



OPEN **Ultrasound-assisted aqueous two-phase extraction of flavonoids from *erigeron breviscapus*: process optimization, structural characterization, antioxidant study, and DFT calculation**

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The well-known medicinal plant *Erigeron breviscapus* has long been used to treat cerebral embolism, cerebral thrombosis, and cerebral hemorrhage. Response surface methodology (RSM) was applied to optimize the ultrasonic-assisted extraction process of total flavonoids from *Erigeron breviscapus* (EBTF) using aqueous two-phase system. The flavonoids from *E. breviscapus* were qualitatively identified using UPLC-Q-TOF-MS/MS. The capacity of EBTF to scavenge $\cdot\text{OH}$ was used to assess its antioxidant activity. To determine the active sites in the primary bioactive components that scavenge $\cdot\text{OH}$, density functional theory (DFT) calculations were conducted. Total flavonoid content (TFC) from *E. breviscapus* was 48.53 mg/g under ideal conditions with PEG2000 mass fraction of 16%, $(\text{NH}_4)_2\text{SO}_4$ mass fraction of 14%, ultrasound time of 41 min, and liquid-solid ratio of 35 mL/g. 28 flavonoids have been tentatively identified in *E. breviscapus* via ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS). Furthermore, EBTF demonstrated moderate hydroxyl radical scavenging capacity, with scavenging rate of 60.68% at 3.9 mg/mL. The 6-OH site of scutellarin was the core active site for scavenging hydroxyl radicals. The findings provide both theoretical and experimental support for the in-depth development of EBTF as a natural antioxidant.

Keywords *Erigeron breviscapus*, Flavonoids, Ultrasonic, Response surface methodology, Density functional theory

Erigeron breviscapus (*E. Breviscapus*) is a perennial herb that belongs to genus *Erigeron* in the Asteraceae family. The whole herb is used medicinally as Dengzhanxixin. It possesses the effects of promoting blood circulation, relieving pain, dispelling wind, and dispelling cold, which can be used for the treatment of stroke-induced hemiplegia, chest pain due to angina pectoris, rheumatic arthralgia, headache, and toothache. *E. Breviscapus* is currently mentioned in the 2025 edition of the Pharmacopoeia of the People's Republic of China. Dengzhanxixin injection has been traditionally used in China for the clinical treatment of cerebral hemorrhage, cerebral thrombosis, and cerebral embolism¹. The existing research has found flavonoids, volatile oils, coumarins, and other compounds in *E. breviscapus*, of which flavonoids are the primary chemical constituents². Li Y et al. systematically established a method for ultrasonic-assisted extraction and macroporous resin enrichment of flavonoids from *E. breviscapus*. The key parameters optimized for ultrasonic-assisted extraction of total flavonoids were a liquid-solid ratio of 38:1 mL/g, 55% ethanol, 90 min, and 52 °C. Among six macroporous resins, AB-8 resin was identified as the optimal enrichment material. Its optimal dynamic adsorption/desorption parameters were further determined as a sample volume of 5.5 bed volume, a concentration of 1.14 mg/mL, and 70% ethanol as the eluent. The total flavonoid concentration after enrichment reached 5.16 times that of the crude extract³. Furthermore, it has been revealed that *E. breviscapus* total flavonoids (EBTF) have anti-pseudorabies virus efficacy⁴, estrogenic and neuroprotective properties⁵, and an inhibitory action on GABA shunt enzymes⁶. Notably, scutellarin was reported to be the primary flavonoid component of *E. breviscapus*², which could be useful

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in the treatment of Alzheimer's disease, cancer, diabetic vascular complications, *Helicobacter pylori* infection, and various other illnesses^{7,8}. Taken together, it is obvious that EBTF has substantial research value.

The aqueous two-phase system (ATPS) is a common technique for liquid-liquid extraction, which occurs when the two different polymers or one polymer fuse with salts in an aqueous solution. ATPS extraction is a combination of extraction and separation. Due to its simplicity, high extraction efficiency, and environmental friendliness, it is widely used in the separation and purification of natural products⁹. Using the high-pressure effects produced by ultrasonic waves, ultrasound-assisted extraction (UAE) breaks down an organism's cell walls to release intracellular components. Simultaneously, its high-frequency vibrations speed up the target chemicals' release, diffusion, and dissolution. This method works in concert with an ATPS to produce a synergistic effect that allows for simultaneous purification while also greatly reducing the extraction time and improving the effectiveness of natural product active chemicals. Research on the extraction of EBTF using an ultrasonic-assisted ATPS remains largely unexplored. Therefore, this study employed an response surface methodology (RSM) to optimize the ultrasonic-assisted extraction process of EBTF using a PEG2000-(NH₄)₂SO₄ dual-water-phase system. Concurrently, the flavonoids from *E. breviscapus* were qualitatively identified using UPLC-Q-TOF-MS/MS. Additionally, the antioxidant activity of EBTF was evaluated by measuring its hydroxyl radical scavenging capacity. Lastly, DFT calculations were carried out to identify the active sites in its primary bioactive components responsible for hydroxyl radical scavenging. This study provided theoretical support for the green extraction of EBTF and the subsequent development and utilization of plant resources.

Materials and methods

Materials and Chemicals

The aerial part of *E. breviscapus* was collected in August 2024 from Luxi County, Kunming City, Yunnan Province, P.R. China (geographic coordinates: 25°32'N, 103°45'E). *E. breviscapus* is not listed as an endangered or protected species under these frameworks, and the collection process did not cause damage to the local ecological environment. All collection activities strictly complied with relevant regulations, including the Regulations on the Protection of Wild Plant Resources of the People's Republic of China, the IUCN Policy Statement on Research Involving Species at Risk of Extinction, and the provisions of the Convention on the Trade in Endangered Species of Wild Fauna and Flora (CITES). The collected plant specimens were morphologically identified by Prof. Yanling Li (Department of Chemistry of Traditional Chinese Medicine and Natural Medicines, North Henan Medical University). A voucher specimen has been deposited in the North Henan Medical University under the collection number YN-8123.

Ascorbic acid was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Rutin was purchased from the National Institutes for Food and Drug Control. Salicylic acid, ferrous sulfate (FeSO₄), 30% hydrogen peroxide (H₂O₂), sodium hydroxide (NaOH), sodium nitrite (NaNO₂), aluminium nitrate (Al(NO₃)₃), and ethanol were purchased from Tianjin Kermel Chemical Reagent Co. All other reagents used were of analytical grade.

