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Process optimization and antioxidant efficacy evaluation of total flavonoids from *Bougainvillea glabra* leaves: an integrated approach combining ultrasonic-assisted extraction with response surface methodology

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Abstract: This study systematically investigated the optimization of total flavonoid extraction from the leaves of *Bougainvillea glabra* Choisy (*B.glabra*) and evaluated its antioxidant potential. The impact of three critical parameters (ultrasound time, heating reflux time, and ethanol concentration) on extraction efficiency was investigated using ultrasonication-assisted heat reflux extraction. A three-factor, three-level Box-Behnken design (BBD) was implemented to optimize the extraction procedure, using total flavonoid yield (TFY) as the response variable. The extracted flavonoids' antioxidant potential was measured quantitatively in vitro. The ideal conditions were 53% ethanol concentration, 10 minutes of ultrasonication, and 41 minutes of reflux. This procedure produced a TFY of (6.21 ± 0.26)%. The extracts exhibited significant free radical scavenging action against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical. When the concentration exceeded 1.0 mg/ mL, the scavenging rate of DPPH free radicals surpassed 84.1%. When the concentration was more than 0.6 mg/ mL, the ability of total flavonoids to scavenge ABTS free radicals was over 93.8%, which was very similar to how well vitamin C worked. This optimized protocol proves effective for flavonoid extraction, with the obtained products exhibiting significant antioxidant properties in standardized assays.

Keywords: *Bougainvillea*; ultrasound-assisted extraction; total flavonoids; Box-Behnken response surface methodology; antioxidant activity.

1. Introduction

Bougainvillea glabra Choisy (*B.glabra*), a perennial flowering shrub of the *Nyctaginaceae* family, is commonly known as "leaf flower" or "paper flower." Cultivated in Fujian, China, for over two centuries, its leaves and petals have been traditionally used in medicinal diets for their heat-clearing and detoxifying

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properties. Modern phytochemical and pharmacological studies reveal that the leaves of *B.glabra* are rich in flavonoids and phenolic acids [1-3], exhibiting biological activities such as dispelling blood stasis, reducing swelling, nourishing skin, and promoting blood circulation [4]. Flavonoids from these leaves demonstrate notable efficacy in regulating glucose homeostasis and suppressing inflammation [5]. Flavonoids may also exert anticancer effects by remodeling gut microbiota [6], and preliminary evidence supports their potential role in mitigating Alzheimer's disease [7]. Collectively, these findings highlight a strong correlation between *B.glabra* leaf flavonoids and their traditional medicinal applications. Building on this foundation, this study contributes to the use of ultrasound-assisted heat reflux extraction to isolate total flavonoids from the leaves of *B.glabra*. A Box-Behnken response surface methodology was applied to analyze multi-factor interactions and establish optimal extraction conditions. For the first time, the antioxidant activity of the extracts was investigated using vitamin C as a positive control. This research not only enhances the extraction efficiency of *B.glabra* flavonoids but also provides a theoretical basis for developing high-value natural products. Furthermore, it expands the potential of *B.glabra* in food, nutraceutical, and pharmaceutical industries. The study bridges traditional medicinal knowledge with evidence-based health product development, offering both scientific significance and practical applications.

2. Materials and Methods

2.1. Plant Material

B.glabra were collected from the Chinese outstanding Botanical Garden (GPS: 26.082°N, 119.234°E) at Fujian Agriculture and Forestry University in September 2024. The botanical identity was authenticated by Prof. Xiaoxing Zou from the College of Forestry. *B.glabra* Specimen (No. 20240913) was stored at the Sample Room of the Fujian Province University Engineering Research Center for Conservation and Utilization of Natural Biological Resources at Fujian Agriculture and Forestry University.

