

Extraction, Purification, and *in vitro* Antioxidant Activity Assessment of Total Flavonoids from the Aerial Parts of *Tetrastigma hemsleyanum*

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Abstract

Background: The effective extractives of *Tetrastigma hemsleyanum* (*T. hemsleyanum*) are useful, but this plant was not fully utilized in rural China. In this study, flavonoids from the aerial parts of *T. hemsleyanum* were extracted, and the purification method and the antioxidant capability were explored.

Purpose: This study is to explore the optimized purification method and the biological capability of the total flavonoids extracted from *T. hemsleyanum*.

Materials and Methods: The optimal extraction conditions were determined by single-factor and Box-Behnken design experiments. The antioxidant activities were assessed by analyzing their capability to scavenge 2,2-diphenyl-1-picrylhydrazyl and OH free radicals.

Results and Discussion: The optimal conditions for the extraction of the total flavonoids were as follows: ethanol volume fraction 60%, liquid-to-material ratios 35:1 mL/g, temperature 45°C, and time 85 min. The extraction rate of the total flavonoids was (3.59 ± 0.03)%. NKA-9 is an ideal macroporous resin for purification. The optimized purification process conditions of flavonoids were mass concentration of adsorption solution 1.50 mg/mL, pH value of adsorption solution 4.0, sample loading flow rate 2 BV/h, ethanol volume fraction in desorption process 60%, desorption flow rate 2 BV/h and elution volume 4 BV. After crude extracts were purified by NKA-9 macroporous resin, the content of the total flavonoids enhanced from (21.76 ± 0.18)% to (58.92 ± 0.22)%. The results of the experiments for antioxidant activity showed that the half maximal inhibitory concentration (IC₅₀) values of the purified flavonoids extracted were 33.1 and 42.7 µg/mL, respectively.

Conclusion: The results of this study indicated that the flavonoids in *T. hemsleyanum* have good antioxidant activity *in vitro* and deserve exploring in further pharmacological studies.

Keywords

Tetrastigma hemsleyanum, flavonoids, extraction, purification, antioxidant activity

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Introduction

Tetrastigma hemsleyanum (*T. hemsleyanum*) is a plant of the Vitaceae family. It is edible and has medicinal value. *T. hemsleyanum* grows in the Yangtze River Basin in China (Ji et al., 2024; Lin et al., 2023). Multiple pharmacological studies have shown that the bulbous roots of this plant have antiviral, anti-inflammation, and antitumor functions, and relieve rheumatic pain and cold (Ding et al., 2019; Han et al., 2023; Ji et al., 2024; Xia et al., 2018). The extracts from the bulbous roots are used as therapeutic agents in China. *T. hemsleyanum* leaves (THL) are also very useful when their medicinal values are considered. However, THLs in rural China are waste biomass and are usually burned.

Numerous studies have reported the extraction of active ingredients including polyphenols, flavonoids, amino acids, and terpene compounds from THL (Liu et al., 2023; Wang et al., 2023; Zhou et al., 2023). Modern pharmacological

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studies show that its ethanol extracts have antibacterial, anti-falling sickness, anti-tumor, spasmolytic, and anti-inflammatory activities (Waghole et al., 2022; Wu et al., 2021; Xiao et al., 2023; Sun et al. 2017; Yong et al., 2018). The results of our preliminary experiments including color reaction and UV spectrum indicated that the ethanol extracts of THL might be rich in flavonoids.

Flavonoids are one of the most widely distributed natural compounds in the plant kingdom. They possess biological functions including antioxidant, antibacterial, and anti-inflammatory. Besides, they can regulate blood sugar, blood lipids, and blood pressure (Hamed et al., 2019; Kousar et al., 2023). Herein, the THL were used as experimental material, the response surface method was applied to optimize the extraction procedure by ethanol for total flavonoid extraction. Macroporous resin column chromatography was used to purify flavonoids. The antioxidant capability of the compounds was measured by assessing their capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) and OH free radicals.

Materials and Methods

Plant Material and Chemicals

The fresh THL (4.2 kg) were collected in August 2022 from Ningbo Shengwang Biotechnology Corporation, China. In the experiment, the leaves were first dried at 45°C for 72 h. Then the dry leaves were ground into fines (1.2 kg), sieved by a screen of 60-mesh, and preserved in a desiccator. The specimens were identified by Prof. Xin Peng, Zhejiang University. The voucher specimen was stored at the Biotechnology Institute of Zhejiang Pharmaceutical University, China. Rutin and macroporous resins of D101, HP-20 and HPD 100 were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Macroporous resins of NAK-9 and AB-8 were obtained from Nankai Hecheng Science & Technology Co., Ltd. (Tianjin, China). DPPH was purchased from Solarbio Technology Co., Ltd. (Beijing, China).

Assessment of Total Flavonoid Content

The total flavonoid content (TFC) was measured by NaNO_2 – $\text{Al}(\text{NO}_3)_3$ – NaOH colorimetric method (Ma et al., 2023) with minor modification. A rutin solution of 1.05 mg/mL was

prepared and served as the standard solution. The standard rutin solution in an appropriate volume was added into seven volumetric flasks (10 mL). Afterward, solutions including 5% NaNO_2 (0.5 mL), 10% $\text{Al}(\text{NO}_3)_3$ (0.5 mL), and 4% NaOH (4 mL) were added in each flask at 5-min intervals. Then, the mixed solution was diluted to 10 mL with 70% ethanol. Finally, the rutin concentrations were 0, 10, 20, 30, 40, 50, and 60 $\mu\text{g/mL}$. The absorbance was measured thrice at 510 nm by a MAPADA P5 spectrophotometer. The absorbance was in direct proportion to rutin concentration, which ranges from 10 to 60 $\mu\text{g/mL}$. The formula of absorbance: $A = 0.0172C + 0.0081$ ($R^2 = 0.9995$). A represented the average absorbance, and C was the rutin concentration. The TFC was evaluated by replacing the rutin solution with the extracted solution. The calibration curve was used for the calculation of the extraction rate.

Single-factor Experiments

The impacts of the volume fraction of ethanol, liquid-to-solid ratio (L/S ratio), and extraction temperature and time on the extraction rate of flavonoids from THL were explored. In the exploration of the optimal ethanol volume fraction, a series of volume infractions were set at 30%, 40%, 50%, 60%, 70%, 80%, and 90%. The L/S ratio, temperature, and time were 40:1 mL/g, 50°C, and 60 min, respectively. In the exploration of the optimal L/S ratio, a series of ratios were set at 10:1, 20:1, 30:1, 40:1, 50:1, 60:1, and 70:1 mL/g. In this exploration, the previously determined optimal ethanol volume fraction was taken, and the extraction temperature and time were at 50°C, and 60 min, respectively. In the exploration of the optimal temperature, a series of temperatures were set at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C. The previously determined optimal ethanol volume fraction and L/S ratio were taken, and the extraction time was 60 min. In the exploration of the optimal extraction time, the determined optimal ethanol volume fraction, L/S ratio, and extraction temperature were taken.

Box-Behnken Experimental Design

Box–Behnken design was used to optimize the extraction conditions. There were four independent variables of three levels: ethanol volume fraction (a) of 60%, 70%, and 80%; L/S

Table 1. Factors and Levels of Response Surface Method.