Methods

Ultrasound-assisted extraction

The materials were ground up, put through a 60-mesh sieve, and kept at 4 °C after being dried at 60 °C. After adding 40 mL of the two-phase aqueous system to a container containing 1.0 g of the sample, the container was sealed and put in an ultrasonic processor (KQ-300DE, Kun Shan Ultrasonic Instruments Co., Ltd, Jiangsu, China) with ultrasound power of 200 W, ultrasound temperature of 30 °C, and ultrasound time of 30 min. Following cooling, the extraction solution was centrifuged for 30 min at room temperature at 4,000 rpm, and the filtration was then transferred to a stoppered measuring cylinder. After stratification, the filtration was placed in a stoppered measuring cylinder and given some time to settle before the volumes of the upper and lower phases were measured.

Determination of the total flavonoids

Utilizing the NaNO₂-Al(NO₃)₃-NaOH colorimetric method (a technique adapted from an earlier study¹⁰), the total flavonoid content (TFC) was ascertained. After adding 100 µL of the upper phase solution to a 10 mL flask, 0.4 mL of 5% NaNO₂ was added, and the flask was thoroughly shaken. After six minutes, 0.4 mL of 10% Al(NO₃)₃ was added, and the mixture was well mixed. Four milliliters of 4% NaOH were added after six minutes. After adding water to a final amount of 10 mL, the mixture was evenly shaken and allowed to stand for 15 min. Then, the absorbance was determined by UV spectrophotometry at 510 nm. The standard curve was computed using rutin reference solutions at various concentrations (0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, and 0.07 mg/mL). With a linear regression equation of $y = 13.863x + 0.0336$ and $r = 0.9992$, the results showed a positive linear connection between rutin concentration and absorbance over the investigated concentration range. The total flavonoid concentrations were calculated using the standard curve formula previously mentioned. The findings were displayed as mg rutin equivalents per g of dry weight of *E. breviscapus* (mg/g).

Single-factor experiment

One factor was examined, but all other parameters remained the same. The following factors and their levels were observed: PEG molecular weight (600, 1000, 2000, 3500, and 4000), PEG2000 quality fraction (10%, 12%, 14%, 16%, and 18%), (NH₄)₂SO₄ mass fraction (12%, 13%, 14%, 15%, and 16%), ultrasound time (10, 20, 30, 40, and 50 min), and liquid-solid ratio (20, 30, 40, 50 and 60 mL/g).

Response surface experimental design

Based on a one-way test, using a four-factor, three-level test with PEG2000 mass fraction, ammonium sulphate mass fraction, liquid-solid ratio, and ultrasound time as the investigating factors and total flavonoid content as the response value, the Box-Behnken response surface methodology was employed to determine the optimal extraction process for EBTF. The experimental factors and levels are shown in Table 1.

UPLC-Q-TOF-MS/MS analysis

Sample preparation

Under optimal extraction conditions, the top-phase sample solution in the extract was enriched for flavonoids in *E. breviscapus* using macroporous adsorption resin chromatography. *E. breviscapus*'s enriched flavonoid solution was vacuum evaporated to dryness at 60 °C. The obtained residues were dissolved in 10 mL of methanol, filtered through a 0.22 µm filter, and the filtrates were analyzed by UPLC-Q-TOF-MS/MS.

UPLC-Q-TOF-MS/MS condition

The UPLC analysis was performed on a Dionex UltiMate 3000 UHPLC-Standard (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Chromatographic separation was also performed on an ACQUITY UPLC HSS T3 column (2.1 mm×100 mm, 1.8 µm) (Waters Co., Milford, Massachusetts, USA). Acetonitrile (A) and water with 0.1% formic acid (B) in positive ion mode made up the binary gradient elution system. Acetonitrile (A) and water containing 2 mM ammonium acetate (B) made up the binary gradient elution system in negative ion mode. The gradient elution condition was as follows: 0–1.5 min, 5% A; 1.5–2.5 min, 5–10% A; 2.5–14 min, 10–40% A; 14–22 min, 40–95% A; 22–25 min, 95% A; 25–26 min, 95–5% A; 26–30 min, 5% A. The volume of injection was 3 µL. The flow rate and column temperature were 400 µL/min and 40 °C, respectively.

An AB SCIEX 5600 quadrupole time-of-flight mass spectrometer (AB SCIEX Co., Boston, Massachusetts, USA) that operated in negative electrospray ionization mode was used for the mass spectrometric study. The MS acquisition parameters were as follows: curtain gas 35 psi, ion source gas 1 60 psi, and ion source gas 2 60 psi, temperature 650 °C, ion spray voltage floating –4000 V. Data processing was acquired by information-dependent acquisition (IDA) mode with a TOF-MS survey scan of 100 ms and 10 dependent TOF-MS/MS experiments of 50 ms accumulation time. The mass range was m/z 50–1200 and m/z 100–1200 for MS and MS/MS mode, respectively. A collision energy of 30 eV was used for the MS/MS scans. Analyst TF 1.7 software (AB Sciex) was used to collect mass spectrometric data, and PeakView 2.2 software (AB Sciex) with integrated MasterView 1.1 software and MultiQuant 3.0 software (AB Sciex) were utilized to process the data. The components evaluated by UPLC-Q-TOF-MS/MS were identified based on retention time, precise molecular weight, secondary mass spectrometry fragments, and integrated with literature data.

Determination of hydroxyl radical scavenging ability

A significantly modified version of previously published methods was used to evaluate EBTF's ability to scavenge hydroxyl radicals¹¹. 30 µL of 9 mmol/L FeSO₄ solution, 30 µL of 9 mmol/L salicylic acid-ethanol solution, and 120 µL of 20 mmol/L H₂O₂ solution were added to 30 µL of EBTF solution containing concentrations of 1.3, 1.7, 1.9, 2.3, 2.7, 2.9, 3.1, 3.4, and 3.9 mg/mL. The mixtures were then shaken for 10 s and incubated at 37 °C for 30 min. The absorbance of the solution was subsequently measured at 510 nm. In the test, distilled water served as the blank control and vitamin C as the positive control. The following formula was used to get hydroxyl radical scavenging rate:

$$\text{Hydroxyl radical scavenging rate (\%)} = (1 - \frac{(A_1 - A_2)}{A_0}) * 100$$

Where A₀ is the absorbance of a mixture of all reagents without a sample, A₁ is the absorbance of a mixture of all reagents along with the sample, and A₂ is the absorbance of a mixture of deionized water and the sample.