2.2. Chemicals and Instruments

Rutin standard (purity ≥ 98%, Beijing Jinyin Biotechnology Co., Ltd.); Sodium nitrite solutions (5% w/v); aluminum nitrate solutions (10% w/v, Shenzhen Fulin Standard Titration Solution Co., Ltd.); 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical assay kits (Beijing Solarbio Science & Technology Co., Ltd.); Anhydrous ethanol,sodium hydroxide of analytical grade (Sinopharm Chemical Reagent Co., Ltd.)

Analytical balance (PX124ZH, OHAUS Corp.); UV-Vis spectrophotometer (UVmini-1240, Shimadzu Scientific); Thermostatic water bath (DK-98-ⅡA, Taisite Instruments); Forced-air drying oven (WGL-125B, Tianjin Test Instruments); Digital ultrasonic cleaner (KQ-500DE, Kunshan Ultrasonic Instruments); Circulating thermostatic bath (HX-1050, Beijing Biocare Experimental Equipment); Water-circulating vacuum pump (SHB-Ⅲ, Shanghai Yujie Instruments)

2.3. Sample Preparation

The leaves of *B.glabra* underwent initial purification with sterile distilled water. Subsequent to surface sanitization, the specimens were dehydrated in a forced-air drying oven at 60 ± 1 °C for 5 hours until a consistent weight was attained. The dehydrated material was equilibrated to ambient laboratory conditions (25 ± 2 °C) in a laminar flow cabinet. We used a high-speed grinder to reduce the particle size before sieving through a standard 100-mesh sieve. The resultant homogenized powder (particle size ≤ 150 μm) was hermetically stored in desiccators prior to extraction procedures.

2.4. Rutin Standard Curve Establishment

Studies have used rutin as a reference and the sodium nitrite-aluminum nitrate-sodium hydroxide colorimetric method to determine the total flavonoid content in plums, successfully validating that the rutin standard curve exhibits a good linear range, enabling flavonoid content determination. Similarly,

this study used rutin as a standard reference to construct the rutin standard curve [8]: A stock solution (0.8 mg/ mL) was prepared by dissolving 80.0 mg of rutin standard in anhydrous ethanol, followed by volumetric dilution to 100 mL with vigorous vortex mixing. Exact aliquots (0.625, 1.25, 1.875, 2.5, 3.125 mL) of the stock solution were dispensed into separate 25 mL volumetric flasks. 0.5 mL of 5% sodium nitrite and 0.5 mL of 10% aluminum nitrate solutions were added successively, followed by a 6-minute incubation after mixing. Following the addition of 5 mL of 1 mol/ L sodium hydroxide solution, each flask was filled to the designated mark with ethanol, thoroughly mixed, and permitted to stand for 15 minutes. Absorbance at 510 nm was measured in triplicate using a UV-Vis spectrophotometer, with average values recorded [9]. The standard curve was generated by plotting absorbance (y-axis) against rutin concentration (x-axis).

2.5. Fundamental Extraction Protocol

Exactly 1.000 g of *B.glabra* leaf powder was measured and placed into a round-bottom flask. An ethanol-water solvent system was introduced based on established parameters (extraction time, heating reflux time, and ethanol concentration). Subsequent to extraction, the filtrate was volumetrically adjusted to 30 mL. A 0.5 mL sample was transferred into a 25 mL volumetric flask, where chromogenic development was conducted following the rutin standard curve process. Triplicate absorbance measurements were acquired and averaged. The total flavonoid extraction yield was determined using the subsequent method [10]:

$$\text{Total flavonoid extraction rate (\%)} = \frac{V \times n \times C}{m \times 1000} \times 100\%$$

Here, V denotes the extract volume, n is the dilution factor, C is the concentration derived from the linear regression equation, and m is the powder mass.