Level	Factor			
	Ethanol Volume (A) Fraction (%)	L/S Ratio (B) (mL/g)	Extraction Time (C) (min)	Extraction Temperature (D) (°C)
	60	20:1	60	40
–1	70	30:1	80	50
0	80	40:1	100	60
1				

ratio (b) of 20, 30, and 40 mL/g; extraction time (c) of 60, 80, and 100 min; and extraction temperature (d) of 40, 50, and 60°C. The extraction rate was the dependent variable (Table 1).

Purification Process of Total Flavonoids

Five kinds of resin (HPD100, D101, HP-20, AB-8, and NAK-9) were employed, and the best resin for the purification of flavonoid was investigated. All the resins were immersed in 95% ethanol solution for 24 h, soaked until fully swelled, and then fully washed with distilled water until no ethanol flavor, with the washing liquid clear, not turbidified. Then, the resin was immersed in a 5% HCl solution for 5 h and thoroughly washed with distilled water through a 100 mesh sieve until the pH paper showed that the effluent wash solution was neutral. Then, the resin was immersed in 5% NaOH solution for 5 h and thoroughly washed with distilled water until the wash solution showed neutral.

Two grams of each of the five pre-treated resins were weighed and placed in a 50 mL conical flask, and 30 mL crude flavonoids of THL (mass concentration is C_0) were added. The mixture was placed in an uniform temperature (30°C) oscillation chamber and fully shaken at 150 r/min for 24 h, and then filtered with suction, fixed the volume of the filtrate and determined the mass concentration of flavonoids (C_1). Afterward, the adsorption resins were placed in a conical flask of 50 mL. Thirty milliliters ethanol solution (80%), which is the desorption solution, was added to the rotary mixer to desorb for 24 h. After vacuum filtration, the volume of the filtrate was fixed, and the mass concentration of flavonoids (C_2) was determined. The calculation formulas for adsorption capacity, adsorption rate, and desorption rate are as follows:

$$Q = (C_0 - C_1) \times V/M \quad (1)$$

$$Q_1/\% = (C_0 - C_1)/C_0 \times 100 \quad (2)$$

$$Q_2/\% = C_2/(C_0 - C_1) \times 100 \quad (3)$$

Q denotes adsorption amount, mg/g; Q_1 denotes adsorption rate, %; Q_2 denotes desorption rate, %; C_0 denotes the flavonoid mass concentration in the initial adsorption solution, mg/mL; C_1 denotes the flavonoid mass concentration in the adsorption equilibrium solution, mg/mL; C_2 denotes the mass concentration in the desorption solution, mg/mL; V denotes the volume, mL; M denotes the wet weight of the resin, g.

According to the selected adsorption resin, the optimization of the purification conditions was based on adsorption time, ethanol concentration, desorption time, and sample pH value, and dynamic adsorption and desorption, including sample concentration, sample loading flow speed, elute flow speed, and elution volume.

Determination of Antioxidant Activity In Vitro of Total Flavonoids

DPPH Radical Scavenging Assay

The DPPH radical scavenging assay for assessing the antioxidant capability of the extracted total flavonoids was based on a study previously reported (Li et al., 2021). At room temperature, 1.0-mL sample solution (1–100 µg/L) of different concentrations was mixed with 2.0 mL of DPPH (0.1 mM in ethanol). After half an hour of incubation, the absorbance of the mixture solution was recorded at 517 nm and expressed as A_i . The absorbance when the DPPH solution was replaced by ethanol solution was also recorded and expressed as A_j . In addition, the absorbance when the sample solution was replaced by ethanol solution was recorded as expressed as A_0 . Ascorbic acid served as a positive control. The formula for the scavenging rate ($I\%$) was as below:

$$I\% = [1 - (A_i - A_j) / A_0] \times 100\% \quad (4)$$

The results were expressed by half maximal inhibitory concentration (IC_{50}).

Hydroxyl Radical Scavenging Assay

The *in vitro* antioxidant activity of the extracted total flavonoids was also measured by hydroxyl free radical scavenging assay (Kang et al., 2022). Salicylic acid was selected as a trapping agent for hydroxyl radicals produced by the Fenton reagent reaction to evaluate the antioxidant activity of the flavonoids. To start the reaction, 2.0-mL sample solution of different concentrations of flavonoids was added to the test tube, and then 2.0 mL of 1.8 mmol/L ferrous sulfate solution and 2.0 mL of 1.8 mmol/L salicylic acid ethanol solution, and finally 0.1 mL of hydrogen peroxide (0.03%). The absorbance recorded at 510 nm was expressed as A_i . The absorbance recorded when the hydrogen peroxide was replaced by distilled water was expressed as A_0 . The absorbance recorded when the sample solution was replaced by 70% ethanol solution was expressed as A_j . Ascorbic acid served as a positive control. The formula for the scavenging rate was the same as Formula (4).

Results and Discussion

Results of a Single-factor Experiment for Flavonoid Extraction

The results of the assessment of optimal ethanol volume fraction, L/S ratio, extraction temperature, and extraction time are presented in Figure 1. The extraction rate increased to the highest level when the volume fraction of ethanol was 70%, while declined sharply when the fraction was greater than 70%. This phenomenon was due to the polarity of the

mixer change. The polarities of the mixer and total flavonoids were nearby when the volume fraction of ethanol was 70% (Yuan et al., 2014). Thus, the ethanol volume fractions of 60%, 70%, and 80% were taken as the three levels in the response surface experiment. As illustrated in Figure 1B, with the increase in the liquid-to-material ratio, the extraction rate increased first and then decreased with a further increase in the L/S ratio. This was because a larger L/S ratio corresponds to a larger difference in the concentrations between the plant and the exterior extraction solvent, thus the diffusion of the target compounds was more rapid. When the extract reaches saturation, too much ethanol may cause the dissolution of fat-soluble substances (Yao & Li, 2020). Besides, it is not cost-effective to use a large amount of extraction solvent, thus the L/S 1 ratios of 20:1, 30:1, and 40:1 g/mL were taken as the three levels in the response surface experiment. As illustrated in Figure 1C, the extraction rate of flavonoids gradually increased from 20 to 80 min, whereas after 80 min, the extraction rate declined, which may be due to that the flavonoid compounds of THL were unstable under the long-term heating (Wang et al., 2017), thus the extraction time of

60, 80, and 100 min were taken as the three levels in the response surface experiment. As illustrated in Figure 1D, when the temperature increases, the extraction rate of flavonoids first increased and then decreased sharply, which may be due to that the increase in temperature promotes molecular movement and accelerates the leaching of flavonoids, while the too-high temperature may destroy the structure of the flavonoid (Wang et al., 2022). Thus, the temperatures of 40°C, 50°C, and 60°C were used as the three levels in the subsequent response surface experiment.