DFT Predictions

Since scutellarin is the primary active component of *E. breviscapus* and possesses antioxidant activity^{2,12}, we selected scutellarin for subsequent density functional theory (DFT) analysis of hydroxyl radical scavenging. The B3LYP functional with the 6–31 + G(d, p) basis set in the Gaussian 16 software package was used in this study. Frontier molecular orbital theory and Fukui function analysis were used to predict active sites, while molecular geometry optimization was used to identify stable configurations. The bond dissociation enthalpies (BDEs) of

Symbol	Independent variable	Actual levels at coded factor levels		
		–1	0	1
A	PEG2000 mass fraction (%)	15	16	17
B	(NH ₄) ₂ SO ₄ mass fraction (%)	13	14	15
C	Ultrasound time (min)	30	40	50
D	Liquid-solid ratio (mL/g)	20	30	40

Table 1. Level and factors of the response surface analysis.

different phenolic hydroxyl sites were calculated to assess the thermodynamic viability. The aquatic environment was simulated using the solvation model based on density (SMD) solution model. Through frequency analysis, all optimized structures were confirmed to be minimal or transition states on the potential energy surface, and zero-point energy adjustments were incorporated into all energy calculations.

Statistical analysis

All experiments were conducted in triplicate. The mean $\pm$ standard error of the mean was used to display the data. Statistical analysis was conducted using SPSS 26.0. The One-way analysis of variance (ANOVA) was used to assess the statistical significance of the data, and differences between treatments were shown when $P \leq 0.05$. Design Expert 8.0.6 was used to forecast the response surface optimization and visualized the response surface and contour plots. Origin 2021 was used to visualize the figures.

Results and discussion

Single-factor experiment

Effect of PEG molecular weight

The effect of PEG molecular weight on the total flavonoid content (TFC) of *E. breviscapus* is shown in Fig. 1a. The TFC grew in tandem with the PEG molecular weight and started to decline when the PEG molecular weight exceeded 2000.

The hydrophobicity of PEG strengthened with increasing molecular weight, which could form stronger hydrophobic interactions with the hydrophobic aromatic moieties of flavonoids. Thus, it facilitated the desorption of flavonoids from *E. Breviscapus* powder and their dissolution in the PEG/ammonium sulfate system. However, as PEG's molecular weight increased, certain flavonoid glycosides became less soluble due to the excessive enhancement of their hydrophobicity. Further, the increase in PEG molecular weight generated a rise in solution viscosity, which greatly inhibited the diffusion and mass transfer of flavonoids from the *E. Breviscapus* powder into the PEG phase. This was the same as what Gao et al. reported¹³. Hence, the optimal PEG molecular weight for extracting flavonoids from *E. breviscapus* was 2000.

Effect of PEG2000 quality fraction

When the PEG2000 mass fraction was between 10% and 16%, the TFC and PEG2000 mass fraction showed a positive connection, with a maximum TFC of 45.53 ± 0.37 mg/g (Fig. 1b). As the mass fraction of PEG2000 grew, its capacity for capturing water in the salt phase was reinforced, and the volume of the upper phase and its hydrophobicity were raised, which promoted the distribution of hydrophobic flavonoids in the upper phase. When the mass fraction of PEG2000 exceeded 16%, high concentrations of PEG2000 caused micellization at the biphasic interface, which hindered the diffusion of flavonoids across the phase. This was identical to what

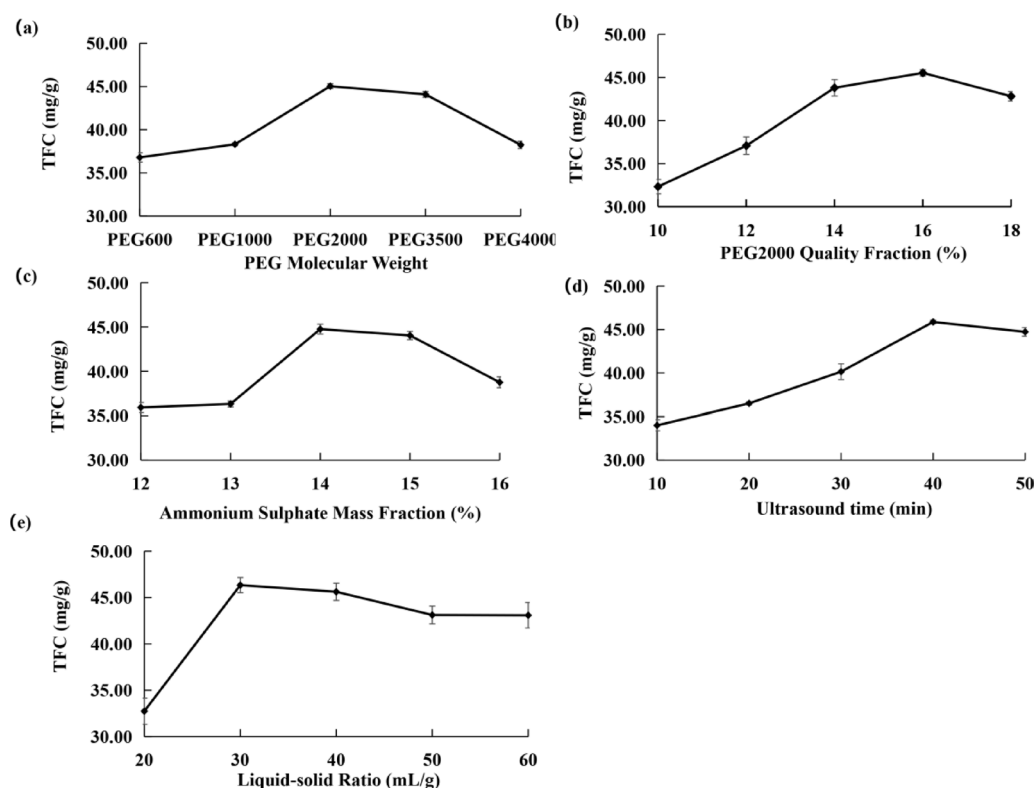


Fig. 1. Effect of single factors on the TFC from *E. breviscapus*. (a) PEG molecular weight, (b) PEG2000 quality fraction, (c) $(\text{NH}_4)_2\text{SO}_4$ mass fraction, (d) Ultrasound time, (e) Liquid-solid ratio ($n = 3$).

was reported by Chong et al.¹⁴. The optimal single-factor condition was determined to be 16% PEG2000 mass fraction.