2.6. Experimental Design

2.6.1. Single-Factor Optimization

The study investigated the impact of ultrasound time, heating reflux time, and ethanol concentration on the yield of total flavonoid from *B.glabra* leaves. The extraction yield served as the response variable to ascertain the ideal circumstances for each specific parameter. A rotational univariate approach was implemented:

Specifically, when probing ultrasound time, we varied it across 5 discrete levels (5, 10, 15, 20, 25 min) with heating reflux time fixed at 30 min and ethanol concentration at 60%.

Similarly, when analyzing heating reflux time, it was tested at 5 levels (10, 20, 30, 40, 50 min) under a fixed ultrasound time of 15 min and ethanol concentration of 60%.

Finally, for ethanol concentration, 5 discrete values (40%, 50%, 60%, 70%, 80%) were evaluated while maintaining ultrasound time at 15 min and heating reflux time at 30 min.

2.6.2. Box-Behnken Response Surface Methodology

The Box-Behnken design (BBD) allows for accurate mathematical modeling of interactions between multiple factors while reducing the number of experiments needed, leading to better predictions compared to traditional orthogonal arrays [11]. This approach is particularly applicable for optimizing complex botanical extraction systems. Building upon single-factor optimization results, a three-level BBD (Table 1) was implemented via Design-Expert 13.0 software. Ultrasound time (A), heating reflux time (B), and ethanol concentration (C) were selected as independent variables, with total flavonoid content (Y) as the response parameter.

Table 1. Factor level of response surface

Factorse		Level		
		-1	**0**	**1**
Ultrasound time (min)	A	5	10	15
Heating reflux time (min)	B	30	40	50
Ethanol concentration（%）	C	40	50	60

2.7. Antioxidant Activity Assessment

2.7.1. DPPH Radical Scavenging Assay

It took exactly 25 μL of flavonoid extract (0.2, 0.4, 0.6, 0.8, 1.0 mg/ mL) to be added to 2 mL microcentrifuge tubes that already had 950 μL of either DPPH radical solution (for the test group) or ethanol (for the control group). The mixtures were homogenized by vortexing and incubated in darkness at 25 ± 1 °C for 30 min. Absorbance at 515 nm was measured in triplicate using a UV-Vis spectrophotometer with ethanol as a blank reference. The average of three readings was recorded. We replaced the flavonoid solution with distilled water for the blank controls. Ascorbic acid (vitamin C) served as a positive control following identical procedures.

$$\text{DPPH radical scavenging rate } D_{VC}(\%) = \frac{A_{blank}\text{-}A_{control}}{A_{blank}} \times 100\%$$

$$\text{DPPH radical scavenging rate D}(\%) = \frac{A_{blank}\text{-}(A_{test}\text{-}A_{control})}{A_{blank}} \times 100\%$$

Here, A_{blank} is the absorbance of the blank controls, A_{test} is the absorbance of the test group and $A_{control}$ is the absorbance of the control group.

2.7.2. ABTS Radical Scavenging Assay

Just 50 μL of flavonoid extract (0.2, 0.4, 0.6, 0.8, 1.0 mg/ mL) was put into 2 mL microcentrifuge tubes that already had 950 μL of either ABTS radicals solution (for the test group) or ethanol (for the control group). The mixtures were homogenized by vortexing and incubated under light-protected conditions at 25 ± 1 °C for exactly 6 min. Absorbance at 405 nm was measured in triplicate using a UV-Vis spectrophotometer with ethanol as a blank. Triplicate readings were averaged for data analysis. Blank controls were prepared by replacing the flavonoid solution with distilled water. Vitamin C was used as a positive control to conduct the test according to the same method described above.

$$\text{ABTS radical scavenging rate } D_{VC}(\%) = \frac{A_{blank}\text{-}A_{control}}{A_{blank}} \times 100\%$$

$$\text{ABTS radical scavenging rate D}(\%) = \frac{A_{blank}\text{-}(A_{test}\text{-}A_{control})}{A_{blank}} \times 100\%$$

Here, A_{blank} is the absorbance of the blank controls, A_{test} is the absorbance of the test group and $A_{control}$ is the absorbance of the control group.

