Response Surface Methodology for Optimization of Ultrasound-Assisted Extraction of Flavonoids from Lotus Leaf and Its Antioxidant Activity

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Abstract. In this study, ultrasonic-assisted extraction was adopted to extract total flavonoids from lotus leaves. The optimal conditions were obtained through single-factor experiments. Subsequently, four factors with significant effects on the flavonoid extraction yield were selected: extraction time, ultrasonic power, ethanol concentration, and solid-liquid ratio. A response surface analysis experiment with four factors and three levels was conducted. After optimization, the optimal conditions were determined as follows: ethanol concentration of 38%, solid-liquid ratio of 1:41, extraction temperature of 50°C, extraction time of 72 min, and ultrasonic power of 225 W. Under these conditions, the total flavonoid extraction rate could reach 108.532 mg/g. Furthermore, the in vitro antioxidant activity of the extract was systematically evaluated by measuring its scavenging capacities against hydroxyl radicals (OH^-) and DPPH radicals, as well as its reducing power. The results indicated that the extract exhibited excellent scavenging effects on the aforementioned radicals and possessed moderate reducing power. Collectively, this study established a stable and reliable extraction and detection method, elucidated the optimal extraction process, major active components, and in vitro antioxidant activity of total flavonoids from lotus leaves. The method established in this study is stable and reliable. It identifies the optimal extraction process and antioxidant activity of total flavonoids from lotus leaves, providing experimental evidence and reference for the development of lotus leaf as an edible and medicinal resource and the modern utilization of by-products of medicinal and edible plants.

Keywords: Lotus leaf, Response surface methodology (RSM), Antioxidant activity.

1 Introduction

The dried leaves of Lotus Leaf, a plant of the genus Nelumbo, have been a valuable dietary herb for thousands of years. [1] This plant is distributed throughout the country,

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and the National Health Commission of the People's Republic of China has listed Lotus Leaf as a food with medicinal properties. In the Chinese Pharmacopoeia, it is clearly recorded that Lotus Leaf has the functions of clearing summer heat and dampness, promoting the rise of clear yang, cooling the blood and stopping bleeding. [2] Modern research has shown that the extracts of Lotus Leaf exhibit various biological activities, such as antibacterial, anti-cancer, [3] anti-obesity, anti-hyperlipidemic, [4] anti-viral, anti-gingival inflammation, [5] antioxidant, hepatoprotective, [6] and cardioprotective activities. [7] Currently, the extraction and separation of active components from Lotus Leaf and the study of their pharmacological effects have attracted much attention. Phytochemical research has confirmed that Lotus Leaf contains active components such as alkaloids, flavonoids, polysaccharides, terpenoids and amino acids, among which flavonoids are one of the core substances for its efficacy. These active components have significant free radical scavenging and antioxidant potential, providing an important material basis for Lotus Leaf to intervene in the body's oxidative stress state. However, deficiencies still exist in current research. For one thing, the process parameters of ultrasonic-assisted extraction of flavonoids from lotus leaves lack systematic optimization, and insufficient multi-factor collaborative research has led to low extraction rates and inefficient resource utilization. For another, the evaluation of their antioxidant capacity is fragmented, failing to systematically clarify their antioxidant potential and mechanism of action. This study optimizes the ultrasonic extraction process of lotus leaf flavonoids and determines their antioxidant activity, providing a basis for the development and utilization of lotus leaf resources.

During human metabolism, the action of oxygen free radicals and carbohydrate metabolism processes both promote the accumulation of carbonyl toxins, among which unsaturated carbonyl compounds are highly accumulated and harmful organic metabolic toxins in the human body. The antioxidant system (including antioxidants and antioxidant enzymes) is the first line of defense against sub-health and stress-related diseases, and is crucial for enhancing immunity and improving physical fitness. [8] Currently, the problem of sub-health is becoming increasingly severe. In this state, the antioxidant function of the body decreases, leading to excessive generation of free radicals, accelerating cell aging and reducing immunity. Studies have shown that antioxidant drugs can alleviate oxidative stress and improve sub-health, thus promoting the development of antioxidant products from various raw materials. At the same time, natural plants are a rich source of antioxidant resources, containing active components such as flavonoids, polyphenols, terpenoids and anthocyanins, which can protect cells through pathways such as scavenging free radicals, chelating metal ions and activating the antioxidant enzyme system. Typical examples include spinach [9], purple sweet potatoes [10], and mugwort leaves [11]. Antioxidants are divided into natural and synthetic types. Natural antioxidants, due to their wide sources, low prices, large reserves, strong activities, and their derivation mostly from food, have better safety and have become the mainstream direction in antioxidant research. Currently, research on natural antioxidants mainly focuses on Chinese herbal medicines and endogenous substances[12]. At present, research on natural antioxidants has mostly focused on Chinese herbal medicines and endogenous substances. Although lotus leaves have been confirmed to contain antioxidant flavonoids, relevant studies remain preliminary, and both

extraction processes and antioxidant evaluation methods are inadequate, making it difficult to meet the needs for their resource development.