Effect of ammonium sulphate mass fraction

The effect of $(\text{NH}_4)_2\text{SO}_4$ mass fraction on the TFC from *E. breviscapus* is shown in Fig. 1c. When the $(\text{NH}_4)_2\text{SO}_4$ mass fraction ranged from 12 to 14%, the TFC from *E. breviscapus* was positively correlated with the $(\text{NH}_4)_2\text{SO}_4$ mass fraction, and the maximum TFC from *E. breviscapus* was 44.76 ± 0.56 mg/g. As the $(\text{NH}_4)_2\text{SO}_4$ mass fraction increased, the capacity of salt ions (NH_4^+ , SO_4^{2-}) to capture water was increased. This led to an expansion of the lower phase volume and an increase in the mass fraction of PEG2000 in the upper phase, which made it easier for hydrophobic flavonoids to dissolve the more hydrophobic upper phase. When the $(\text{NH}_4)_2\text{SO}_4$ mass fraction exceeded 14%, the TFC showed a downward trend. This was because the continuing increase in the mass fraction of $(\text{NH}_4)_2\text{SO}_4$ severely affected the stability of the entire dual-water-phase system. Moreover, the extraction of flavonoids from *E. breviscapus* was hampered when the excess ammonium sulfate solution approached saturation and a tiny amount of ammonium sulfate precipitated. This was identical to what was reported by Wang et al.¹⁵. Thus, a mass fraction of 14% $(\text{NH}_4)_2\text{SO}_4$ was chosen for further research.

Effect of ultrasound time

The impact of ultrasound time on the TFC from *E. breviscapus* is shown in Fig. 1d. With an increase in ultrasound time from 10 to 40 min, the TFC from *E. breviscapus* rose and peaked at 40 min. This is due to the fact that the ultrasonic cavitation effect and mechanical vibration were enhanced in the 10–40 min range, which facilitated the solubility of flavonoids in coarse powder and increased the TFC from *E. breviscapus*. After 40 min, the TFC from *E. breviscapus* decreased as the ultrasound time was increased. This because that the dissolved flavonoid structure was destroyed or other impurities were dissolved out by the prolonged influence of ultrasound, which lowered the TFC from *E. breviscapus*. This was analogous to the findings of Lai J. et al.¹⁶. Therefore, 40 min was determined as the optimal ultrasound time.

Effect of liquid-solid ratio

According to Fig. 1e, when the liquid-solid ratio was between 20 and 30 mL/g, the TFC from *E. breviscapus* rose as the liquid-solid ratio grew. These trends were attributed to the fact that the difference in concentration of flavonoid between the solvent and the surface of *E. breviscapus* coarse powder increased as the liquid-solid ratio increased. This resulted in a faster rate of flavonoid diffusion, a lower mass transfer resistance, and a higher TFC from *E. breviscapus*. The solvent volume, however, dramatically rose when the liquid-solid ratio surpassed 30 mL/g, which caused a noticeable dilution of the flavonoid concentration in the upper phase, and lowered the TFC from *E. breviscapus*. A similar phenomenon has been found in other reports^{17,18}. Therefore, the optimal liquid-solid ratio was determined to be 30 mL/g.

Response Surface Experimental results

Experimental design and the results

For the Box-Behnken design, 29 runs were conducted to optimize the four parameters: PEG2000 mass fraction, ammonium sulfate mass fraction, ultrasound time, and liquid-solid ratio. Table 2 presents the experimental conditions of the Box-Behnken design and their corresponding results.

The response value (TFC) and test variables (A, B, C, and D) were ascertained using multivariate regression analysis utilizing the second-order polynomial equations shown below, after the implementation of the response surface regression program:

$$Y = 47.7 - 0.2872A - 2.87B + 2.81C + 1.37D + 0.3939AB + 0.1925AC - 3.25AD + 0.7577BC - 0.02BD + 0.6453CD - 2.35A^2 - 1.35B^2 - 5.04C^2 - 3.32D^2$$

Model fitting and statistical analysis

The results of the ANOVA for evaluating second-order models are shown in Table 3. The ANOVA showed that the *P*-value for the regression model was highly significant ($P < 0.0001$), while the model's lack of fit was not significant ($P = 0.1138 > 0.05$), indicating that the model effectively fitted the linear relationship between TFC from *E. breviscapus* and the tested factors. The coefficient of determination (R^2) from the ANOVA was 0.9808, signifying that the model, with high statistical significance, fitted well with the experimental data. The *P*-values for the one terms (B, C, and D), interaction terms (AD), and quadratic terms (A^2 , B^2 , C^2 , and D^2) were all less than 0.01, indicating that the model was applicable for optimizing the extraction process of EBTF. Besides, the influence of each factor on TFC was ranked in descending order as B ($(\text{NH}_4)_2\text{SO}_4$ mass fraction) > C (ultrasound time) > D (liquid-solid ratio) > A (PEG2000 mass fraction).

*: $P < 0.05$; **: $P < 0.01$.

Response surface analysis

The impact of the interaction effect between two factors on the response surface results of TFC from *E. breviscapus* is illustrated in Fig. 2. As depicted in Fig. 2a and f, the TFC from *E. breviscapus* presented a trend of increasing first and then decreasing with the increase in the mass fraction of PEG2000 and $(\text{NH}_4)_2\text{SO}_4$, ultrasound time, and liquid-solid ratio. All response surface curves showed a noticeable peak within the experimental field, confirming that the chosen factor range was suitable. The density of contour plots rose, and the shape became more elliptical when the gradient changes on the response surface of two interacting factors became steeper, suggesting a significant interaction between these two factors¹⁹. The steepness of the response surface, as well

Run No.	Factor				Response
	A: (%)	B: (%)	C: (min)	D: (mL/g)	Y: (mg/g)
1	17	13	40	30	45.95 ± 0.28
2	16	14	30	40	36.77 ± 0.48
3	16	14	40	30	48.26 ± 0.60
4	15	14	30	30	38.62 ± 0.61
5	17	14	40	40	40.98 ± 0.18
6	16	15	50	30	41.68 ± 0.87
7	16	14	40	30	47.66 ± 0.70
8	17	14	40	20	44.56 ± 0.58
9	15	13	40	30	47.85 ± 0.39
10	16	13	40	40	47.35 ± 0.18
11	16	14	50	40	44.53 ± 0.79
12	16	14	50	20	40.58 ± 0.49
13	15	15	40	30	41.23 ± 1.03
14	16	14	40	30	48.12 ± 0.83
15	16	13	40	20	44.65 ± 0.80
16	16	14	40	30	47.22 ± 1.01
17	16	13	30	30	42.51 ± 0.20
18	17	14	30	30	36.20 ± 0.59
19	16	15	30	30	35.54 ± 0.92
20	17	14	50	30	42.38 ± 0.41
21	15	14	40	40	46.06 ± 0.82
22	16	15	40	20	38.76 ± 0.38
23	15	14	40	20	36.63 ± 0.31
24	17	15	40	30	40.91 ± 0.23
25	16	14	30	20	35.40 ± 0.31
26	16	14	40	30	47.25 ± 0.54
27	16	13	50	30	45.62 ± 0.62
28	16	15	40	40	41.38 ± 0.96
29	15	14	50	30	44.03 ± 0.81

