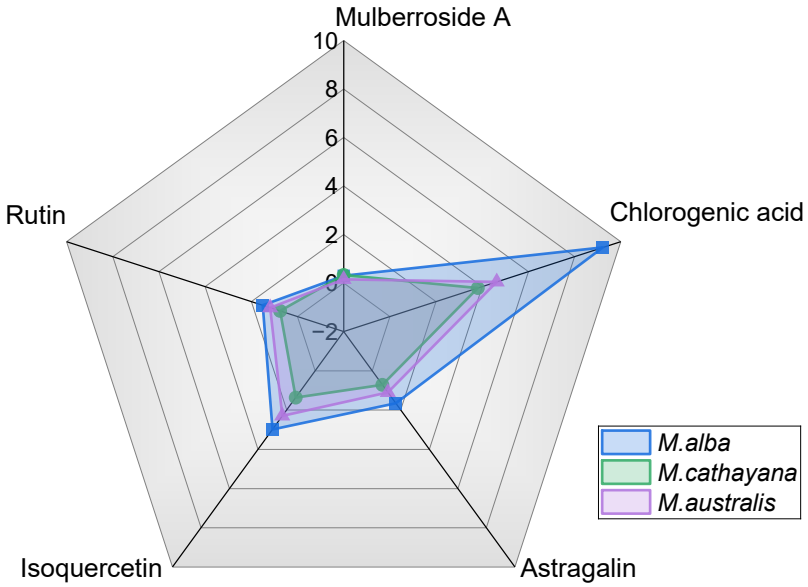

Figure 3: Radar chart of five active components in mulberry leaves from different varieties.

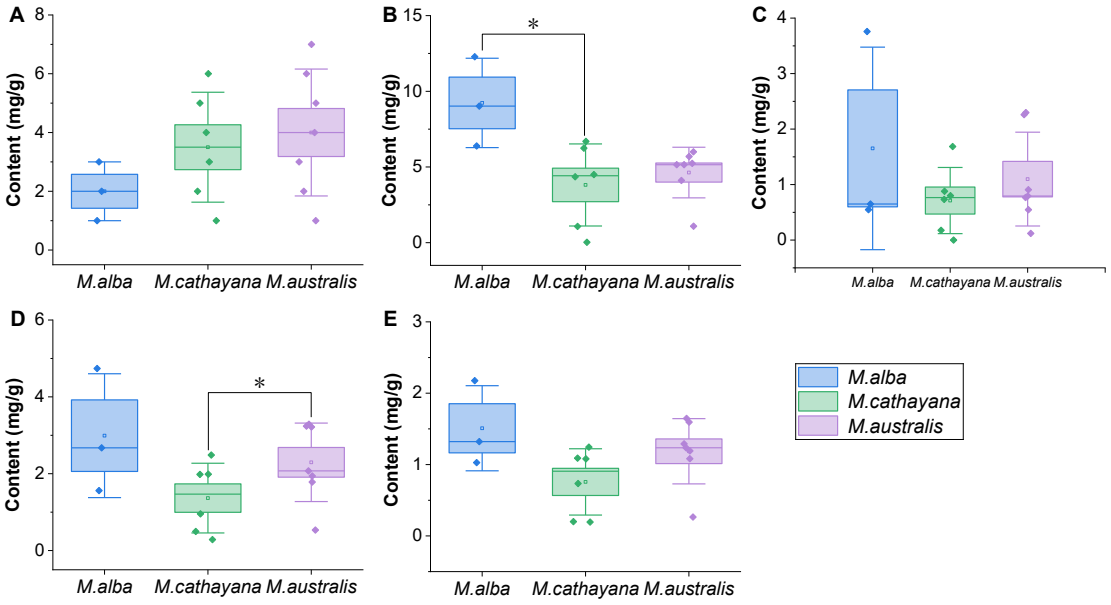

Figure 4: Box plots of five active components in mulberry leaves from different varieties. (**A**) Mulberroside A; (**B**) Chlorogenic acid; (**C**) Rutin; (**D**) Isoquercitrin; (**E**) Astragalin. Note: $^{*}p < 0.05$.

3.4 Comparison among Different Geographical Origins

In this study, the contents of five bioactive components, namely mulberroside A, chlorogenic acid, rutin, isoquercitrin and astragalin, in mulberry leaves collected from six producing areas in Chongqing, namely Wanzhou District, Yunyang County, Fengjie County, Kaizhou District, Banan District, and Nan'an District, were determined. The results are shown in Figs. 5 and 6.