2.8. Statistical Analysis

Primary data curation was conducted utilizing Microsoft Office Excel 2021. A one-way analysis of variance (ANOVA) was conducted using the SPSS 27.0 statistical software. A three-factor, three-level experimental matrix was created using Design-Expert 13.0. All graphical representations were generated utilizing Origin 2021 software.

3. Results and Analysis

3.1. Rutin Calibration Curve

As depicted in Figure 1A), a linear dose-response relationship (0.02-0.12 mg/ mL) was established between rutin concentration and absorbance. The regression equation was determined as Y=8.464X+0.0821, demonstrating exceptional linear correlation (R^2 = 0.9995). This calibration model satisfied method validation criteria and was subsequently applied for accurate flavonoid quantification.

3.2. Single-Factor Optimization Outcomes

3.2.1. Impact of Ultrasound Time

Figure 1(B) shows that the total flavonoid output first increased and then gradually decreased as the sonication time got longer. The breakdown of the cell wall was mainly helped by stronger cavitation effects at the beginning of ultrasonication, which significantly improved how well the solvent could get in and transfer mass. The optimal extraction period enabled the total release of flavonoids from the plant matrix [12]. Due to differences in solubility within the extraction solvent, ultrasonic treatment can produce hydroxyl and peroxy radicals that degrade flavonoids and react with antioxidants in the extract, leading to variations in flavonoid flavonoid yield (TFY) [13]. Thus, we recognized precise temporal management as a vital factor for optimizing total flavonoid yield while maintaining phytochemical integrity.