Response Surface Optimization

The extraction procedure of total flavonoids was designed by combining four factors and three horizontal based on the results of the single-factor experiment. The experimental design and response results are presented in Table 2. Design Expert 10.0 software was employed to conduct regression model variance analysis on the data in Table 2. By regression fitting the experimental data of each factor, the regression formula was obtained as follows:

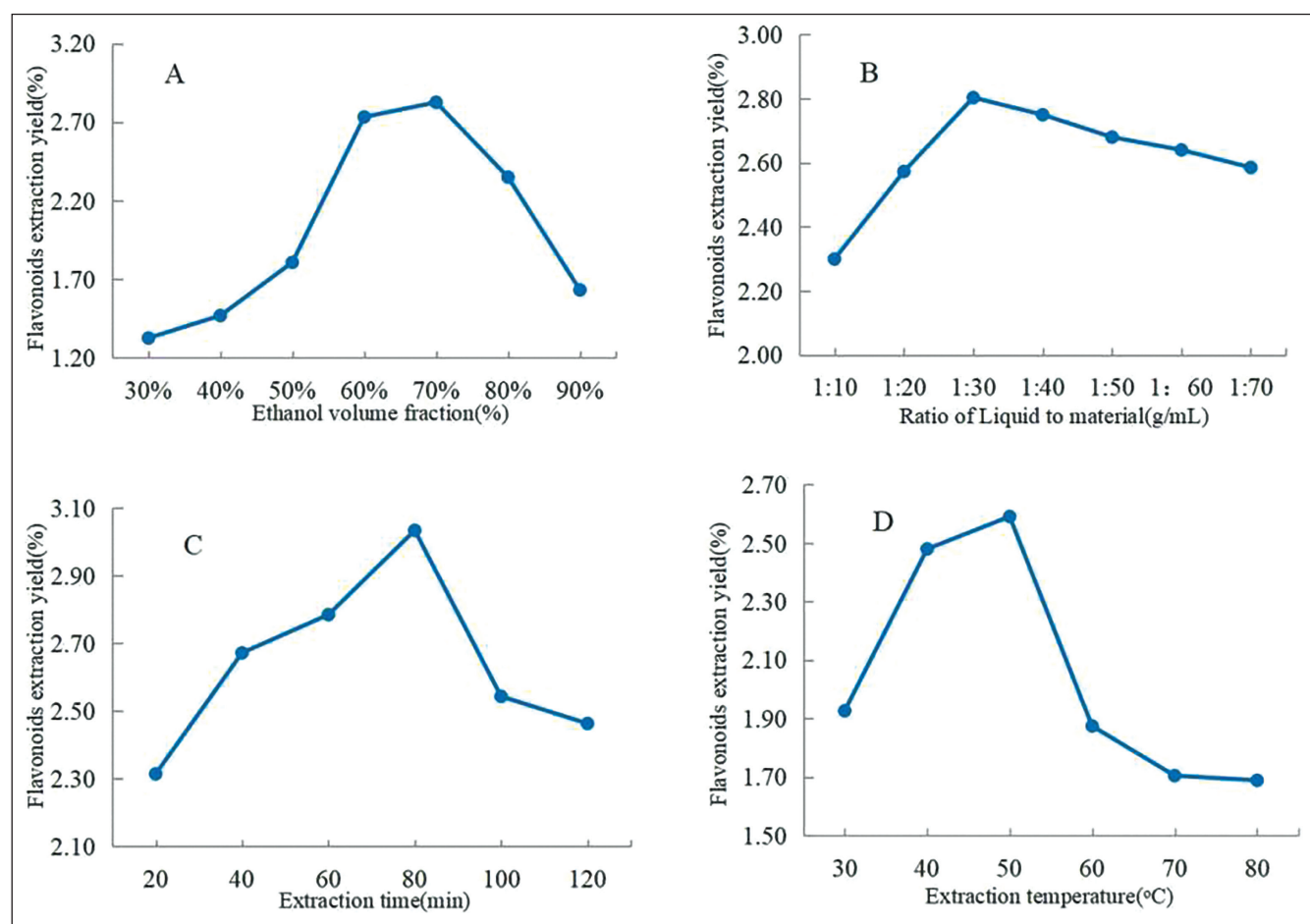


Figure 1. Effect of Sample Concentration (A), Ethanol Concentration (B), Liquid-Solid Ratio (C), and Extraction Temperature (D) on the Extraction of THL.

Table 2. Response Results of Box–Behnkenn Test.

Test Number	Code Value of Box–Behnkenn Test				Total Flavone (%)
	Ethanol Volume Fraction (%)	Liquid-to-Solid Ratio (g/mL)	Extraction Time (min)	Extraction Temperature (°C)	
1	I	0	0	−I	2.85
2	−I	0	0	I	3.16
3	I	I	0	0	2.52
4	0	0	I	I	2.81
5	I	0	I	0	2.61
6	−I	0	0	−I	2.15
7	0	I	−I	0	2.66
8	−I	0	−I	0	2.73
9	I	−I	0	0	2.34
10	0	0	0	0	2.84
11	0	−I	I	0	2.75
12	0	−I	−I	0	2.52
13	−I	0	I	0	2.88
14	0	I	0	I	2.78
15	0	0	0	0	2.85
16	0	I	0	−I	2.11
17	0	0	0	0	2.95
18	0	I	I	0	3.21
19	I	0	0	I	1.83
20	0	−I	0	−I	2.22
21	0	−I	0	I	2.58
22	0	0	0	0	3.04
23	0	0	I	−I	2.03
24	0	0	−I	−I	2.25
25	0	0	−I	I	1.93
26	I	0	−I	0	2.64
27	−I	−I	0	0	2.84
28	0	0	0	0	3.15
29	−I	I	0	0	2.75

Total flavonoids yield (%) = $2.97 - 0.1433A + 0.0650B + 0.1300C + 0.1233D + 0.0675AB - 0.0450AC - 0.5075AD + 0.0800BC + 0.0775BD + 0.2750CD - 0.1184A^2 - 0.1209B^2 - 0.1534C^2 - 0.4434D^2$.

Table 3 shows that the regression ANOVA significance test showed that the model had significant regression ($p = .0005 < .01$), while the missing fitting item was not significant ($p = .1919 > .05$), and the model had statistical significance. The first-order terms A, C, and D have considerable impacts on the response value, and the second-order terms D^2 have extremely remarkable impacts on the response value. The interaction term AD has a very significant effect on the response value, while CD has a significant influence. The F -value of each factor in the test can directly reflect the impact of each factor. The larger the F -value, the greater the effect on the response value. By analyzing the influence degree of each factor, it can be found that $FA = 6.83$, $FB = 1.40$, $FC = 5.62$, $FD = 5.06$, thus it can be seen that the effect of each factor on the total flavonoids of THL was ethanol

concentration > extraction time > extraction temperature > liquid-to-material ratio. The response surface diagram and contour diagram of the interaction between the test influencing factors were shown in Figure 2, which was in line with the results of the variance analysis of the regression model shown in Table 3. Response surface is an effective statistical and optimization method, which has been widely used in the exploration of optimal extraction conditions of flavonoids from plant sources. Compared with the traditional orthogonal design method, data analysis in the response surface method is more detailed, and the significance of each influencing factor and the interaction between the two factors can be intuitively seen (He, 2020; Yuan et al., 2019; Zhang et al., 2023).

Determination of Optimal Process Conditions and Model Verification

Further analysis by response surface software indicated that the optimal conditions for total flavonoid extraction were ethanol

Table 3. Variance Analysis of Regression Model.

