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Study on Optimization of the Extraction Process of Total Flavonoids from Yao Medicine *Gnetum parvifolium* and Their Antioxidant Activity

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Abstract

Objective: To optimize the extraction protocol for total flavonoids from *Gnetum parvifolium*, determine their content, and investigate their *in vitro* antioxidant activity. **Methods:** Using rutin as a standard, key influencing factors were identified through single-factor experiments. An orthogonal experimental design was then employed to establish the optimal extraction protocol for total flavonoids from *Gnetum parvifolium*. The *in vitro* antioxidant activity was evaluated via a DPPH radical scavenging assay. **Results:** Scientific analysis of the experimental data indicated that the optimal conditions were ultrasound-assisted extraction with 90% ethanol as the solvent, a sample-to-solvent ratio of 1:60 (w/v), and a total of three extraction cycles. Under these conditions, the highest extraction yield of total flavonoids from *Gnetum parvifolium* was achieved, reaching 47.79 mg/mL. Moreover, the *Gnetum parvifolium* total flavonoid extract demonstrated significant radical scavenging activity, with a DPPH radical scavenging rate of 79% in the concentration range of 0.6 - 3.0 μg/mL. **Conclusion:** This study provides a scientific basis for the further extraction, utilization, and development of total flavonoids from *Gnetum parvifolium*.

Keywords

Gnetum parvifolium, Total Flavonoids, UV-Vis Spectrophotometry, DPPH Free Radical Scavenging Rate, Orthogonal Experiment

1. Introduction

Gnetum parvifolium, known as Mai Ma Teng, is a medicinal material consisting of the dried vine stems of the large-leaf and small-leaf species of *Gnetum* (Gnetaceae). These plants are widely distributed throughout southwestern China [1].

First documented in the Supplement to the Compendium of Materia Medica, it is also known by the alias Magufeng. As one of the "seventy-two winds", it is highly esteemed in Yao medicine [2] and is officially listed in the Quality Standards of Yao Medicinal Materials in Guangxi Zhuang Autonomous Region. With a medicinal history in China spanning over a millennium, the entire plant is considered to have therapeutic value [3]. Modern pharmacological studies indicate [4] that *Gnetum* possesses multiple therapeutic effects—including anti-rheumatic, blood-activating, stasis-resolving, anti-inflammatory, and analgesic properties—beneficial for treating rheumatic and hematological diseases. In folk medicine, it is commonly employed for conditions such as chronic bronchitis, rheumatic arthralgia, closed soft tissue injuries, and hyperuricemic arthritis. The literature indicates [5]-[7] that medicinal plants of the *Gnetum* genus are rich in stilbene derivatives, flavonoid glycosides, and alkaloids. These compounds exhibit significant biological activities, including anti-inflammatory, antibacterial, anti-tumor, anti-rheumatic, and cardioprotective/cerebroprotective effects [8]-[10]. To date, research on *Gnetum* as a traditional Chinese medicine has primarily centered on the analysis of its chemical constituents and the pharmacological activities of its main components, the stilbenes. Therefore, optimizing the extraction process is of great practical importance for the enhanced development and utilization of *Gnetum* as a medicinal resource. Accordingly, this study aims to optimize the extraction process of total flavonoids from *Gnetum* and evaluate their *in vitro* antioxidant activity, thereby providing an experimental and theoretical basis for future research.

2. Materials and Instruments

2.1. Materials

Dried stems of *Gnetum parvifolium* from Yulin, Guangxi, are sun-dried, pulverized to pass through a No. 3 sieve, and the powder is stored for later use.

2.2. Reagents

Rutin reference standard (batch No. RP220522, >99% purity; Chengdu Medson Technology Co., Ltd.); DPPH solution (batch No. 20240514; Feijing Biotechnology Co., Ltd.); ascorbic acid (analytical grade, batch No. 2024002; Tianjin Beichen Fangzheng Reagent Factory); aluminum nitrate (analytical grade, batch No. 4104-20-9; Tianjin Kemiou Chemical Reagent Co., Ltd.); sodium nitrite (analytical grade, batch No. 1401061; Xilong Chemical Co., Ltd.); and sodium hydroxide (analytical grade, batch No. 2112131; Xilong Chemical Co., Ltd.). All other reagents were of analytical grade, and deionized water was used throughout the experiments.