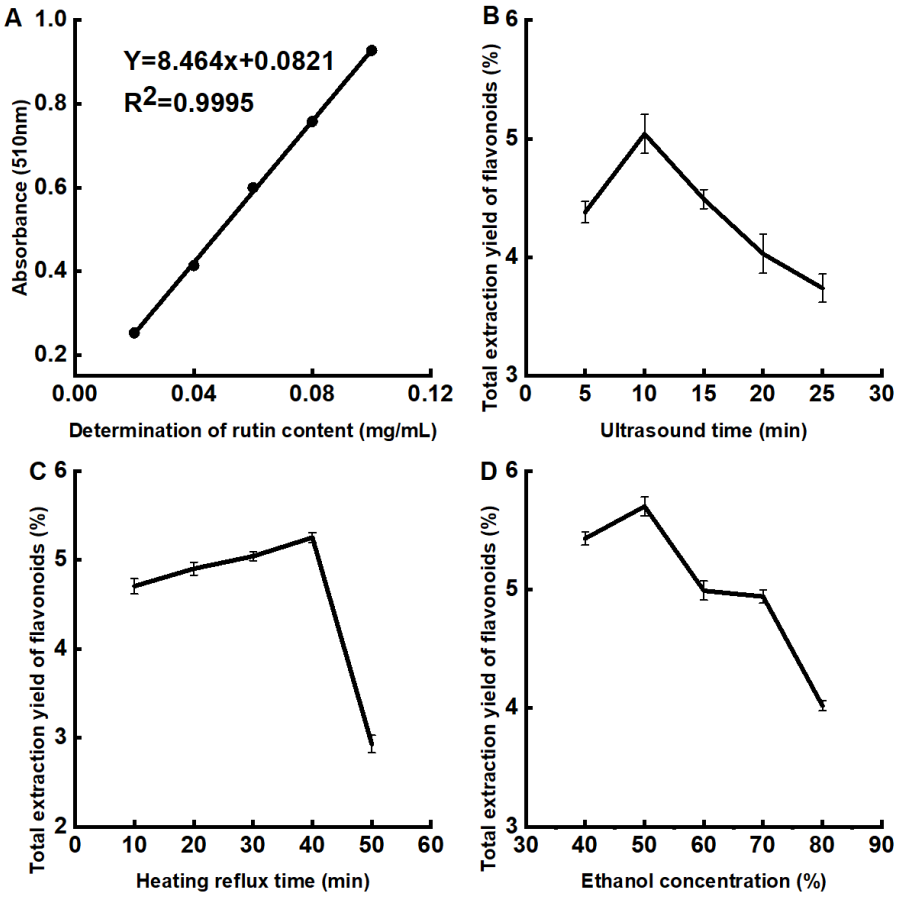

Figure 1. The result of standard curve (A), and the effect of ultrasound time (B), heating reflux time (C), ethanol concentration (D) on the TFY

3.2.2. Impact of Heating Reflux Time

As shown in Figure 1(C), the TFY initially increased and then decreased with prolonged heating reflux duration. This biphasic pattern can be attributed to the progressive dissolution of flavonoids during the initial extraction stages [14]. However, extended heating reflux time caused flavonoid degradation,

resulting in reduced extraction efficiency [15]. These results highlight the importance of selecting optimal reflux duration.

3.2.3. Impact of Ethanol Concentration

Figure 1(D) demonstrates a concentration-dependent biphasic response in TFY. At low ethanol concentrations, the high-polarity solvent preferentially extracted polar flavonoid glycosides. Conversely, elevated ethanol levels facilitated selective dissolution of non-polar aglycone derivatives [16]. Additionally, high ethanol concentrations can denature other proteins in the bracts, Preventing the dissolution of flavonoid compounds thus impacts the extraction rate [17]. At low ethanol concentrations, water causes the bract matrix to swell, increasing the contact surface area. However, the extraction efficiency is lower in a water-based solvent compared to organic solvents [18]. Maximal TFY was achieved at 50% ethanol, enabling simultaneous extraction of both glycosylated and aglycone flavonoid species. This phenomenon underscores the paramount importance of ethanol concentration modulation in flavonoid fractionation processes.

3.3. Experimental Design

3.3.1. Multiple Comparisons in Single-Factor Analysis

Multiple comparison processes help accurately identify differences between groups, making sure the results are statistically reliable for parametric optimization. Table 2 delineates the outcomes of Duncan's multiple range tests concerning ultrasound time, heating reflux time, and ethanol concentration in an organized format.

Table 1. The results of multiple comparisons of different parameter

Parameters	I (VAR)	J (VAR)	Mean difference (I-J)	Std. error	Sig.	95% confidence interval	
						Lower bound	Upper bound
Ultrasound time (min)	10	5	0.66	0.0559	< 0.001	0.5355	0.7845
		15	0.55	0.0559	< 0.001	0.4255	0.6745
		20	1.01	0.0559	< 0.001	0.8855	1.1345
		25	1.30333	0.0559	< 0.001	1.1788	1.4279
Heating reflux time (min)	40	10	0.54667	0.06092	< 0.001	0.4109	0.6824
		20	0.35	0.06092	< 0.001	0.2143	0.4857
		30	0.21	0.06092	0.006	0.0743	0.3457
		50	2.32	0.06092	< 0.001	2.1843	2.4557
Ethanol concentration (min)	50	40	0.27	0.05266	< 0.001	0.1527	0.3873
		60	0.71	0.05266	< 0.001	0.5927	0.8273
		70	0.76	0.05266	< 0.001	0.6427	0.8773
		80	1.68	0.05266	< 0.001	1.5627	1.7973

3.3.2. Response Surface Optimization Outcomes

We performed three experimental replicates for each run, using the arithmetic mean values of TFY as response parameters.

Table **2** neatly organizes all the data from the 17 experimental runs created using Box-Behnken response surface methodology.