Source of Variance	Sum of Squares	Freedom	Mean Square	F-value	p Value
Model	3.41	14	0.2433	6.74	.0005**
A	0.2465	1	0.2465	6.83	.0204*
B	0.0507	1	0.0507	1.40	.2557
C	0.2028	1	0.2028	5.62	.0327*
D	0.1825	1	0.1825	5.06	.0412*
AB	0.0182	1	0.0182	0.5048	.4891
AC	0.0081	1	0.0081	0.2244	.6430
AD	1.03	1	1.0300	28.54	.0001**
BC	0.0256	1	0.0256	0.7091	.4139
BD	0.024	1	0.0240	0.6655	.4283
CD	0.3025	1	0.3025	8.38	.0118*
A ²	0.091	1	0.0910	2.52	.1348
B ²	0.0948	1	0.0948	2.63	.1274
C ²	0.1527	1	0.1527	4.23	.0589
D ²	1.28	1	1.2800	35.33	.0001**
Residual	0.5054	14	0.0361		
Lack of fit	0.4365	10	0.0437	2.53	.1919
Pure error	0.0689	4	0.0172		
Cor total	3.91	28			

Note: *Significant ($p < 0.05$); **very significant ($p < 0.01$).

volume fraction of 60.06%, L/S ratio of 34.39:1 mL/g, extraction time 84.65 min, and extraction temperature of 45.02°C. The predicted value of TFC was 3.64%. Due to the operability of the conditions, the optimal extraction conditions of the total flavonoids were revised as 60% ethanol volume fraction, 35:1 mL/g L/S ratio, 85 min extraction time, and 45°C extraction temperature. After three repeated tests, the TFC of THL was $(3.59 \pm 0.03)\%$ under the adjusted technological conditions. The relative deviation from the predicted value was 1.39%, which has an ideal practical application value.

Selection of Macroporous Adsorption Resin

Macroporous resin is broadly used for the preliminary purification of flavonoid compounds due to its good selectivity, low cost, large adsorption capacity, fast adsorption rate, and convenient regeneration treatment (Ke et al., 2021; Wang et al., 2021). The absorption results of different resins on total flavonoids are shown in Table 4. Under the same conditions, different types of macroporous resins have different adsorption and desorption capacities. The adsorption and desorption rates of NKA-9 resins were 90.71% and 77.26%, respectively. NKA-9 is an ideal macroporous resin for the separation and purification of total flavonoids from THL.

Static Adsorption and Desorption

The research results of the factors influencing the purification process of flavonoids in THL are shown in Figure 3A–D. Within

1.5 h from the beginning of adsorption, the adsorption of NKA-9 resin increased exponentially. After 1.5 h of adsorption, the static adsorption of NKA-9 resin began to be stable. The static adsorption rate at 1.5 h was 90.40%. Within 2.0 h after desorption, the desorption rate of NKA-9 resin increased rapidly over time. After that, the desorption of NKA-9 resin stagnated, and there was little change in the overall long-term range. The results indicated that the static desorption time of NKA-9 resin was 2.0 h, and the static desorption rate was 76.82%.

When PH increased, the adsorption rate increased at the beginning and then declined, and the maximum adsorption rate was observed when the pH was 4. When the pH increases, the phenolic hydroxyl group in the flavonoids will dissociate to H⁺ and the corresponding anion, which will affect the adsorption and selection performance of the macroporous resin, resulting in a decrease in the flavonoid adsorption rate. Thus the optimal pH was 4.0 (Hong et al.). The desorption rate of total flavonoids gradually increased when the ethanol concentration increased from 40% to 60%. The maximum desorption rate (81.05%) was reached when the ethanol concentration was 60%, indicating that the total flavonoids were easily eluted at this concentration. When the concentration exceeded 60%, the desorption rate decreased gradually. Thus 60% served as the eluent ethanol concentration.

Dynamic Adsorption and Desorption

The research results of the factors influencing the purification process of flavonoids in THL are shown in Figure 4A–D. The impact of sample concentration on the adsorption rate is shown

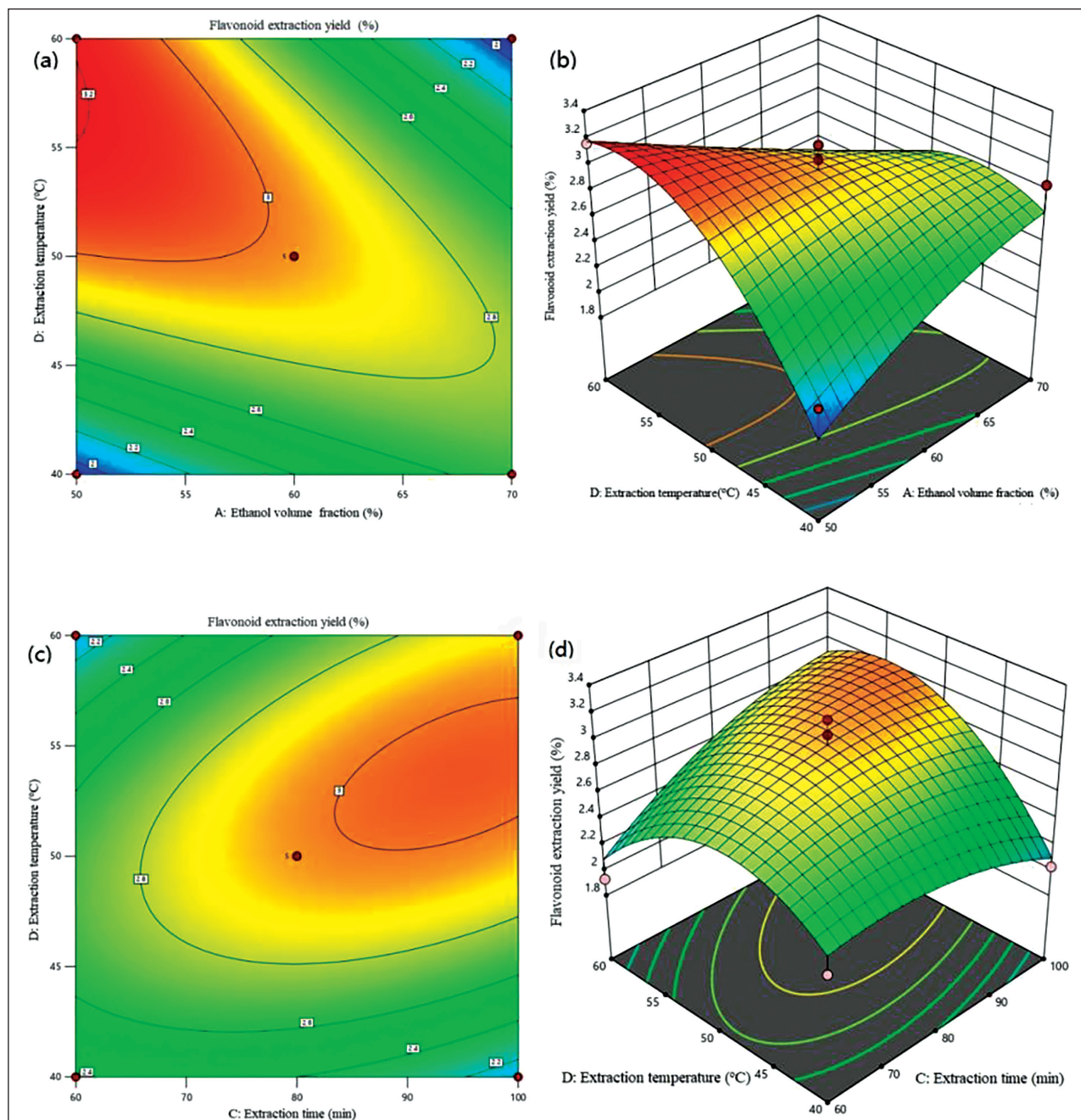


Figure 2. Contour and Response Surface of Effects of Two Factors on Extraction Yield of Total Flavonoids. (A) Contour of Effects of Ethanol Volume Fraction and Extraction Temperature. (B) Response Surface of Effects of Ethanol Volume Fraction and Extraction temperature. (C) Contour of Effects of Extraction Time and Extraction Temperature. (D) Response Surface of Effects of Extraction Time and Extraction Temperature.