2.3. Instrument

TU-1950 double-beam UV-Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd.); KQ-800KDV ultrasonic cleaner (Kunshan Jielimei Ultrasonic Instrument Co., Ltd.); FA1104N electronic analytical balance (Shanghai Sunny

Hengping Scientific Instrument Co., Ltd.).

3. Determination of Total Flavonoid Content

3.1. Preparation of Standard Solution

Accurately weigh 0.1 g of rutin reference standard into a 50 mL clean and dry volumetric flask, add 70% ethanol solution to dissolve and dilute to the mark to obtain a 0.02% rutin reference standard solution.

3.2. Preparation of Sample Solution

Accurately weigh 0.2 g of *Gnetum parvifolium* fine powder, add 20 mL of 70% ethanol solution to a 50 mL conical flask and mix well, then sonicate at room temperature for 20 min, perform suction filtration with a Büchner funnel, and the filtrate is the sample solution.

3.3. Determination of Measurement Wavelength

Aliquots of the standard solution (1.0 mL; Section "3.1") and the sample solution (0.5 mL; Section "3.2") were accurately pipetted into separate 25 mL volumetric flasks. To each flask, 1 mL of 5% sodium nitrite solution was added, and the contents were mixed and allowed to stand for 5 min. Subsequently, 1 mL of 10% aluminum nitrate solution was added, followed by mixing and an additional 5-minute standing period. Next, 5 mL of 4% sodium hydroxide solution was added. The solution was then brought to volume with 70% ethanol, mixed thoroughly, and allowed to stand for 15 min. A reagent blank was prepared in the same manner, excluding the standard or sample solution. The absorption spectrum for each solution was then recorded from 200 to 600 nm against the reagent blank.

3.4 Method Validation

3.4.1. Linear Relationship Investigation

Aliquots of a 0.02% rutin standard solution (0.40, 0.60, 0.80, 1.00, and 1.20 mL) were individually transferred into five clean, dry 25 mL volumetric flasks. Color was developed using the aluminum nitrate colorimetric method, and the absorbance of each solution was measured with a spectrophotometer at the characteristic absorption wavelength of 345 nm. A standard curve was then constructed by plotting absorbance against rutin mass concentration [9].

3.4.2. Repeatability Investigation

In accordance with the guidelines of the Chinese Pharmacopoeia, six 0.2 g samples of *Gnetum parvifolium* fine powder were precisely weighed using an analytical balance (0.1 mg sensitivity). The samples were processed following the procedure described in Section 3.2. After color development via the aluminum nitrate colorimetric method, the absorbance of each solution was measured with a UV-visible spectrophotometer at a characteristic absorption wavelength of 345 nm. The total flavonoid content of each sample was then calculated, and the relative standard

deviation (RSD) was determined. The resulting RSD was 1.67%, which falls within the industry standard of ≤2.0% for good repeatability. This demonstrates that the experimental method possesses high repeatability and reliability [10]. These results are presented in Table 1.

Table 1. Results of repeatability experiments.

Number	Sample powder weight (g)	Absorbance A	Total flavonoid content (mg/g)	Average content (mg/g)	RSD (%)
1	0.2002	0.549	29.71		
2	0.2003	0.551	29.79		
3	0.2004	0.570	30.69	30.33	1.67
4	0.2002	0.568	30.62		
5	0.2001	0.566	30.54		
6	0.2002	0.568	30.62		

3.4.3. Precision Investigation

A 1.0 mL aliquot of the standard solution described in Section 3.1 was precisely pipetted into a 25 mL clean, dry volumetric flask. A color development procedure was then performed according to the aluminum nitrate colorimetric method. The absorbance of the resulting solution was measured at 345 nm. Six replicate measurements were performed, and the data were recorded. Statistical analysis of the results yielded a relative standard deviation (RSD) of 1.32%, indicating that the instrument possesses good precision and meets the requirements of the experiment. These results are presented in Table 2.

Table 2. Results of precision test.

Number	Absorbance A	Average absorbance A	RSD (%)
1	0.573		
2	0.575		
3	0.573	0.569	1.32
4	0.572		
5	0.563		
6	0.556		

3.4.4. Stability Study

An aliquot of 0.5 mL of the sample solution was accurately pipetted into a 25 mL volumetric flask for color development using the aluminum nitrate colorimetric method. The absorbance of the solution was measured and recorded at a wavelength of 345 nm with a spectrophotometer. Readings were taken at 0.5-hour intervals over a continuous 3-hour period. Analysis of the absorbance data yielded a relative standard deviation (RSD) of 1.48%, indicating that the sample solution remained stable for 3 hours and met the requirements for subsequent analysis. These results are presented in Table 3.