Table 2. The Box-Behnken experimental design with three independent variables

Number	Ultrasound time （min）	Heating reflux time （min）	Ethanol concentration （%）	Total flavonoid yield （%）
1	5	30	50	3.98
2	15	30	50	4.66
3	5	50	50	4.52
4	15	50	50	4.28
5	5	40	40	4.08
6	15	40	40	4.27
7	5	40	60	4.83
8	15	40	60	5.08
9	10	30	40	4.45
10	10	50	40	4.82
11	10	30	60	4.92
12	10	50	60	5.32
13	10	40	50	5.96
14	10	40	50	6.05
15	10	40	50	6.22
16	10	40	50	6.27
17	10	40	50	6.13

3.3.3. Model Fitting and ANOVA

The influence of ultrasonication time (A), heating reflux time (B), and ethanol concentration (C) on the extraction yield (Y) of total flavonoids from the leaves of *B.glabra* was investigated and modeled utilizing Design-Expert 13.0 software, resulting in the following multiple regression equations:

$Y=6.13+0.1100A+0.1163B+0.3163C-0.2300AB+0.0150AC+0.0075BC-1.04A^2-0.7268B^2-0.5217C^2$

We used Fisher's test to confirm the model's significance, and

Table **3** provides the full ANOVA results for the quadratic polynomial model.

Table 3. ANOVA for response surface quadratic model analysis of variance table

Source	Sum of squares	Degree of freedom	Mean square	*F*-Value	*p*-Value
Model	9.97	9	1.11	50.71	< 0.0001
A	0.0968	1	0.0968	4.43	0.0733
B	0.1081	1	0.1081	4.95	0.0615
C	0.8001	1	0.8001	36.62	0.0005
AB	0.2116	1	0.2116	9.68	0.017
AC	0.0009	1	0.0009	0.0412	0.8449
BC	0.0002	1	0.0002	0.0103	0.922
A^2	4.55	1	4.55	208.13	< 0.0001
B^2	2.22	1	2.22	101.78	< 0.0001
C^2	1.15	1	1.15	52.46	0.0002
Residual	0.1529	7	0.0218		
Lack of fit	0.09	3	0.03	1.91	0.2697
Pure error	0.0629	4	0.0157		
Adequate precision	10.12	16			

Table 4 shows that the model was very effective (F=50.71, P < 0.001) and did not have a significant lack of fit (P=0.2697), which means it is good for predicting how flavonoids are extracted from *B.glabra* leaves. There were also significant effects from the AB interaction (P < 0.05), and strong significance for the linear term C and the squared terms A^2, B^2, and C^2 (P < 0.01). Substantial impacts were noted for the AB interaction (P < 0.05), as well as pronounced significance for the linear term C and the quadratic terms A^2, B^2, and C^2 (P < 0.01). The linear terms A, B, and the interaction terms AC and BC exhibited no statistical significance (P> 0.05). We established the influence hierarchy as follows: C (ethanol concentration) exceeds B (heating reflux time), which in turn exceeds A (ultrasound time).

3.3.4. Response Surface

The AB interaction (ultrasound-reflux time) in Figure 2 showed the sharpest slope and closest contour lines, indicating it had the strongest combined effect on the TFY. Maximum TFY was achieved at 10-min sonication and 40-min reflux. The AC interaction (ultrasound-ethanol concentration) showed some importance with a moderate increase, reaching its highest point at 10 minutes of ultrasound and 50% ethanol concentration. This hierarchical interaction potency (AB > AC > BC) aligns precisely with the regression coefficient significance hierarchy established above.