Table 4. Adsorption Properties of Different Resins on Total Flavonoids from THL.

Resin Type	Adsorption Capacity (mg/g)	Adsorption Rate (%)	Desorption Rate (%)
HPD100	11.29	88.64	76.39
DI01	10.73	86.22	72.39
HP-20	11.68	91.85	64.28
AB-8	11.49	89.38	59.80
NKA-9	11.67	90.71	77.26

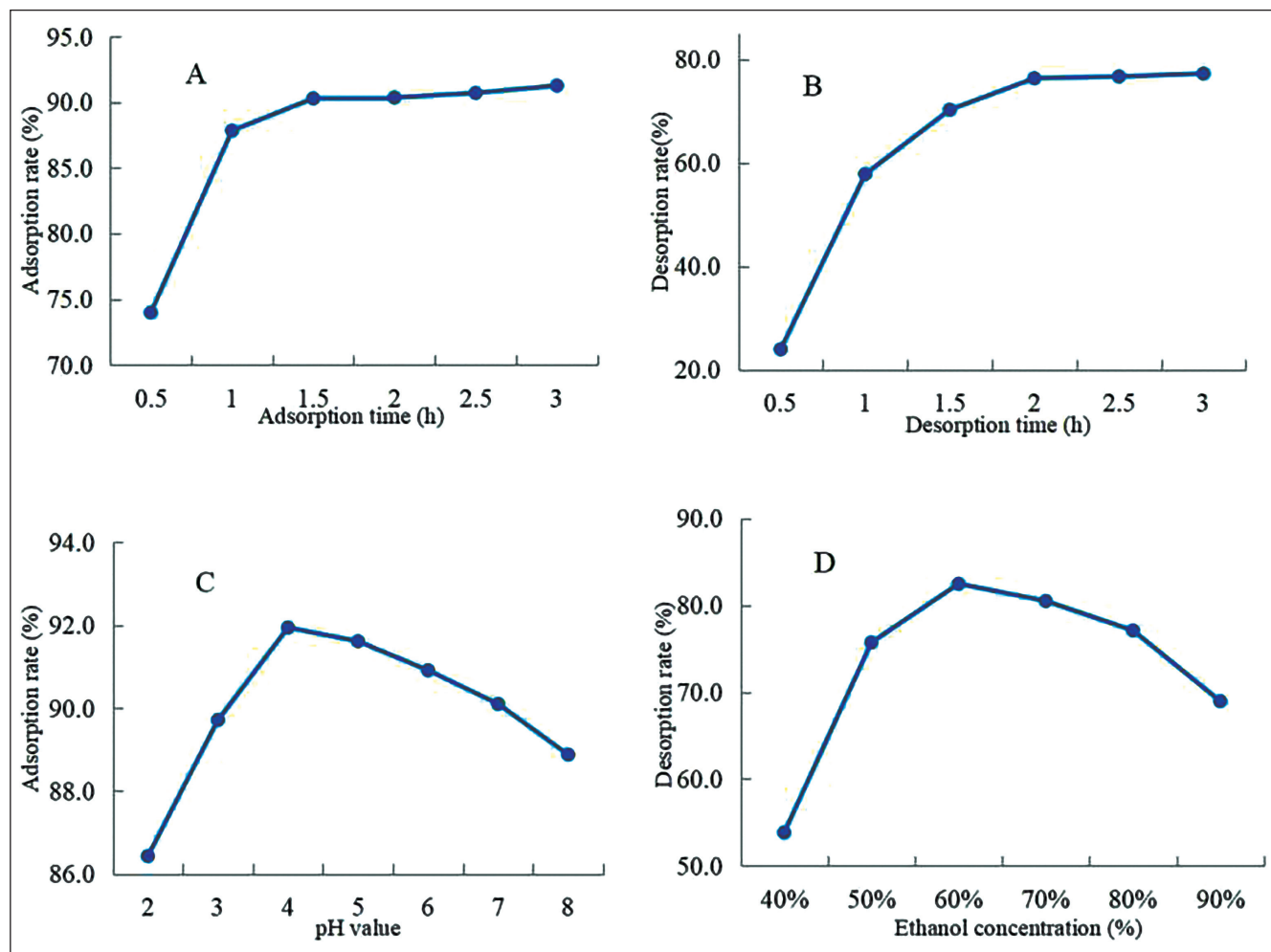


Figure 3. Effect of Adsorption Time (A), Desorption Time (B), pH Value (C), and Ethanol Concentration (D) on the Purification Process of Flavonoids in THL.

in Figure 4A. When the concentration was 0.5 to 1.5 mg/mL, the adsorption rate of the resin increased with the increase in sample solution concentration. When the sample concentration exceeds 1.5 mg/mL, the adsorption rate decreases. This was because the flow rate of low-concentration flavonoid solution was greater than the mass transfer rate when it passed through the column bed, and the mass transfer adsorption was not thorough. However, the flow rate of the carrier liquid at high concentration through the column bed slowed down, and the mass transfer rate to the interior slowed down, resulting in the leakage of some flavonoid components and not being adsorbed in time (Yang, Huang, et al., 2020). In addition, excessive concentration of the loading solution may also lead to a short service period of the adsorbent resin, increase the number of regenerations, and affect the service life of the resin. When the sample solution was 1.5 mg/mL, the adsorption rate reached 67.84%, thus the concentration of 1.5 mg/mL of the sample solution was taken in the subsequent test.

The impact of sample loading flow speed on the adsorption rate of NKA-9 resin is shown in Figure 4B. When the loading

flow speed was from 1.0 to 2.0 BV/h, the total flavonoid adsorption rate increased with the flow rate. When the loading flow speed exceeded 2.0 BV/h, the total flavonoid adsorption rate decreased with increasing flow speed. This was because when the flow speed was too low, the contact time between the solution and the resin was longer and the adsorption rate gradually increased. However, if the flow speed was too high, the active components could not be adsorbed on the resin, resulting in increased leakage of flavonoids and decreased adsorption rate (Yang, Liu, et al., 2020). Therefore, the sample loading flow speed of 2.0 BV/h was chosen for subsequent tests.