Table 3. Stability test results.

Determination time (min)	Absorbance A	Average absorbance A	RSD (%)
30	0.540		
60	0.530		
90	0.537	0.539	1.48
120	0.547		
150	0.550		
180	0.532		

3.4.5. Investigation of Spike Recovery

Six 0.1 g samples of fine *Gnetum parvifolium* powder were precisely weighed and transferred to 50 mL ground-mouth conical flasks. To each sample, 4.0 mg of rutin standard, dried to a constant weight, was added. Total flavonoids were extracted according to the procedure in Section "3.2", followed by color development using the aluminum nitrate colorimetric method. The absorbance of the resulting solution was measured at 345 nm with a UV-visible spectrophotometer. Statistical analysis determined the average sample recovery rate to be 98.07% with a relative standard deviation (RSD) of 1.85%. This result indicates that the method provides good accuracy and reliable measurements, as detailed in **Table 4**.

Table 4. Sample recovery test result.

Number	Medicinal material sampling amount (g)	Original Amount (mg)	Amount added (mg)	Measured amount (mg)	Recovery rate (%)	Average recovery rate (%)	RSD (%)
1	0.1003	4.0710	4.0000	8.0298	98.97%		
2	0.1004	4.0422	4.0000	7.9146	96.81%		
3	0.1004	4.1286	4.0000	7.9434	95.37%	98.07%	1.85%
4	0.1005	4.2294	4.0000	8.2313	100.05%		
5	0.1006	4.3733	4.0000	8.2745	97.53%		
6	0.1004	4.1862	4.0000	8.1737	99.69%		

3.5. Single-Factor Investigation

With ethanol as the extractant, the total flavonoid yield from *Gnetum parvifolium* was used as the evaluation index to compare three methods: room temperature cold maceration (24 h), water bath reflux (0.5 h), and ultrasonic-assisted extraction (0.5 h). Subsequently, a single-factor experimental design was conducted to sequentially analyze the influence of several parameters on the flavonoid yield. With baseline conditions set at a 70% ethanol concentration, 20 minutes of sonication, a 1:100 sample-to-solvent volume ratio, and a single extraction, the effects of the following factors were investigated: solvent concentration (30%, 50%, 70%, 90%), sonication time (20, 30, 40, 60 min), sample-to-solvent volume ratio (1:40, 1:60, 1:80, 1:100), and number of extractions (1, 2, or 3).

3.6. Orthogonal Experiment

Based on the results of the single-factor experiments, an L^9 (3^4) orthogonal experiment was designed to investigate the three main factors that significantly affect the total flavonoid yield from *Gnetum parvifolium* (extraction solvent concentration, sample-to-solvent volume ratio, and number of extractions) and their interactions. The factor levels for this experiment are presented in Table 5.

Table 5. Factor level table.

Level	A	B	C
	Ethanol concentration/%	Sample-solution volume ratio/mL	Number of extractions
1	50	60	1
2	70	80	2
3	90	100	3

3.7. DPPH *in Vitro* Antioxidant Activity Study

The experimental procedure was based on the method of Xu Xianghao *et al.* [11]. Aliquots (1 mL) of total flavonoid extracts from *Gnetum parvifolium* were prepared at various concentrations (0.60, 0.90, 1.20, 1.50, 2.00, and 3.00 μg/mL). Each aliquot was added to 3 mL of 0.200 mmol/L DPPH solution in a brown test tube. The mixtures were incubated for 0.5 h in the dark. Subsequently, absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Vitamin C (Vc) served as the positive control, and the DPPH free radical scavenging rate was calculated using Equation (1).

$$\text{DPPH radical scavenging rate} = \left[A_0 - \left(A_1 - A_2\right)\right] / A_0 \times 100\% \quad (1)$$

In the formula: A_0 is the absorbance value of 1.0 mL anhydrous ethanol + 3.0 mL DPPH solution; A_1 is the absorbance value of 1.0 mL flavonoid extract + 3.0 mL DPPH ethanol solution; A_2 is the absorbance value of 1.0 mL flavonoid extract + 3.0 mL anhydrous ethanol.