Notably, mulberry leaves from Wanzhou District exhibited the highest contents of chlorogenic acid (8.72 mg/g), rutin (2.15 mg/g), isoquercitrin (3.34 mg/g) and astragalin (1.68 mg/g) among all sampling sites. The nearly 8-fold difference in chlorogenic acid content between Wanzhou District (8.72 mg/g) and Nan'an District (1.08 mg/g) is particularly noteworthy. While environmental parameters were not synchronously monitored, this divergence can be tentatively interpreted in light of the distinct geographical characteristics of these localities. Combined with the altitude data of sampling sites, the elevation of samples from Wanzhou, Yunyang and Fengjie (ranging from 415.3 m to 848.2 m) was significantly higher than that of urban areas such as Nan'an District (141.8–154.2 m). Generally, higher altitude is accompanied by stronger ultraviolet radiation, sufficient natural light and larger diurnal temperature differences, which are typical ecological factors conducive to activating the phenylpropanoid metabolic pathway in mulberry leaves and stimulating the biosynthesis and accumulation of phenolic acids and flavonoids. Wanzhou, situated in the northeastern mountainous region with relatively high elevation and open terrain, would presumably experience enhanced UV-B exposure and greater diurnal temperature amplitude—conditions known to upregulate phenylpropanoid metabolism and promote chlorogenic acid accumulation. In contrast, Nan'an lies within the core urban built-up area, where reduced sky openness, the heat island effect, and chronic air pollution may collectively suppress the metabolic capacity for phenolic acid synthesis. These interpretations remain speculative and await validation through synchronous environmental monitoring. These contrasting habitat conditions are consistent with previous reports that altitude, light intensity and ambient pollution status can substantially modulate the biosynthesis of phenolic acids and flavonoids in mulberry leaves [28]. Accordingly, the varying accumulation of target compounds observed in the

present study is tentatively hypothesized to be associated with divergent habitat conditions across sampling locations, though further field investigation with synchronous collection of site-specific environmental parameters will be required to validate such habitat-dependent variation in subsequent research.

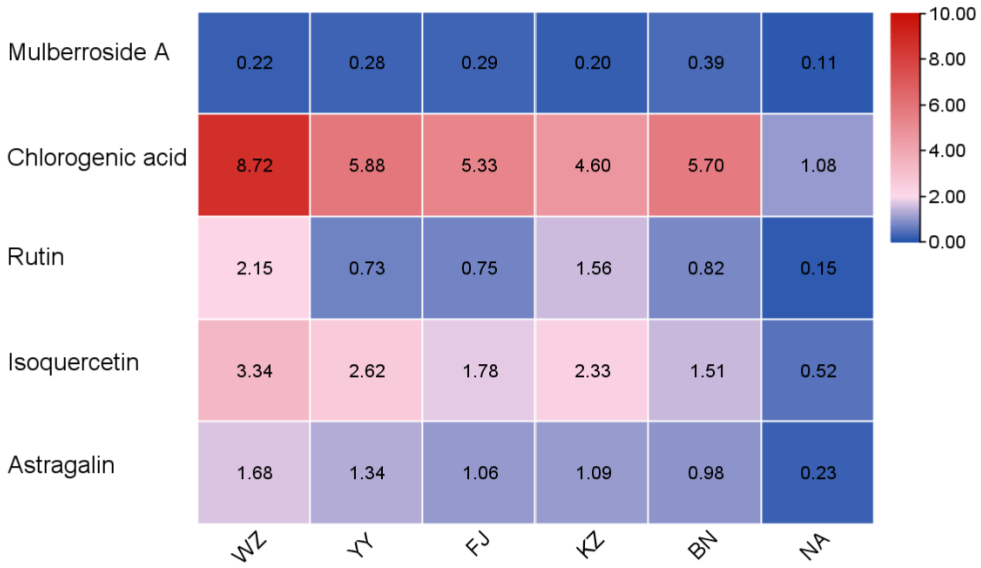

Figure 5: Heatmap of five active components in mulberry leaves from different regions. Note: Origin code refers to Table 1.

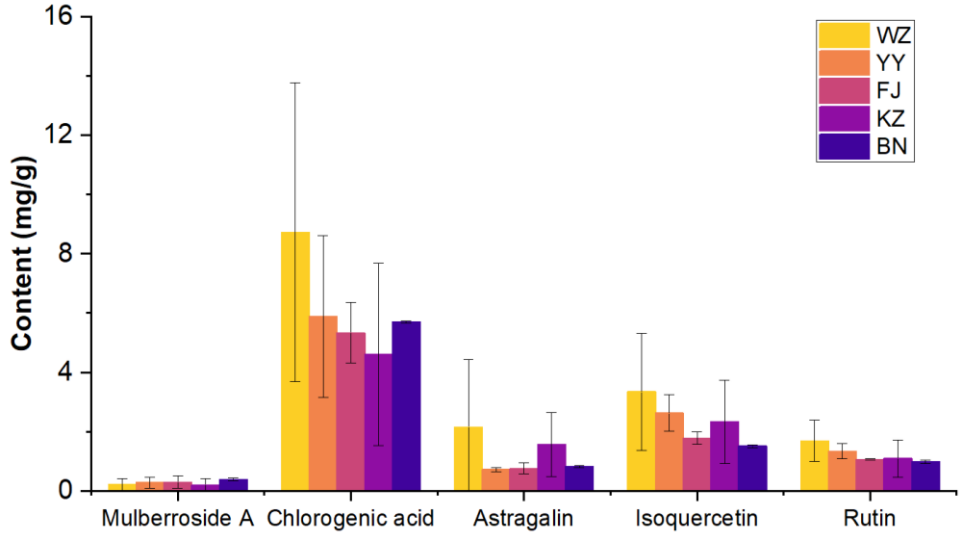

Figure 6: Comparison of contents of five active components in mulberry leaves from different producing areas. Note: Origin code refers to Table 1.