The impact of elute flow speed on the desorption rate of NKA-9 resin is shown in Figure 4C. The elute flow speed was usually required to be slow because too fast a flow rate will lead to poor elution performance, elution bandwidth, and serious tailing and incomplete elution. However, too slow a flow rate will prolong the whole elution period (Shi & Zhang, 2023). When the elute flow speed was 2.0 BV/h, the desorption rate reached the maximum value of 71.24%. To use as little solvent as possible to fully elute the flavonoids, it

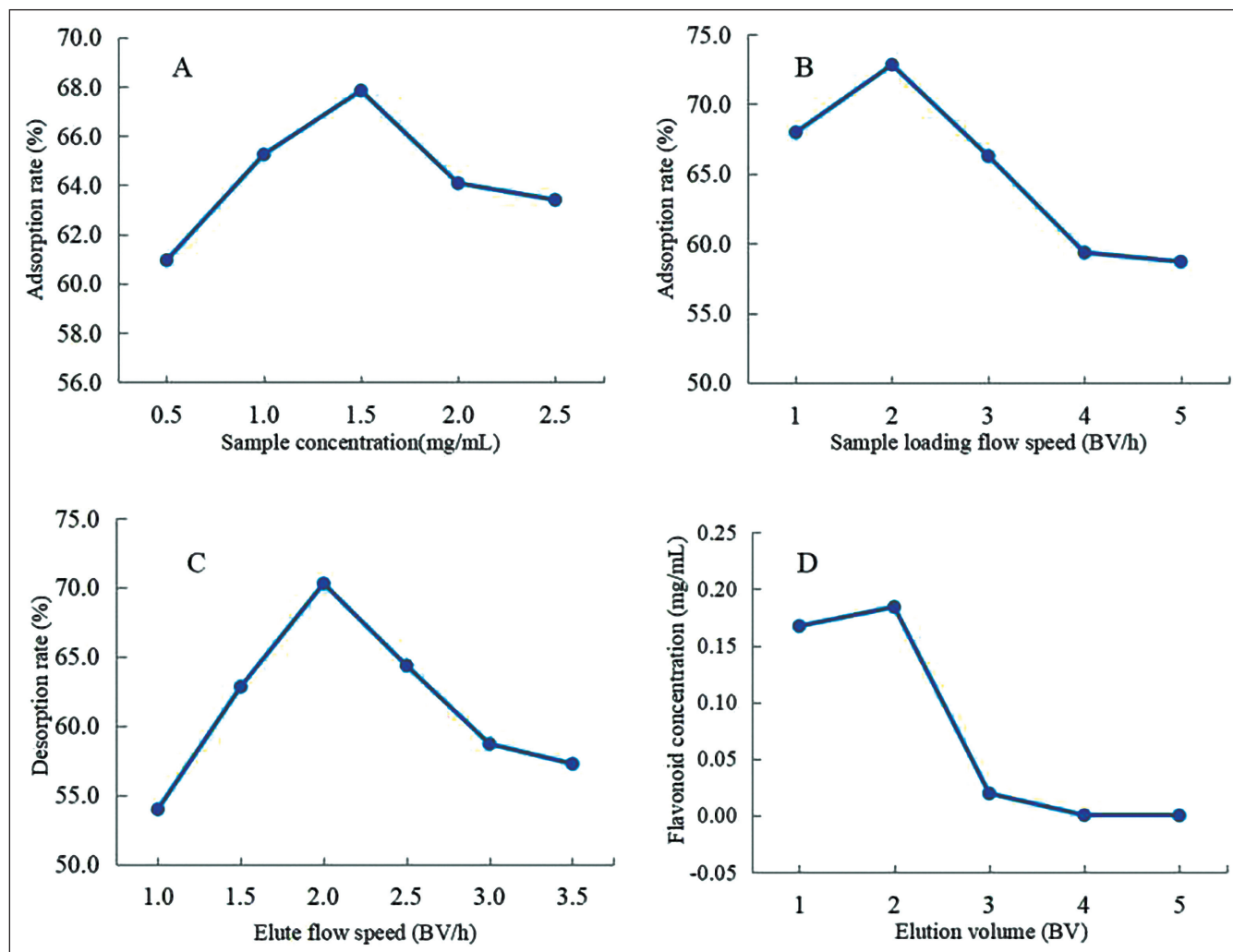


Figure 4. Effect of Sample Concentration (A) Sample Loading Flow Speed, (B) Elute Flow Speed, (C) and Elution Volume, (D) on the Purification Process of Flavonoids in THL.

was necessary to explore the influence of the elution volume. The influence of volume is shown in Figure 4D. When the elution volume increased, the total flavonoid mass concentration in the eluent decreased accordingly. When the elution volume reached 4 BV, the total flavonoid concentration in the eluent was less than 0.01 mg/mL, indicating that the flavonoids on the resin had been fully eluted at this time, so the elution volume was determined to be 4 BV.

Calculation of Purity

The total flavonoids of THL were purified by NKA-9 macroporous adsorption resin. The purity was calculated as follows:

$$\text{Purity} / \% = \text{Total flavonoid mass (mg)} / \text{Sample mass (mg)} \times 100 \quad (5)$$

After purification by NKA-9 resin, the purity of the refined product was $(58.92 \pm 0.22)\%$, which was ideal.

Determination of the Antioxidant Activity In Vitro of Flavonoids in THL

This assessment was based on the method reported previously (Wang, 2022). The scavenging effect of crude extracts and purified flavonoids on the DPPH radical is illustrated in Figure 5A. Ascorbic acid served as a control. The results showed that the DPPH scavenging rate and flavonoid concentration were positively correlated, implying that the purified flavonoids had higher activity, which was beneficial to the scavenging capability. When the concentration reached 60.0 $\mu\text{g} / \text{mL}$, the scavenging rate became stable. When the concentration was 5.0–120.0 $\mu\text{g} / \text{mL}$, the scavenging rate of DPPH in flavonoid solution was between 6.1% and 82.7%, and its IC_{50} was 33.1 $\mu\text{g} / \text{mL}$; the reason was that NKA-9 adsorbed some of the nonflavonoid impurities, making the content of flavonoid in THL higher. The IC_{50} value of Vitamine C (VC) for the positive control was 7.2 $\mu\text{g} / \text{mL}$. The

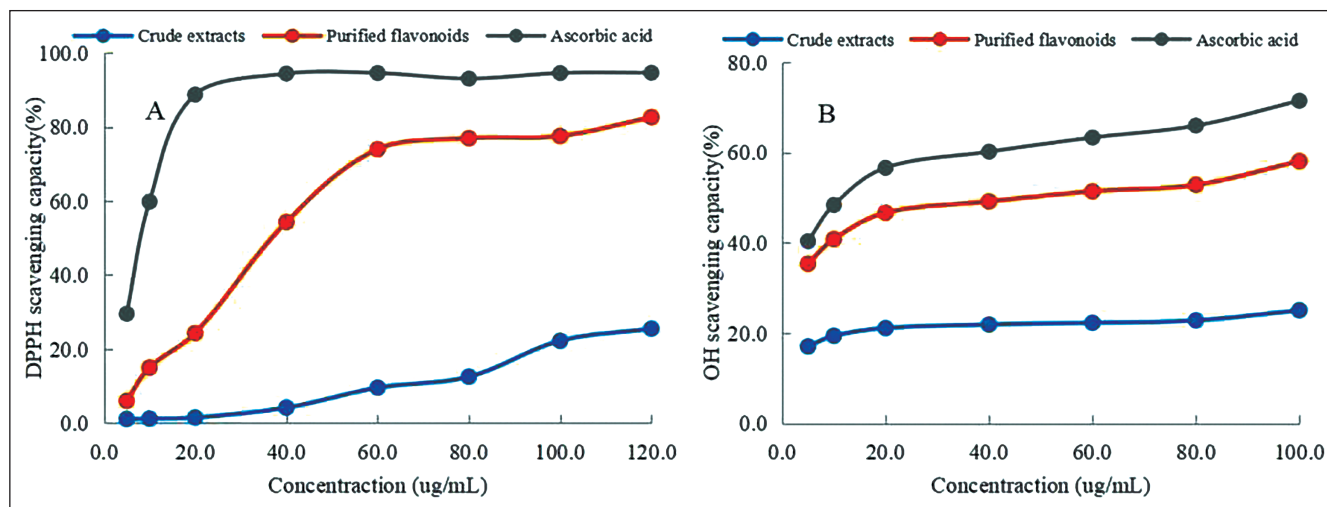


Figure 5. Determination of Antioxidant Activity *In Vitro* of Flavonoids in THL. (A) DPPH Radical Scavenging Activity, (B) OH Radical Scavenging Activity.

order of DPPH radical scavenging ability was ascorbic acid > purified flavonoids > crude extracts.