3.8. Data Analysis

All experiments were repeated 3 times, orthogonal experimental design and analysis were performed using software IBS SPSS Statistics 24.0, and data were processed using software Microsoft Excel.

4. Experimental Results

4.1. Wavelength Determination Results

Spectral scan analysis revealed that both the rutin standard solution and the sample solution exhibited a distinct absorption maximum at a wavelength of 345 nm (Figure 1). Given the significant absorption response of the target component at this wavelength, 345 nm was selected as the determination wavelength for this experiment to ensure effective detection.

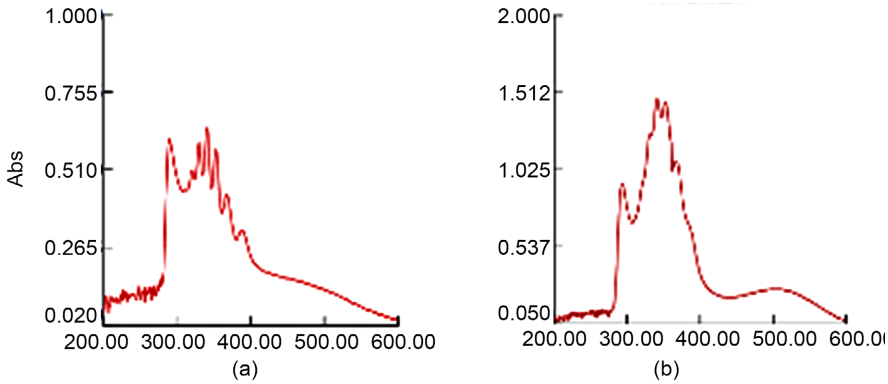

Figure 1. UV spectrum of aluminum nitrate colorimetric method. (a) Sample solution of *Gnetum parvifolium*; (b) Rutin control group solution.

4.2. Standard Curve Plotting

As shown in **Figure 2**, linear regression analysis of the rutin standard curve yielded the equation y = 0.1042x − 0.0708 and a correlation coefficient (R^2) of 0.9995. This value indicates a highly significant linear relationship between rutin mass concentration and absorbance within the 0.0032 - 0.0096 mg/mL range, which validates the standard curve as a reliable basis for quantification [12].

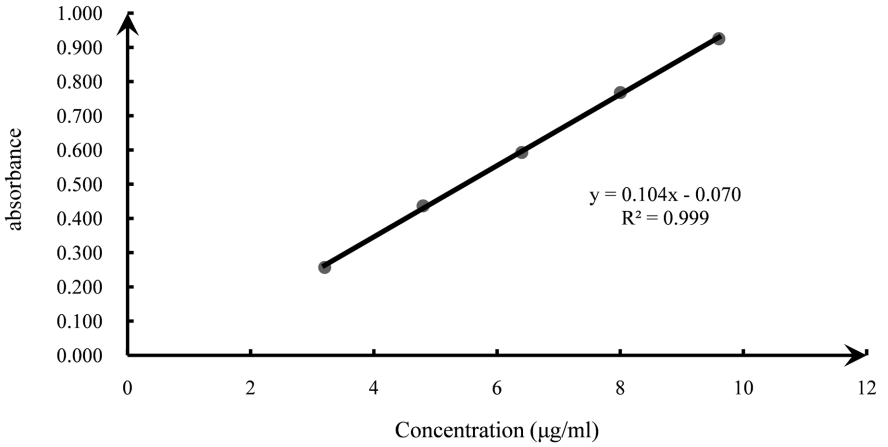

Figure 2. Standard curve of rutin.

4.3. Single-Factor Experiment Results

As shown in **Figures 3-7**, a systematic analysis of the single-factor variables indicated that the maximum yield of total flavonoids from *Gnetum parvifolium* was obtained using ultrasound-assisted extraction under the following conditions: a 90% ethanol solvent, a sample-to-solvent volume ratio of 1:100, sonication for 60 minutes, and three extraction cycles. However, prolonged sonication can damage the instrument, and the yield increase from 20 to 60 minutes was minimal. Therefore, to balance extraction efficiency and equipment preservation, a standard sonication time of 20 minutes was adopted for all subsequent experiments.