Therefore, in this experiment, lotus leaves were used as the research material, and ultrasonic-assisted extraction was employed to extract flavonoids from lotus leaves. The optimal extraction conditions within this experiment were obtained through single-factor experiments and response surface methodology, so as to improve the extraction yield of lotus leaf flavonoids. Based on systematic research, this experiment determined the optimal extraction conditions for total flavonoids in lotus leaves, and investigated the antioxidant capacity of flavonoids from lotus leaves by measuring their DPPH radical scavenging effect and hydroxyl radical inhibitory ability. The analytical results of this experiment can provide an experimental basis for further research and utilization of lotus leaves, as well as for exploring the development potential of lotus leaves as edible and medicinal resources. It is of great significance for human health protection, enriches the product categories using lotus leaves as raw materials, and reduces resource waste.

2 Experimental Materials and Instruments

Rutin standard; Hydroxyl Radical (OH·) Assay Kit; DPPH Free Radical Scavenging Capacity Kit; 80% ethanol solution; 70% ethanol; 5% sodium nitrite solution; 1 mol/L sodium hydroxide solution; 10% aluminum nitrate solution; other reagents are of analytical grade; Lotus Leaf; Multiskan Spectrum Automatic Full-Wavelength Microplate Reader (Thermo Fisher Scientific China Laboratory Products Division); TopPette Pipette (Dragon Lab (Beijing) Co., Ltd.); QVINTIX-1CN Electronic Balance (Ohaus Instruments Changzhou Co., Ltd.); XPR Analytical Balance (Mettler Toledo); BC3-2S Crusher (Hebei Benchen Technology Co., Ltd.); Ultrasonic-Microwave Reactor; Oven.

3 Experimental Methods

3.1 Sample Preparation

Fresh lotus leaves were picked, impurities were removed, and surface dust was washed off with distilled water. The leaves were then dried to constant weight in a constant-temperature drying oven. The dried lotus leaves were crushed in a crusher, and the crushed powder was sieved through a 40-mesh standard test sieve. The undersieve powder was collected as lotus leaf powder, which was sealed and stored in a desiccator for later use. A certain mass of lotus leaf powder was accurately weighed and placed in an Erlenmeyer flask, an appropriate amount of extraction solvent was added, and the mixture was subjected to ultrasonic extraction at a set temperature.

After extraction, the mixture was quickly suction-filtered with qualitative filter paper to collect the filtrate; the residue was re-extracted 1-2 times under the same conditions, and all filtrates were combined. The combined filtrate was cooled to room temperature, transferred to a volumetric flask, fixed to volume with the above extraction solvent,

shaken well, and the lotus leaf flavonoid extract was obtained, which was stored away from light for later use.

3.2 Determination of Total Flavonoids

0.5 mL of the test sample solution was taken and placed in a 10 mL volumetric flask. 70% ethanol was added to make the volume 5 mL, and the mixture was shaken well. Then 0.3 mL of 5% sodium nitrite was added, shaken well, and reacted for 6 minutes. Subsequently, 0.3 mL of 10% aluminum nitrate was added, shaken well, and reacted for 6 minutes. Finally, 4.4 mL of 1 mol/L sodium hydroxide was added, shaken well, and reacted for 12 minutes. The absorbance was measured at 510 nm. The concentration of total flavonoids in the sample solution was calculated using a linear regression equation, and then the content of total flavonoids in lotus leaves was determined. The formula for calculating the extraction amount of total flavonoids is: $A = c \times N \times v/m$, where: A = total flavonoid extraction amount (mg/g); c = measured total flavonoid concentration (mg/mL); N = dilution multiple; v = extract volume (mL); m = sample mass (g).

3.3 Single-Factor Experiment

A single-factor experiment was designed to investigate the effects of extraction power, extraction time, solid-liquid ratio, temperature, and ethanol concentration on the extraction rate of total flavonoids. The optimal conditions of each factor were determined. The factors and levels of the single-factor experiment are shown in Table 1. According to the parameter settings in the table, total flavonoids in lotus leaves were extracted by ultrasonic-assisted extraction, and the test sample solution was obtained. Its absorbance was determined according to the method described in "3.2" to calculate the extraction amount of total flavonoids, so as to screen out the optimal conditions of each single factor.