Hydroxyl radicals are a type of reactive oxygen species, meaning they contain at least one unstable oxygen atom, one of which has extra electrons, causing it to become too active to react with other substances. Hydroxyl radicals can react with active substances and convert them into inactive substances, thus preventing oxidation reactions. Hydroxyl radical scavenging helps to prevent cell damage caused by free radicals, protect cellular structures, and resist damage caused by oxides (Ge et al., 2023). The scavenging effects of crude extracts and purified flavonoids on the Hydroxyl radical are displayed in Figure 5B. Ascorbic acid was the control. The scavenging rate of hydroxyl radicals gradually enhanced along with the increase of flavonoid concentration. The change in the concentration of the crude extract has little effect on the scavenging of hydroxyl radicals, which may be because of the large content of impurities in the crude extract. The purified flavonoids were significantly better at scavenging hydroxyl radicals than the crude extract. When the concentration was 5.0–100.0 µg/mL, the scavenging rate of hydroxyl radicals in purified flavonoid solution was between 35.4% and 58.1%, and its IC_{50} was 42.7 µg/mL. The IC_{50} value of VC for the positive control was 12.2 µg/mL. The ability of hydroxyl radical scavenging was ascorbic acid > purified flavonoids > crude extracts.

In summary, for maximizing extraction of flavonoids from THL, the following optimum extraction conditions were determined: ethanol concentration of 60%, liquid-solid ratio of 35:1 mL/g, extraction time of 85 min, and temperature of 45°C. Under the optimized conditions, the extraction rate of flavonoids in THL could reach $(3.59 \pm 0.03)\%$. As compared with other process optimization designs (Yu et al., 2021), the extraction rate of flavonoids was significantly improved. The most suitable resin was NKA-9 resin, and the optimized

purification process conditions of flavonoids in THL were mass concentration of adsorption solution 1.50 mg/mL, pH value of adsorption solution 4.0, sample loading flow rate 2 BV/h, ethanol concentration in the desorption process 60%, desorption flow rate 2 BV/h, and the elution volume 4 BV. After the extracts were purified by NKA-9 macroporous resin, the TFC increased from $(21.76 \pm 0.18)\%$ to $(58.92 \pm 0.22)\%$, which was increased by 2.71 times. The results showed that flavonoids in THL have excellent antioxidant capability, with the IC_{50} values of 33.1 µg/mL for scavenging DPPH radical and 42.7 µg/mL for scavenging hydroxyl radical.

Conclusion

In this study, an optimized purification method for the total flavonoids from THL was explored. Based on the conditions determined, a high extraction rate was obtained. The antioxidant capability assessment indicated that it could be developed as potential antioxidants. The contents and results of this study have important reference value for the research on the active components and efficacy of THL, especially the development and utilization of the flavonoids of THL.

Abbreviations

DPPH: 2,2-Diphenyl-1-picrylhydrazyl; IC_{50} : Half maximal inhibitory concentration; L/S ratio: Liquid-to-solid ratio; *T. hemsleyanum*: *Tetrastigma hemsleyanum* Diels et Gilg; THL: *T. hemsleyanum* leaves; TFC: Total flavonoid content.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Not applicable.

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Informed Consent

Not applicable.

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References

- Ding, F., Liu, J., Du, R., Yu, Q., Gong, L., Jiang, H., & Rong, R. (2019). Qualitative and quantitative analysis for the chemical constituents of *Tetragium hemsleyanum* Diels et Gilg using ultra-high performance liquid chromatography/hybrid quadrupole-orbitrap mass spectrometry and preliminary screening for anti-influenza virus components. *Evidence Based Complement Alternative Medicine*, 2019, 9414926. <https://doi.org/10.1155/2019/9414926>
- Ge, B., Wang, W., Gao, Y., & Chen, X. (2023). Optimization of extraction of lycopene from carrot and determination of its antioxidant activity. *Journal of Food Measurement Characterization*, 17(5), 5497–5505.
- Hamed, Y. S., Abidin, M. H., Akhtar, M. S., Chen, D., Wan, P., Chen, G., & Zeng, X. (2019). Extraction, purification by macroporous resin and in vitro antioxidant activity of flavonoids from *Moringa oleifera* leaves. *South African Journal of Botany*, 124, 270–279.
- Han, B., Zhai, Y., Li, X., Zhao, H., Sun, C., Zeng, Y., Zang, W., Jinjian, L., & Kai, G. (2023). Total flavonoids of *Tetragium hemsleyanum* Diels et Gilg inhibits colorectal tumor growth by modulating gut microbiota and metabolites. *Food Chemistry*, 410, 135361. <https://doi.org/10.1016/j.foodchem.2022.135361>
- He, Y. (2020). Extraction optimization of 4 flavones in *Dendranthema Morifolium* by Box-Behnken experimental design. *China Pharmacist*, 23(7), 1344–1349.
- Hong, K., Song, L., Chen, Y., Liu, J., Zheng, Z., & Gu, M. (2009). Study on purification process and antioxidant activity of macroporous resin of total flavonoids in *Potamogeton distinctus*. *China Food Additive*, 32(10), 1–10.
- Ji, W., Zhu, H., Xing, B., Chu, C., Ji, T., Ge, W., Wang, J., & Peng, X. (2024). *Tetragium hemsleyanum* suppresses neuro-inflammation in febrile seizures rats via regulating PKC-delta/caspase-1 signaling pathway. *Journal of Ethnopharmacology*, 318(Pt A), 116912. <https://doi.org/10.1016/j.jep.2023.116912>
- Kang, H., Zhao, X., Zhang, X., Zhang, Q., Geng, Y., & Zhao, W. (2022). Study on the enzyme-assisted extraction process of essential oil from *Ledum palustre* L. and its antioxidant activity. *Natural Product Research & Development*, 34(10), 1678–1689.
- Ke, C., Ren, Y., Gao, P., Han, J., Tao, Y., Huang, J., & Yang, X. (2021). Separation and purification of pyrroloquinoline quinone from fermentation broth by pretreatment coupled with macroporous resin adsorption. *Separation Purification & Technology*, 257, 117962.
- Kousar, M. U., Jabeen, A., Fatima, T., Hussain, S. Z., Zargar, I. A., Amin, T., & Yaseen, M. (2023). Optimization of ultrasonic-assisted extraction of eugenol-rich fraction from basil leaves: Characterization of extract for phenols, flavonoids and antioxidant activity. *Food Chemistry Advances*, 3, 100374.
- Li, C., Chen, S., Sha, J., Cui, J., He, J., Fu, J., & Shen, Y. (2021). Extraction and purification of total flavonoids from *Eupatorium lindleyanum* DC. and evaluation of their antioxidant and enzyme inhibitory activities. *Food Science Nutrition*, 9(5), 2349–2363. <https://doi.org/10.1002/fsn3.1999>
- Lin, Y., Lv, Y., Mao, Z., Chen, X., Chen, Y., Zhu, B., Yu, Y., Ding, Z., & Zhou, F. (2023). Polysaccharides from *Tetragium hemsleyanum* Diels et Gilg ameliorated inflammatory bowel disease by rebuilding the intestinal mucosal barrier and inhibiting inflammation through the SCFA-GPR41/43 signaling pathway. *International Journal of Biological Macromolecules*, 250, 126167. <https://doi.org/10.1016/j.ijbiomac.2023.126167>
- Liu, X., Liu, X., Mao, W., Guo, Y., Bai, N., Jin, L., Shou, Q., & Fu, H. (2023). Tetragium polysaccharide reprogramming of tumor-associated macrophages via PPARgamma signaling pathway to play antitumor activity in breast cancer. *Journal of Ethnopharmacology*, 314, 116645. <https://doi.org/10.1016/j.jep.2023.116645>
- Ma, M., Song, Z., Chn, L., Tang, Z., Kong, X., & Duan, J. (2023). Optimization of extraction and purification process of total flavonoids from *Carthamus tinctorius* L. leaf and its anti-acute liver injury activity. *Natural Product Research & Development*, 35, 108–117.
- Shi, Y., & Zhang, L. (2023). Extraction, purification technology of total flavonoids from white Chenopodium quinoa and separation of chemical components. *China Food Additive*, (8), 94–101.
- Sun, Y., Tsao, R., Chen, F., Li, H., Wang, J., Peng H., Zhang K., & Deng, Z. (2017). The phytochemical composition, metabolites, bioavailability and in vivo antioxidant activity of Tetragium hemsleyanum leaves in rats. *Journal of Functional Foods*, 30, 179–193.
- Waghole, R. J., Misar, A. V., Kulkarni, N. S., Khan, F., Naik, D. G., & Jadhav, S. H. (2022). In vitro and in vivo anti-inflammatory activity of *Tetragium sulcatum* leaf extract, pure compound and its derivatives. *Inflammopharmacology*, 30(1), 291–311. <https://doi.org/10.1007/s10787-021-00897-6>
- Wang, C. (2022). Ultrasonic extraction, composition analysis, in vitro; antioxidant and antiproliferative activities of Mango kernel oil from Jinhuan Mango kernel. *International Journal of Food Properties*, 25(1), 924–935.
- Wang, J., Cao, W., Ji, T., Zhao, M., Liu, T., Wu, J., Feng, F., Zhou, A., & Peng, X. (2023). Gut microbiota and transcriptome profiling revealed the protective effect of aqueous extract of *Tetragium hemsleyanum* leaves on ulcerative colitis in mice. *Current Research in Food & Science*, 6, 100426. <https://doi.org/10.1016/j.crfs.2022.100426>
- Wang, X., Su, J., & Chu, X. (2021). Research progress on separation and purification of flavonoids compounds by macroporous resin. *Food Science & Technology*, 46(08), 208–212.
- Wang, Y., Gao, Y., Ding, H., Liu, S., Han, X., Gui, J., & Liu, D. (2017). Subcritical ethanol extraction of flavonoids from *Moringa oleifera* leaf and evaluation of antioxidant activity. *Food Chemistry*, 218, 152–158. <https://doi.org/10.1016/j.foodchem.2016.09.058>
- Wang, Z., Yang, S., Gao, Y., & Huang, J. (2022). Extraction and purification of antioxidative flavonoids from *Chionanthus*