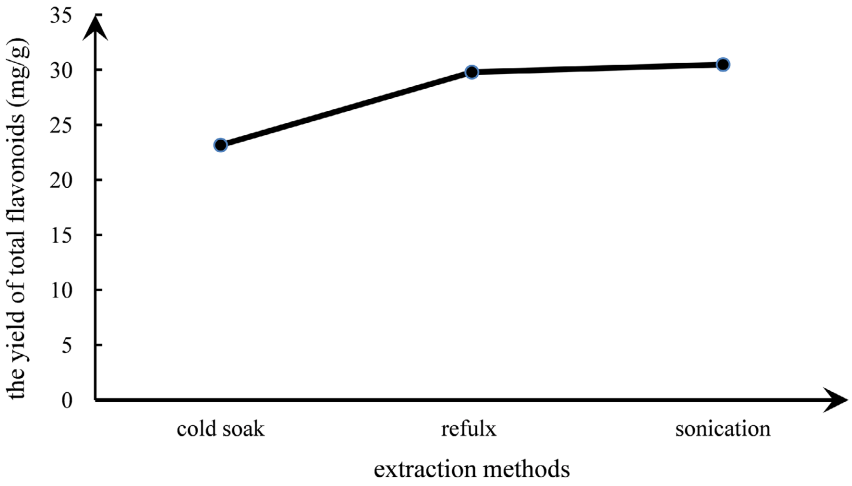

Figure 3. Effect of extraction methods on the yield of total flavonoids from *Gnetum parvifolium*.

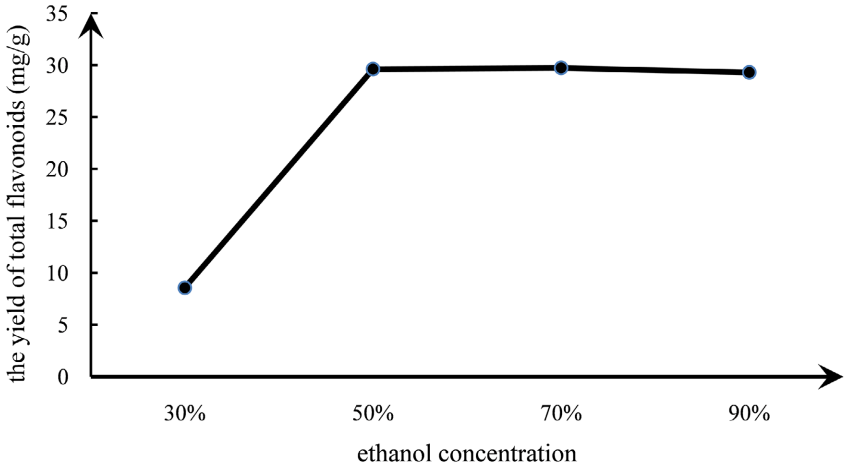

Figure 4. Effect of ethanol concentration on the yield of total flavonoids from *Gnetum parvifolium*.

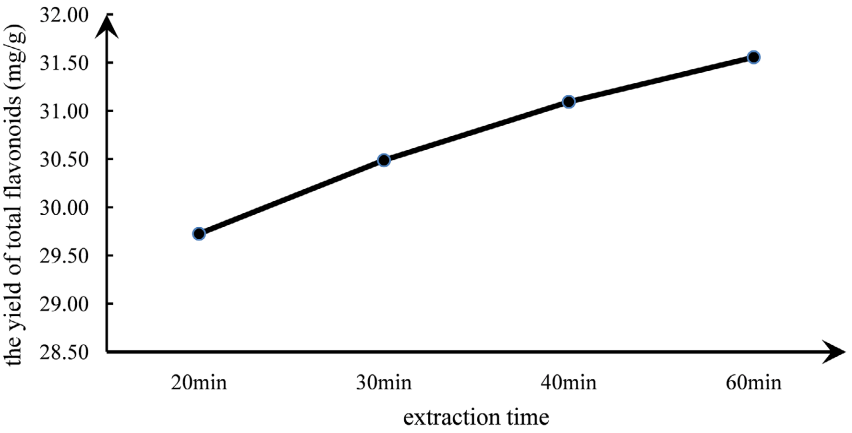

Figure 5. Effect of extraction time on the yield of total flavonoids from *Gnetum parvifolium*.