Table 1. Factors and Levels of Single-Factor Experiment for Lotus Leaf Total Flavonoid Extraction

	Extraction Power/W	Time/min	Solid-Liquid Ratio/g:mL	Temperature/°C	Ethanol Concentration/%
1	60	55	1:20	30	30
2	90	65	1:30	40	40
3	120	75	1:40	50	50
4	150	85	1:50	60	60
5	180	95	1:60	70	70

3.4 Preparation of Standard Curve

Accurately weigh 10.3 mg of rutin standard, add 25mL of 70% ethanol, and dissolve it by ultrasonic treatment for 20 minutes. Precisely measure 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 0.9, and 1mL of the rutin standard solution, and place them into 10mL volumetric flasks respectively. Add reagents according to the method described in "3.2" and measure the absorbance at 510nm.

3.5 Antioxidant Activity of Lotus Leaf Extract

Sample Preparation. Weigh 500.0g of lotus leaf powder and extract it under the optimal extraction conditions determined by the single-factor experiment. Evaporate the organic solvent from the extract by rotary evaporation to obtain the extract paste. Then, use DMSO as the solvent to prepare lotus leaf extract samples with concentrations of 150mg/mL, 75mg/mL, 37.5mg/mL, 18.75mg/mL, 9.38mg/mL, 4.69mg/mL, 2.35mg/mL, and 1.18mg/mL.

Determination of Hydroxyl Radical Scavenging Capacity. The determination was carried out using a Hydroxyl Radical (OH-) Assay Kit. Measure the absorbance according to the operation instructions provided with the kit, and calculate the hydroxyl radical scavenging capacity using the following formula:

Hydroxyl radical scavenging ability (U/ml) = [(A control - A test) / (A standard - A blank)] × Cstandard×1/V sample ×N

Note: C: Concentration of standard solution, 8.824mmol/L; V: Sample volume, 0.2mL; N: Dilution multiple of the sample before testing.

DPPH Free Radical Scavenging Assay. The determination was carried out using a DPPH Free Radical Scavenging Capacity Kit. Measure the absorbance according to the operation instructions provided with the kit, and calculate the DPPH free radical scavenging rate using the following formula:

DPPH Free Radical Scavenging Rate(%)=(1 －(A determination －A control)÷A blank)×100%

4 Experimental Results

4.1 Single-Factor Experiment

Standard Curve. A standard curve was plotted with absorbance as the ordinate and rutin concentration (μg/mL) as the abscissa (as shown in Fig 1). The linear regression equation was obtained as: $y = 0.2895x - 0.0123$, with a linear range of 0～41.79 μg/mL. Rutin showed a good linear relationship with absorbance ($R^2 = 0.9991$).

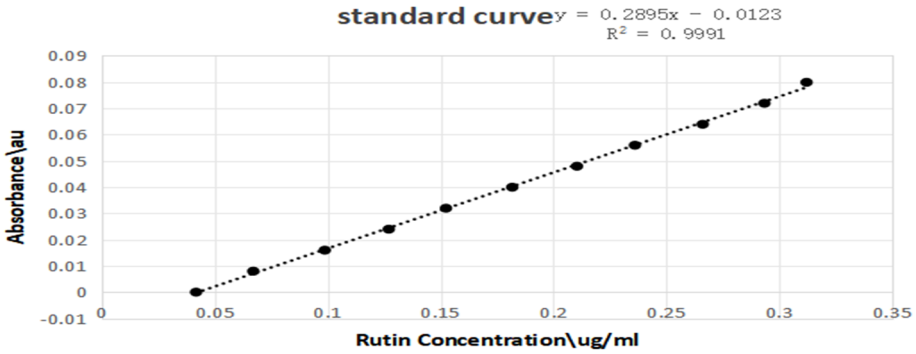

Fig. 1. Rutin Standard Curve

Analysis of Factors Affecting Total Flavonoid Extraction Yield. Accurately weigh 1.0g of lotus leaf powder respectively and divide it into 5 groups (the number of samples in each group is 6, 5, 5, 5, and 5 respectively). Each group is added into the extraction system under corresponding conditions: among them, 6 samples of lotus leaf powder are each added with 40mL of ethanol solutions with different concentrations, and ultrasonic extraction is carried out under the conditions of ultrasonic power 150W, extraction time 90min, and extraction temperature 50℃; the total flavonoid extraction rate is detected and calculated according to the corresponding method. 5 samples of lotus leaf powder are each added with 50% ethanol solutions of different volumes, and ultrasonic extraction is carried out under the conditions of ultrasonic power 150W, extraction time 90min, and extraction temperature 50℃; the extraction rate is detected and calculated. The other two groups of 5 samples each are subjected to ultrasonic extraction under different extraction times, different ultrasonic powers, and different extraction temperatures (other parameters are fixed), and the total flavonoid extraction rate is detected and calculated according to the corresponding method.