Table 2. Response surface test design and results.

as the ellipticity and density of the contour plots were arranged as follows: AD > BC > CD > AB > AC > BD. It indicated that the interaction between PEG2000 mass fraction and liquid–solid ratio had a more significant effect on the TFC from *E. breviscapus*, which was consistent with the results of ANOVA.

(a, g) PEG2000 quality fraction and $(\text{NH}_4)_2\text{SO}_4$ mass fraction, (b, h) PEG2000 quality fraction and ultrasound time, (c, i) PEG2000 quality fraction and liquid–solid ratio, (d, j) $(\text{NH}_4)_2\text{SO}_4$ mass fraction and ultrasound time, (e, k) $(\text{NH}_4)_2\text{SO}_4$ mass fraction and liquid–solid ratio, (f, l) Ultrasound time and liquid–solid ratio.

Optimization and Validation of the Model

The optimum parameters for EBTF extraction were determined using the regression model generated with Design-Expert 13, which produced the following results: PEG2000 mass fraction of 15.95%, $(\text{NH}_4)_2\text{SO}_4$ mass fraction of 13.856%, ultrasound time of 40.904 min, liquid–solid ratio of 35.053 mL/g, and the predicted TFC from *E. breviscapus* of 48.262 mg/g. Based on actual operation, the verification extraction conditions were slightly adjusted to a PEG2000 mass fraction of 16%, $(\text{NH}_4)_2\text{SO}_4$ mass fraction of 14%, ultrasound time of 41 min, and liquid–solid ratio of 35 mL/g. Following three parallel trials with these modified conditions, the average TFC from *E. breviscapus* was 48.53 mg/g. There was a 0.55% relative error between the model predictions and the experimental values. The results demonstrated that the predicted values of TFC from *E. breviscapus* were close to the experimental values, which proved the accuracy and reliability of the model in predicting EBTF extraction.

Compositional Analysis of Flavonoids from *E. breviscapus*

The chemical compositions of flavonoids from *E. breviscapus* were analyzed by UPLC-Q-TOF-MS/MS in negative ion modes. As shown in Fig. 3, the experiment used quasi-molecular ion peaks and distinctive fragment ions to get the total ion chromatogram using full-scan detection in negative ion mode. The flavonoids from *E. breviscapus* were preliminarily confirmed using a comprehensive analysis of the retention time, the mass-to-charge ratio (m/z) in first-stage mass spectrometry (MS1), fragment ion information in second-stage mass spectrometry (MS2) of the compounds, coupled with comparison to relevant literature reports. The identified flavonoids of *E. breviscapus* are listed in Table 4. In the present work, 28 flavonoids were tentatively identified

Source	Sum of Squares	df	Mean Square	F-value	P-value	Significant
Model	474.4	14	33.89	51.21	<0.0001	**
A	0.9898	1	0.9898	1.5	0.2415	
B	98.82	1	98.82	149.32	<0.0001	**
C	95.08	1	95.08	143.68	<0.0001	**
D	22.67	1	22.67	34.26	<0.0001	**
AB	0.6206	1	0.6206	0.9378	0.3493	
AC	0.1482	1	0.1482	0.224	0.6433	
AD	42.3	1	42.3	63.92	<0.0001	**
BC	2.3	1	2.3	3.47	0.0836	
BD	0.0016	1	0.0016	0.0024	0.9615	
CD	1.67	1	1.67	2.52	0.1349	
A ²	35.83	1	35.83	54.14	<0.0001	**
B ²	11.75	1	11.75	17.76	0.0009	**
C ²	164.9	1	164.9	249.18	<0.0001	**
D ²	71.44	1	71.44	107.95	<0.0001	**
Residual	9.26	14	0.6618			
Lack of Fit	8.34	10	0.834	3.61	0.1138	not significant
Pure Error	0.9245	4	0.2311			
Cor Total	483.66	28				
R ²	0.9808					
Adjusted R ²	0.9617					
Predicted R ²	0.8977					

Table 3. ANOVA for response surface quadratic model.

from *E. breviscapus*. The flavonoids in *E. breviscapus*, such as scutellarin, baicalin, quercetin, and quercitrin, were known to be significant therapeutic substances.

Analysis of hydroxyl radical scavenging activity

EBTF demonstrated a moderate scavenging effect on OH radicals in the concentration range of 1.3 to 3.9 mg/mL, as shown in Fig. 4. Its scavenging rate of OH radicals increased with increasing concentration, reaching 60.68% at 3.9 mg/mL. However, EBTF showed less scavenging effect on OH radicals than vitamin C at any tested concentration.

DFT Calculation Results on the ·OH Scavenging Activity of Scutellarin

Molecular Geometry Optimization and Stability

A stable configuration was obtained by optimizing scutellarin's molecular geometry at the B3LYP/6-31 + G(d, p) level (Fig. 5). The optimal structure had no imaginary vibrational frequencies and corresponded to a minimum on the potential energy surface, according to frequency analysis. According to structural research, the scutellarin contained several possible active sites, chiefly the phenolic hydroxyl groups (-OH) on the A and B rings. Subtle differences in the optimized O-H bond lengths at these sites were observed, initially suggesting variations in their reactive activity.

Prediction of Active Sites for ·OH Scavenging

Fukui function calculations and frontier molecular orbital analysis were used to estimate the reactivity order of the phenolic hydroxyl groups in scutellarin. The A rings' phenolic oxygen atoms had the highest occupancy of the highest occupied molecular orbital (HOMO) (Fig. 6), suggesting that these areas had a high electron density and were vulnerable to the electrophilic OH radical's attack.