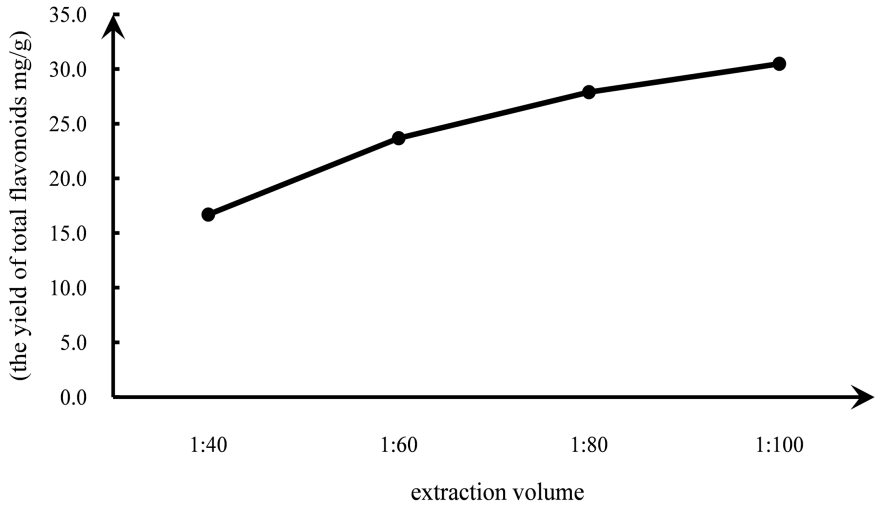

Figure 6. Effect of extraction volume on the yield of total flavonoids from *Gnetum parvifolium*.

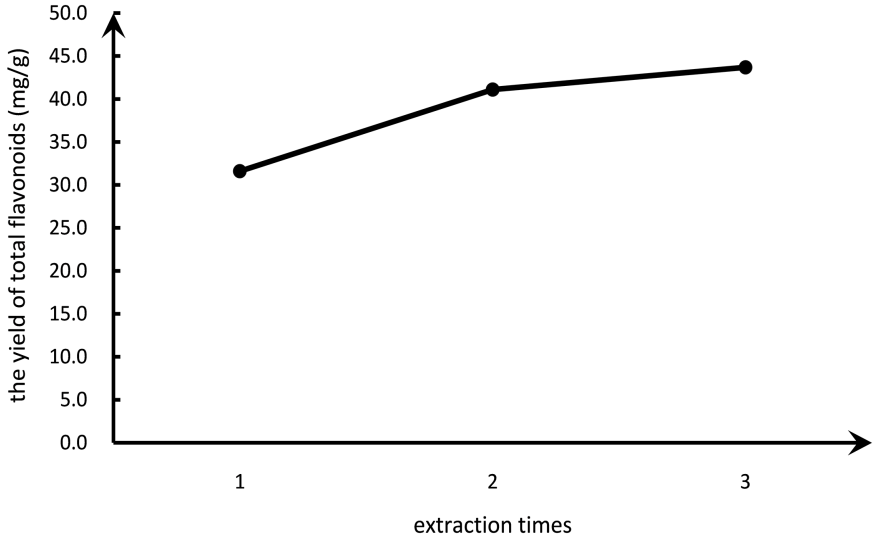

Figure 7. Effect of extraction times on the yield of total flavonoids from *Gnetum parvifolium*.

4.4. Orthogonal Experiment Results

A statistical analysis of the orthogonal test data in Table 6 was performed to determine the influence of three variables—solvent concentration (A), sample-to-solvent volume ratio (B), and number of extractions (C)—on the total flavonoid yield from *Gnetum parvifolium*. The analysis indicated that the order of factor influence was A > C > B. The optimal extraction protocol was identified as $A_3B_1C_3$, which corresponds to using a 90% ethanol solution, a 1:60 sample-to-solvent volume ratio, and performing three extraction cycles. This combination of process parameters effectively enhances the extraction efficiency of total flavonoids from *Gnetum parvifolium* (Table 7).

Table 6. Orthogonal test results.

Trial No.	A/%	B/(mL:g)	C/Count	Total flavonoid content (mg/g)
1	1	1	1	19.41
2	1	2	3	27.47
3	1	3	2	24.50
4	2	1	2	33.61
5	2	2	1	27.53
6	2	3	3	41.27
7	3	1	3	47.18
8	3	2	2	33.60
9	3	3	1	31.57
k1	23.79	33.40	26.17	
k2	34.13	29.53	30.57	
k3	37.45	32.44	38.64	
Range R	13.65	3.87	12.47	

Table 7. Variance analysis of orthogonal experiment.