- retusa* leaf. *Frontiers in Bioengineering & Biotechnology*, 10, 1085562. <https://doi.org/10.3389/fbioe.2022.1085562>
- Wu, T., Wang, X., Xiong, H., Deng, Z., Peng, X., Xiao, L., Jiang, L., & Sun, Y. (2021). Bioactives and their metabolites from *Tetragium hemsleyanum* leaves ameliorate DSS-induced colitis via protecting the intestinal barrier, mitigating oxidative stress and regulating the gut microbiota. *Food & Function*, 12(23), 11760–11776. <https://doi.org/10.1039/d1fo02588k>
- Xia, G. S., Li, S. H., & Zhou, W. (2018). Isoquercitrin, ingredients in *Tetragium hemsleyanum* Diels et Gilg, inhibits hepatocyte growth factor/scatter factor-induced tumor cell migration and invasion. *Cell Adhesion & Migration*, 12(5), 464–471. <https://doi.org/10.1080/19336918.2018.1473664>
- Xiao, L., Xiong, H., Deng, Z., Peng, X., Cheng, K., Zhang, H., Jiang, L., & Sun, Y. (2023). *Tetragium hemsleyanum* leaf extracts ameliorate NAFLD in mice with low-grade colitis via the gut-liver axis. *Food & Function*, 14(1), 500–515. <https://doi.org/10.1039/d2fo03028d>
- Yang, R., Liu, C., Xu, T., Li, R., & Chen, Y. (2020). Investigation of adsorption properties of five macroporous resins for total flavonoids in *Epimedium Folium*. *Chinese Journal of Experimental Traditional Medical Formulae*, 26(6), 113–120.
- Yang, Y., Huang, Y., & Zheng, B. (2020). Separation and purification of total flavonoids from *Artemisia argyi* and their antioxidant activity. *Food Research & Development*, 41(22), 55–62.
- Yao, H., & Li, H. (2020). Extraction and purification process optimization and antioxidant activity in vitro of flavonoids in *Amaranthus caudatus* L. *Medicinal Plant*, 11(4), 48–53.
- Yong, S., Qianru, H., Ran, C., Hongyan, L., Han, P., Fang, C., & Zeyuan, D. (2018). Apoptosis in human hepatoma HepG2 cells induced by the phenolics of *Tetragium hemsleyanum* leaves and their antitumor effects in H22 tumor-bearing mice. *Journal of Functional Foods*, 40, 349–364.
- Yu, W., Cai, W., Chen, D., Xiong, C., & Liao, S. (2021). Study on optimization of preparation technology of total flavonoids from aerial parts of *Tetragium hemsleyanum*. *Fujian Journal of TCM*, 52(5), 32–37.
- Yuan, S., Liu, Y., G., L., Hao, J., & Jin, W. (2019). Optimization of extraction technology of flavonoids in *glycyrrhiza uralensis* by single factor experiment combined with Box-Behnken design-response surface methodology. *China Pharmacy*, 30(3), 355–359.
- Yuan, X. H., Fu, L. N., Gu, C. B., Zhang, Y. D., & Fu, Y. J. (2014). Microwave-assisted extraction and antioxidant activity of vacarin from the seeds of *Vaccaria segetalis*. *Separation and Purification Technology*, 133, 91–98.
- Zhang, X., Lai, W., Lin, X., Huang, Y., Yang, S., Hao, Y., Zhou, J., Fu, C., Hu, C., Dong, Y., Geng, F., & Zhang, Z. (2023). Optimization of decocting process of Simiao Yong'an decoction by Box-Behnken design-response surface methodology. *Chinese Traditional and Herbal Drugs*, 54(10), 3109–3119.
- Zhou, F., Lin, Y., Chen, S., Bao X., Fu, S., Lv, Y., Zhou, M., Chen, Y., Zhu, B., Qian, C., Li, Z., & Ding, Z. (2023). Ameliorating role of *Tetragium hemsleyanum* polysaccharides in antibiotic-induced intestinal mucosal barrier dysfunction in mice based on microbiome and metabolome analyses. *International Journal of Biological Macromolecule*, 241, 124419. <https://doi.org/10.1016/j.ijbiomac.2023.124419>