Thermodynamic parameters analysis

From a thermodynamic standpoint, scutellarin's capacity to scavenge OH radicals were evaluated by computing the bond dissociation enthalpy (BDE) for every phenolic hydroxyl group. Table 5 displays the results of the computation. From a thermodynamic perspective, the 6-OH site of scutellarin was the most advantageous location for OH radical scavenging since it had the lowest BDE value (94.44 kcal/mol), indicating that its O-H bond was most vulnerable to homolytic cleavage, enabling the released proton to readily combine with OH radicals to form H₂O, while scutellarin transformed into a stable phenoxy radical. On the other hand, the 5-hydroxy site displayed the highest BDE value (103.55 kcal/mol) and the lowest reactivity, owing to the formation of an intramolecular hydrogen bond with the nearby carbonyl group.

According to the natural population analysis (NPA) charge calculation results, the oxygen atom charges in the phenolic hydroxyl groups of scutellarin were 4'-OH (-0.74053), 5-OH (-0.74562), and 6-OH (-0.74020). The oxygen atom at the 5-OH site of scutellarin had the most negative NPA charge of all of them, indicating

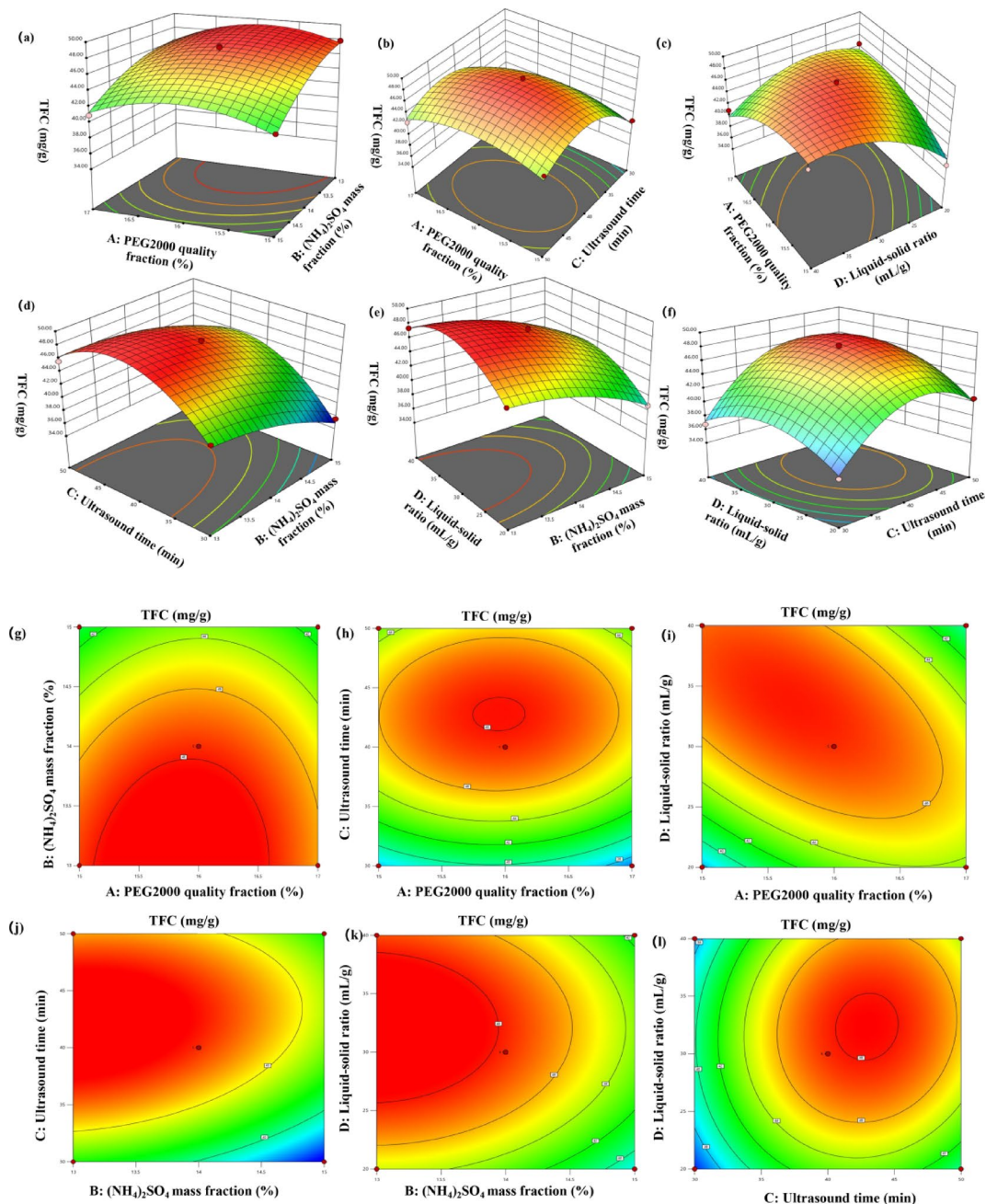


Fig. 2. Response surface plots and contour plots for effects of interaction between various factors on the TFC from *E. breviscapus*.

that the hydrogen atom at this location was most likely to form water through a hydrogen atom transfer (HAT) reaction with hydroxyl radicals.

The oxygen atoms in the phenolic hydroxyl group of scutellarin had the following Fukui function values, according to Fukui function analysis: 6-OH (0.36514), 5-OH (0.34963), 4'-OH (0.3493). 6-OH site of scutellarin had the highest f^- value, suggesting that it exhibited more nucleophilic reactivity toward the -OH group and made electron transfer simpler.

Three important indicators—BDE, NPA, and Fukui function—were examined in the study, which used DFT simulations to examine the active site of scutellarin's scavenging -OH. The proton transfer activity ranking was 6-OH > 4'-OH > 5-OH, according to BDE. NPA charge analysis showed the static electron density ranking of oxygen atoms as 5-OH > 4'-OH > 6-OH. Fukui function analysis indicated the electrophilic reactivity ranking as 6-OH > 5-OH > 4'-OH. The differences in the above results fundamentally originated from the distinct physical dimensions and activation mechanisms represented by each metric. The NPA charge reflected the static electron density distribution of atoms in the ground-state molecule. The oxygen atom at the 5-OH site showed a significant electron-rich impact due to intramolecular hydrogen bonding with the C4 carbonyl group,