Samples from the hilly areas (Kaizhou District and Banan District) showed overall moderate component levels. The rutin content in Kaizhou District (1.56 mg/g) was second only to that in Wanzhou District, and the mulberroside A content in Banan District (0.39 mg/g) was the highest among all regions. Small-scale habitat differences can also significantly alter the levels of bioactive components in mulberry leaves. Although the ecological conditions of outer suburban hilly areas are inferior to those of deep mountainous

regions, they are generally better than urban areas, indicating their potential as sub-suitable production areas for mulberry leaves.

Mulberry leaves from urban producing areas (Nan'an District) displayed the lowest contents of all five components. Specifically, the contents of chlorogenic acid, rutin, isoquercitrin and astragalin in mulberry leaves from Nan'an District were only 1.08 mg/g, 0.15 mg/g, 0.52 mg/g and 0.23 mg/g, respectively, which were substantially lower than those from other regions. Insufficient illumination, the heat island effect and strong environmental stress in urban areas may potentially inhibit the synthesis and accumulation of secondary metabolites in mulberry leaves, making it difficult to meet the production requirements of high-value mulberry leaf raw materials [29,30].

Overall, the contents of the five bioactive components in mulberry leaves from different regions of Chongqing presented a general gradient of outer suburban mountains > outer suburban hills/suburban hills > urban areas. These findings provide preliminary evidence that mulberry leaf quality could be closely related to habitat type. For subsequent standardized cultivation and raw material selection, consideration may be given to prioritizing outer suburban mountainous areas with favorable ecological environments.

It is necessary to clarify the limitations of this part of the research. First, the sample sizes of Wanzhou, Banan and Nan'an Districts were small (n = 2), which restricted further inferential statistical analysis. Second, this study did not synchronously detect environmental indicators including temperature, light intensity and soil properties at each sampling site, so it is impossible to clarify the definite correlation between geographical environment and the variation of secondary metabolite contents. Third, the experimental design failed to realize the repeated layout of each mulberry cultivar in all producing areas, so the observed component differences could not completely separate the independent effects of cultivar and geographical origin. The above limitations should be fully considered when applying the research conclusions to actual production.

4 Conclusion

This study analyzed five main active components in mulberry leaves from different cultivars and producing areas. Overall, cultivar and growing location are closely linked to the levels of phenolic acids and flavonoids in mulberry leaves. *M. alba* had appreciably higher contents of chlorogenic acid, rutin, isoquercitrin, and astragalin than the other two species, while mulberroside A was less affected by cultivar. Spatially, samples from suburban mountainous areas and remote counties such as Wanzhou District, Yunyang and Fengjie County exhibited superior comprehensive quality. By contrast, samples collected from urban areas including Nan'an District had the lowest contents of all detected components, forming a clear spatial gradient. These findings suggest that cultivating *M. alba* in ecologically favorable suburban mountainous regions is an effective way to obtain high-quality mulberry leaf raw materials.

These findings provide a scientific reference for the standardized cultivation of mulberry leaves, the construction of high-quality raw material bases and improvement of quality standards, contributing to the establishment of a sustainable production system that balances yield and quality, and promoting the high-value utilization and industrial development of mulberry leaf resources in Chongqing.

In future work, we will supplement sufficient samples and complete environmental monitoring indicators. Long-term periodic sampling will also be performed to explore the dynamic changes of active components, so as to deepen the understanding of metabolite accumulation characteristics of mulberry leaves.

Acknowledgement: Not applicable.

Funding Statement: This research was supported in part by project of Natural Science Foundation of Chongqing (No. CSTB2024NSCQ-MSX4383), the Science and Technology Research Program of Chongqing Municipal Education Commission (No. KJQN202402727; No. KJQN202302721).

Author Contributions: The authors confirm contribution to the paper as follows: Panpan Wang: conceptualization, validation; Yanjing Liu: methodology, formal analysis, investigation; Haiyan Kong: formal analysis; Ye Li: writing—review and editing, visualization, funding acquisition; Shuang Chen: writing—original draft, supervision; Nong Zhou: project administration, resources, acquisition. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are available from the Corresponding Author, [Ye Li], upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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