Source of variance	Sum of squared deviations	Degrees of freedom	Mean square	F value	P-value
A	680.288	2	340.144	32.777	<0.05
B	88.819	2	44.409	4.279	>0.05
C	167.152	2	83.576	8.054	>0.05
Error	20.755	2	10.378		
Sum	957.014	8			

Note: $P < 0.05$ indicates a significant effect.

4.5. Verification Test

To ensure the accuracy, stability, and reproducibility of the results, validation experiments were conducted in triplicate using the optimal combination of conditions. As presented in **Table 8**, the average yield of total flavonoids from *Gnetum parvifolium* was 47.79 mg/g, which aligns with the predicted outcome. This confirms that the process identified through the orthogonal experiment is stable and viable, establishing $A_3B_1C_3$ as the optimal set of conditions for the extraction process.

Table 8. Verification test results.

ID	Sample powder weight (g)	Absorbance A	Total flavonoid content (mg/g)	Average content (mg/g)
1	0.2004	0.479	47.39	
2	0.2001	0.484	47.90	47.79
3	0.2004	0.487	48.08	

4.6. DPPH *in Vitro* Antioxidant Activity Assay Results

The DPPH free radical scavenging assay is a widely used method for the *in vitro*

evaluation of antioxidant capacity. A DPPH ethanol solution is purple due to the selective light absorption by its stable free radical structure, exhibiting peak absorbance at a wavelength of 517 nm. When an antioxidant reacts with DPPH free radicals via electron or hydrogen atom transfer, the solution's color fades, and its absorbance decreases. This reduction in absorbance allows for the quantitative assessment of antioxidant activity. The experimental data in **Figure 8** indicate that within the concentration range of 0.60 - 3.00 μg/mL, flavonoids from *Gnetum parvifolium* exhibit a scavenging effect on DPPH free radicals. Furthermore, this scavenging ability increases with the concentration of total flavonoids. However, their scavenging rate was significantly lower than that of ascorbic acid at the same mass concentration.

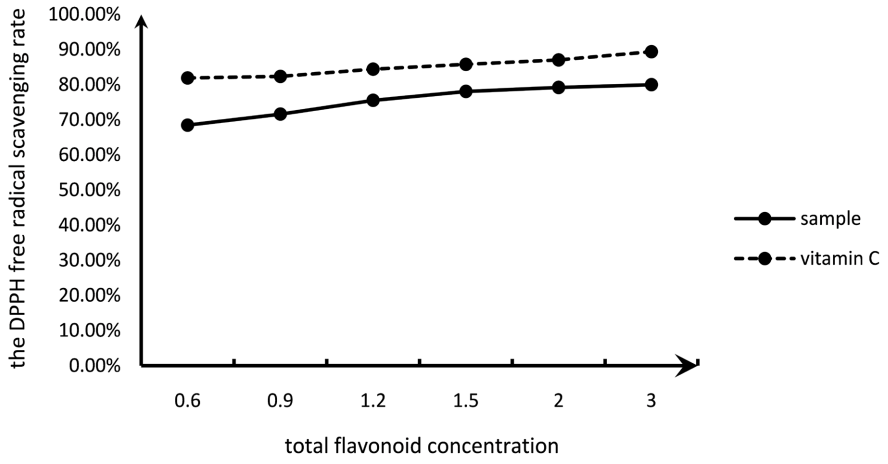

Figure 8. DPPH radical scavenging rate.

5. Discussion

Preliminary phytochemical screening of the medicinal herb *Gnetum parvifolium* revealed the presence of several types of chemical constituents, including flavonoids, alkaloids, saponins, phenols, and tannins. Widely distributed throughout the plant kingdom, flavonoids possess potent antioxidant properties and are a major focus of research in traditional Chinese medicine. Numerous pharmacological studies have demonstrated the multifaceted therapeutic potential of flavonoids [13]-[15], which are particularly effective in the prevention and treatment of cardiovascular diseases [16]. By improving vascular permeability, inhibiting platelet aggregation, and reducing cholesterol synthesis, they exert clear preventive and therapeutic effects against conditions such as hypertension, atherosclerosis, and angina pectoris. Furthermore, flavonoids demonstrate remarkable efficacy in various areas of physiological regulation. Their widely validated pharmacological activities include expectorant, antitussive [17], antiasthmatic, and antifungal [18] effects, as well as efficacy in the treatment of acute and chronic hepatitis and liver cirrhosis [19], indicating broad therapeutic prospects.