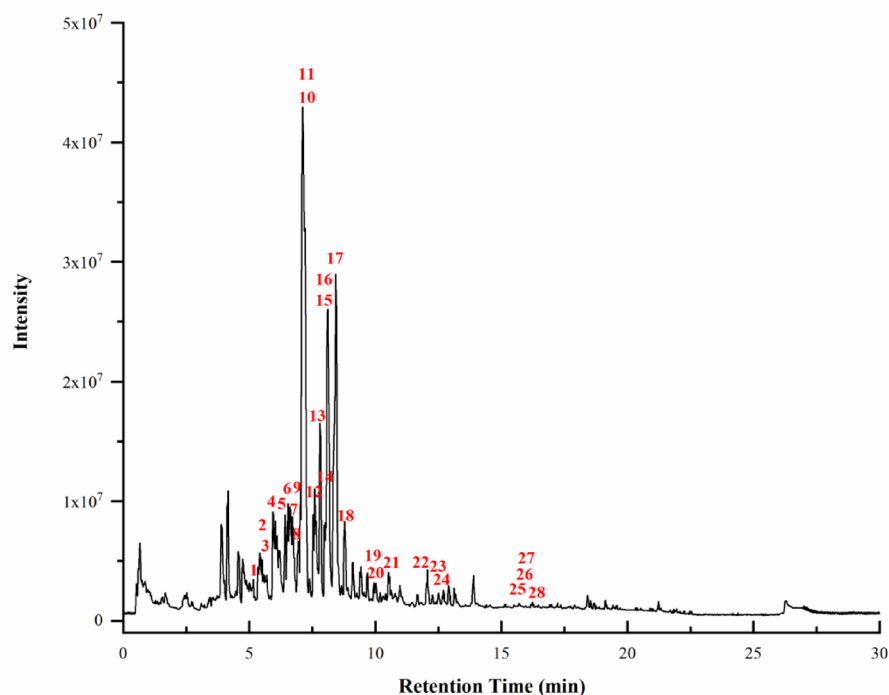


Fig. 3. Total ion chromatogram (TIC) of flavonoids from *E. breviscapus*.

resulting in the greatest static electron density and the strongest negative NPA charge. In contrast, BDE and Fukui function characterized the thermodynamic energy barrier of the proton transfer reaction and the dynamic activity of the electron transfer reaction, respectively. The 6-OH position in the conjugated system of scutellarin's A ring exhibited optimal proton transfer activity and effectively stabilized the phenoxy radical produced after O-H bond cleavage by lowering bond dissociation energy. Furthermore, it increased the dynamic reactivity toward the electrophilic radical $\bullet\text{OH}$ by improving electron delocalization, leading to the greatest Fukui function value. A thorough investigation revealed that the 6-OH site of scutellarin, the primary active site for scavenging hydroxyl radicals, demonstrated optimum proton transfer and electron transfer activity. Although the 5-OH site of scutellarin possessed the highest static electron density, the stabilizing effect of intramolecular hydrogen bonds and steric hindrance hindered its actual reactivity. The 4'-OH site of scutellarin demonstrated intermediate levels of all activity indicators, which was the secondary active site.

Conclusion

The extraction method, component identification, bioactivity evaluation, and mechanism for EBTF were all methodically optimized in this study. RSM was used to optimize the ultrasonic-assisted aqueous two-phase system extraction process, which was shown to be stable and very effective. The total flavonoid yield was 48.53 mg/g under these optimal conditions. Research on the extract's bioactive components was clearly supported by the identification of 28 flavonoids using UPLC-Q-TOF-MS/MS technology. In vitro antioxidant experiments confirmed that EBTF showed moderate hydroxyl radical scavenging capacity. More significantly, the 6-OH site of scutellarin was identified the core active site for scavenging hydroxyl radicals. In summary, the study offers strong theoretical and experimental support for the development of EBTF as a promising natural antioxidant in addition to a dependable technical strategy for the environmentally friendly and effective acquisition of EBTF resources. The antioxidant properties of EBTF were exclusively evaluated in this study using hydroxyl radical scavenging assays. The results might not accurately represent EBTF's antioxidant activity in biological systems or its ability to scavenge additional reactive oxygen species due to analytical techniques and matrix effects. Future research will establish its antioxidant activity using cellular or animal models and complementary tests like DPPH and ABTS.

No	Retention Time (min)	Compound	Formula	Precursor ion (m/z)	Product ion (m/z)	Ref
1	5.21	Vicenin-2	C ₂₇ H ₃₀ O ₁₅	593.15	593.06, 473.05, 383.03, 353.03, 297.04	20
2	5.85	Flavanomarein	C ₂₁ H ₂₂ O ₁₁	449.11	449.05, 287.03, 269.02, 151.00	21
3	5.95	Myricetin	C ₁₅ H ₁₀ O ₈	317.03	317.00, 167.00, 139.00	22
4	6.05	Orientanol E	C ₂₅ H ₂₈ O ₆	423.19	423.13	23
5	6.51	Peltatoside	C ₂₆ H ₂₈ O ₁₆	595.13	595.04, 301.00	24
6	6.64	Apigenin	C ₁₅ H ₁₀ O ₅	269.04	269.02, 193.08, 117.04	25,26
7	6.76	Prunin	C ₂₁ H ₂₂ O ₁₀	433.11	433.05, 271.04, 151.00	27
8	6.97	Rutin	C ₂₇ H ₃₀ O ₁₆	609.15	301.01, 255.01, 217.00, 151.00	28
9	7.03	Isovitexin	C ₂₁ H ₂₀ O ₁₀	431.10	431.04, 341.02, 311.02, 283.04	29
10	7.08	Scutellarin	C ₂₁ H ₁₈ O ₁₂	461.00	461.03, 285.04	30
11	7.18	Scutellarein	C ₁₅ H ₁₀ O ₆	285.04	255.00	31
12	7.53	Eupafolin	C ₁₆ H ₁₂ O ₇	315.05	315.02, 300.00	32
13	7.87	Nictoflorin	C ₂₇ H ₃₀ O ₁₅	593.15	593.06, 285.01	28
14	8.08	Isorhamnetin-3-O-rutinoside	C ₂₈ H ₃₂ O ₁₆	623.16	623.06, 315.02	33
15	8.21	Quercitrin	C ₂₁ H ₂₀ O ₁₁	447.09	271.03	34
16	8.24	Kaempferol	C ₁₅ H ₁₀ O ₆	285.04	285.01	35
17	8.36	Baicalin	C ₂₁ H ₁₈ O ₁₁	445.08	269.02	36
18	8.79	Kaempferol-3-O-alpha-L-arabinoside	C ₂₀ H ₁₈ O ₁₀	417.09	417.03, 285.01	37
19	9.89	Afzelin	C ₂₁ H ₂₀ O ₁₀	431.10	431.04, 283.00	38
20	10.02	Luteolin	C ₁₅ H ₁₀ O ₆	285.04	285.01, 257.02, 199.03, 146.03, 117.04	39
21	10.53	Quercetin	C ₁₅ H ₁₀ O ₇	301.04	178.90, 151.00, 121.03	40
22	12.01	Homoeriodictyol	C ₁₆ H ₁₄ O ₆	301.07	301.04, 151.00	41
23	12.51	Chrysoeriol	C ₁₆ H ₁₂ O ₆	299.06	299.03, 284.01, 256.02	42
24	12.70	Isorhamnetin	C ₁₆ H ₁₂ O ₇	315.05	300.00, 271.00, 164.00, 151.00	35
25	15.59	Chrysin	C ₁₅ H ₁₀ O ₄	253.05	253.03, 143.05, 107.02	43
26	15.75	Pinocembrine	C ₁₅ H ₁₂ O ₄	255.06	213.04, 171.04, 151.00	44
27	15.82	Glycitein	C ₁₆ H ₁₂ O ₅	283.06	283.04, 268.01	45
28	16.18	Velutin	C ₁₇ H ₁₄ O ₆	313.07	298.01, 283.00	46