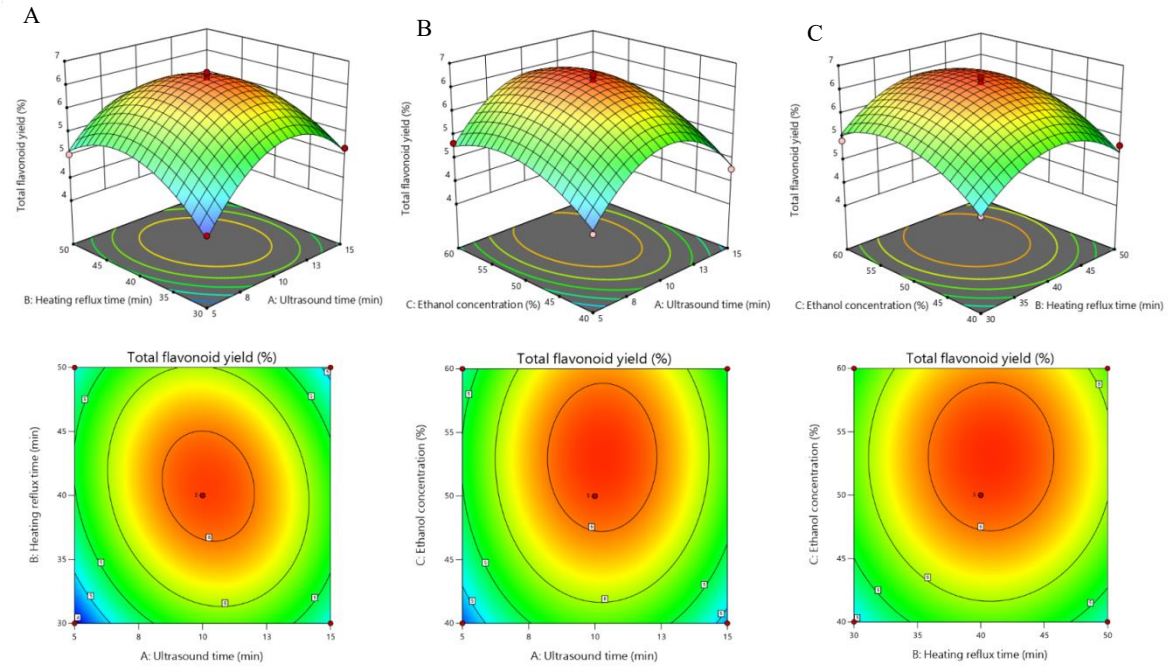

Figure 2. Response surface diagram (above) and contour diagram (below) of the influence of different factors on the TFY. (A) AB; (B) AC; (C) BC

3.3.5. Validation of Optimal Extraction Process

Table 5 illustrates that numerical optimization using Design-Expert 13.0 software produced theoretical best conditions: 10.23 minutes of ultrasound, 40.75 minutes of reflux, and an ethanol concentration of 53.1%, with a predicted TFY of 6.2%. To ensure practical feasibility, settings were modified to include 10 minutes of ultrasound, 41 minutes of reflux, and an ethanol concentration of 53%. Triplicate validation trials revealed TFY of (6.2 ± 0.3)%, affirming the accuracy of the model predictions.

Table 4. Predicted and experimental values of the responses at optimum conditions

Optimum condition			Total flavonoid yield	
Ultrasound time (min)	Heating reflux time (min)	Ethanol concentration (%)	Predicted (%)	Experimental (%)
10.23	40.75	53.05	6.18	(6.21 ± 0.26)

3.4. Antioxidant Activity of Total Flavonoids from B.glabra Leaves

3.4.1. DPPH Radical Scavenging Ability

Figure 3(A) illustrates the concentration-dependent DPPH radical scavenging activity of flavonoids extracted from *B.glabra* leaves. Figure 3(a) illustrates linear dose-response correlations for both experimental groups. The flavonoids in *B.glabra* leaves demonstrated more pronounced concentration-response gradients than the saturating scavenging ability of vitamin C controls. At a concentration of 1.0 mg/ mL, DPPH radical scavenging rates attained 84.1%, indicating efficacy comparable to that of vitamin C. These results show the strong, albeit somewhat poorer, DPPH radical scavenging activity of *B.glabra* flavonoids compared to vitamin C.