In this study, ultrasonic-assisted extraction was employed, which utilizes ultra-

sonic cavitation and mechanical effects to disrupt plant cell walls, thereby promoting solvent penetration and increasing the total flavonoid extraction rate [20]. Single-factor experiments, in conjunction with an orthogonal design method, were used to systematically investigate the effects of four key independent variables—extraction solvent concentration, sonication time, sample-to-solution volume ratio, and number of extractions—on the total flavonoid yield from *Gnetum parvifolium*. An L9 (3^4) orthogonal experiment was conducted to optimize the process parameters. Statistical analysis of the data indicated that the factors affected the extraction rate to varying degrees. The extraction solvent concentration had the most significant impact, followed by the number of extractions, whereas the influence of the sample-solution volume ratio was relatively weak. Based on analysis of variance (ANOVA) and subsequent verification experiments, the optimal conditions were established as follows: 90% ethanol as the extraction solvent, a sample-to-solvent volume ratio of 1:60, and three extraction cycles. As flavonoids are generally polar compounds, a higher concentration of ethanol more effectively disrupts the plant cell structure, facilitating their dissolution. Consequently, the total flavonoid extraction rate increased with rising ethanol concentration. A moderate increase in the sample-to-solvent volume ratio creates a favorable concentration gradient that promotes the diffusion of total flavonoids, whereas an excessively high ratio results in solvent waste. Although extending the sonication time can facilitate flavonoid dissolution, prolonged exposure may lead to their decomposition and oxidation, which in turn reduces the extraction rate and can damage the ultrasonic instrument. Therefore, this study focused on optimizing the number of extractions. Increasing the number of extraction cycles enables a more complete extraction of the total flavonoid content. The optimized number of cycles achieves a balance between extraction efficiency and component stability. Accordingly, this process provides a scientific basis and parameter reference for industrial production.

To investigate antioxidant activity, this study employed the DPPH *in vitro* antioxidant model, using the free radical scavenging rate as the key indicator to assess the capacity of total flavonoids from *Gnetum parvifolium*. Statistical analysis of the experimental data revealed that these total flavonoids exhibited significant free radical scavenging efficacy at various concentrations, confirming their potent antioxidant activity. It is further hypothesized that this activity is attributable to the abundance of phenolic hydroxyl groups within the flavonoid structure. These groups can quench free radicals via a hydrogen atom donation mechanism, which interrupts radical chain reactions and thus produces the antioxidant effect [21].

6. Conclusions

This study conducted an in-depth investigation into the total flavonoids from *Gnetum parvifolium*. By optimizing the ultrasound-assisted extraction process, an efficient system for extracting total flavonoids from *Gnetum parvifolium* was established. The aluminum nitrate colorimetric method was employed to deter-

mine the total flavonoid content, and its methodological validation confirmed high accuracy and precision. Using the extraction yield of total flavonoids from *Gnetum parvifolium* as the evaluation index, the extraction process parameters were optimized through a combination of single-factor experiments and an L9 (3^4) orthogonal design. The optimal extraction conditions for total flavonoids from *Gnetum parvifolium* were determined to be: 90% ethanol as the solvent, a sample-to-solvent volume ratio of 1:60, and three extraction cycles. Under these optimized parameters, the yield of total flavonoids reached 47.79 mg/g. The methodological investigation revealed a relative standard deviation (RSD) of less than 2%, demonstrating the excellent stability and repeatability of the process.

To investigate antioxidant activity, this study employed the DPPH *in vitro* antioxidant assay to establish an evaluation model for the *in vitro* antioxidant capacity of total flavonoids from *Gnetum parvifolium*. Statistical analysis of the experimental data indicated that these total flavonoids exhibit significant free-radical scavenging efficacy at various concentrations, confirming their potent antioxidant activity and providing a theoretical foundation for the development of natural antioxidants.

This research not only further elucidates the medicinal value of *Gnetum parvifolium* but also provides a solid scientific foundation for its comprehensive development and utilization. Future studies should focus on investigating the *in vivo* antioxidant mechanisms of total flavonoids from *Gnetum parvifolium*, while also exploring their practical applications in functional foods and pharmaceuticals to fully realize their potential economic and social value.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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