Table 4. UPLC-Q-TOF-MS/MS analysis of flavonoids from *E. breviscapus*.

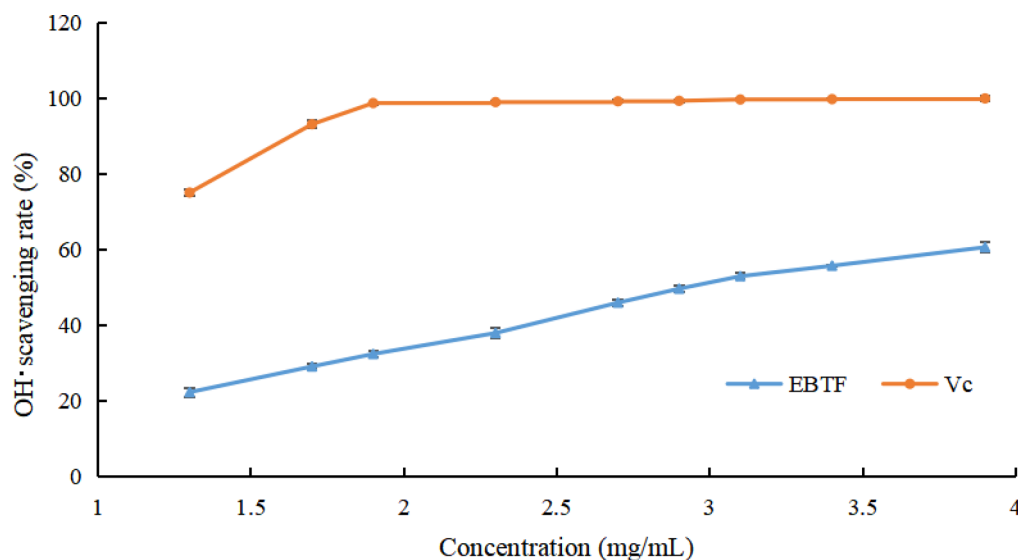


Fig. 4. Antioxidant activity of EBTF on scavenging activity to hydroxyl radicals.

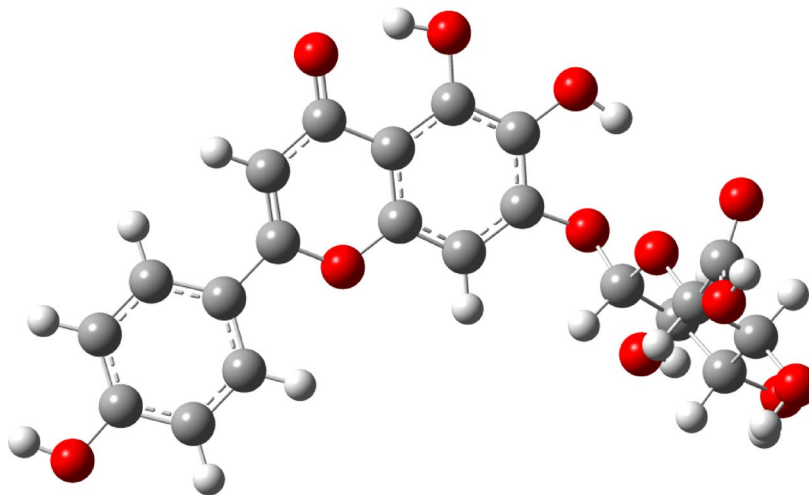


Fig. 5. Molecular structure of scutellarin optimized at the B3LYP/6-31 + G(d, p) level.

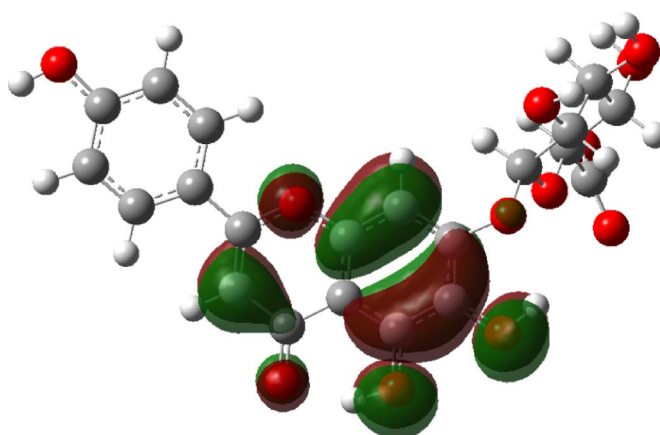


Fig. 6. HOMO distribution of scutellarin.

Site	BDE (kcal/mol)	Relative energy difference (kcal/mol)
4'-OH	99.26	4.82
5-OH	103.55	9.11
6-OH	94.44	0

Table 5. Bond dissociation enthalpy (BDE) values for the phenolic hydroxyl groups of scutellarin.

Data availability

Data is available from the corresponding author on reasonable request.

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Huiqin Qian : Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. Menglin Wang : Supervision, Resources, Conceptualization. Haibo Xu and Kun Feng : Methodology, Formal analysis. Yaxuan Li and Yingfan Hu : Supervision, Conceptualization. Yanling Li : Writing – review & editing, Methodology.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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