3.4.2. ABTS Radical Scavenging Ability

As the amount of flavonoids in *B.glabra* leaves went up, their ability to scavenge ABTS radical also increased, as shown in Figure 3(B). An elevation in flavonoid content shows a strong positive correlation with antioxidant efficacy. The reference vitamin C exhibited analogous linear concentration-response characteristics. Surpassing 0.6 mg/ mL, the scavenging rate stabilized at 93.8%, demonstrating effectiveness comparable to vitamin C controls. The results show that flavonoids in *B.glabra* leaves are very good at removing ABTS radicals, working as well as vitamin C when the concentration is 0.6 mg/ mL or higher.

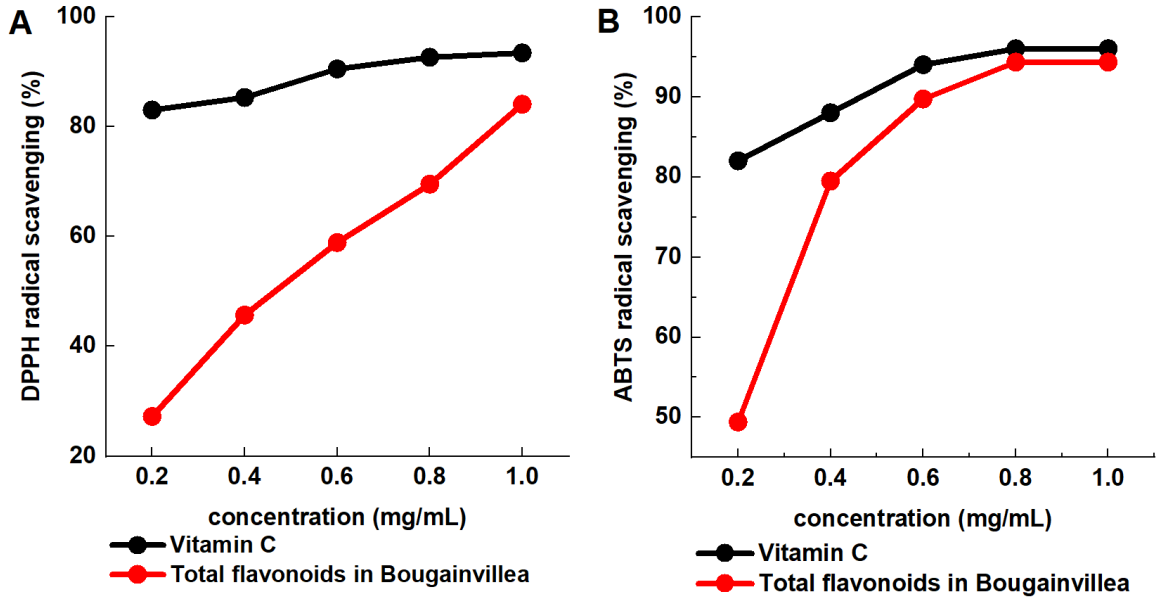

Figure 3. DPPH radical scavenging ability (A), ABTS radical scavenging ability(B) of total flavonoids in *B.glabra* leaves

4. Conclusion

This study presented ultrasound-assisted heat reflux extraction for isolating total flavonoids from *B.glabra* leaves, employing orthogonal experimental design to optimize the extraction process. The results demonstrated that the optimal conditions for extracting total flavonoids from *B.glabra* leaves were an ethanol concentration of 53%, an ultrasonic time of 10 minutes, a reflux time of 41 minutes, and a TFY of (6.21 ± 0.26)% under these parameters. The investigation into the antioxidant capabilities of total flavonoids in *B.glabra* leaves showed their efficacy in eliminating both DPPH and ABTS radicals. When the concentration exceeded 1.0 mg/ mL, the DPPH free radical scavenging rate exceeded 84.1%. At concentrations exceeding 0.6 mg/ mL, total flavonoids scavenged ABTS free radicals by over 93.8%, comparable to vitamin C, indicating potential medicinal use.

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