The experimental results show that the total flavonoid extraction rate is the highest (17.58mg/g) when the ethanol concentration is 50%; both higher and lower concentrations will affect the dissolution of flavonoids due to the change of ethanol polarity, leading to a decrease in extraction rate. The extraction rate is the highest (19.81mg/g) when the solid-liquid ratio is 1:50; after the solid-liquid ratio reaches a certain level, the dissolution of flavonoids tends to be saturated, and the extraction rate has no significant change. The extraction rate is the highest (21.95mg/g) when the extraction time is 85min; exceeding this time will cause the destruction of heat-sensitive flavonoids, resulting in a decrease in extraction rate. The ultrasonic power of 160W is the most suitable, which not only ensures the extraction effect (the extraction rate is close to 22.61mg/g at 180 W) but also reduces energy consumption. The extraction rate is the highest (23.226mg/g) when the extraction temperature is 50℃; too high temperature will damage the cell structure, and too low temperature cannot reach the temperature required for flavonoid dissolution, both of which will reduce the extraction rate. The relationships between each influencing factor are shown in the following Figure 2-6:

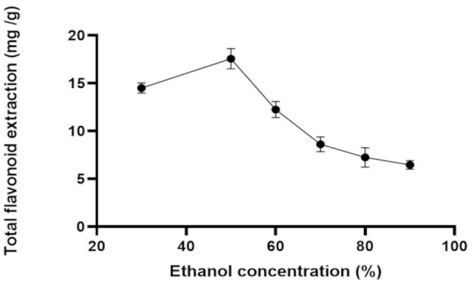

Fig. 2. Relationship between total flavonoid extraction yield and ethanol concentration

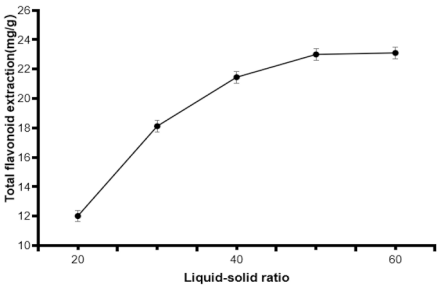

Fig. 3. Relationship between total flavonoid extraction yield and solid-liquid ratio

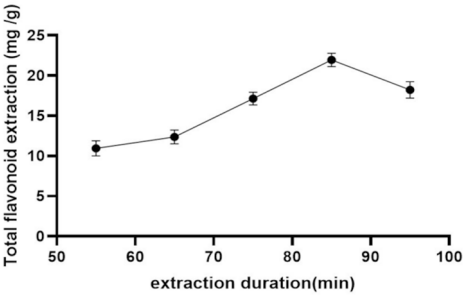

Fig. 4. Relationship between total flavonoid extraction yield and extraction time

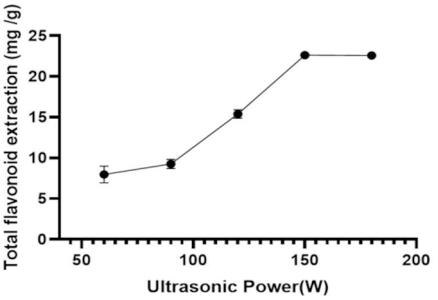

Fig. 5. Relationship between total flavonoid extraction yield and ultrasonic power

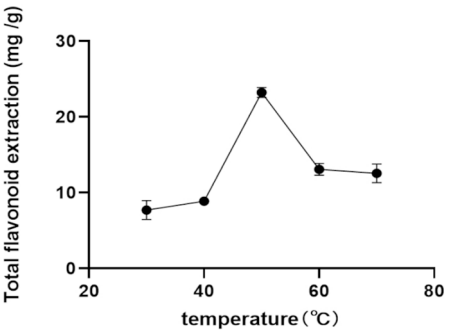

Fig. 6. Relationship between total flavonoid extraction yield and extraction temperature

4.2 Statistical Significance Analysis, Response Surface and Model Fitting of Lotus Leaf Extraction

Determination of Ultrasonic-Assisted Extraction-Response Surface. Based on the single-factor experiment, a central composite experiment with Optimal (Custom) design was adopted, and four factors with significant effects on the flavonoid extraction rate were selected: power (A), extraction time (B), solid-liquid ratio (C), and ethanol concentration (D). Each factor included three levels, encoded as −1, 0, and 1 respectively, as shown in the table.

Table 2. Factors and Levels Methodology in Response Surface

Factor	Level		
	-1	0	1
Power	150	210	270
Time	60	70	80
Solid-liquid Ratio	10	25	60
Ethanol Concentration	0	30	90

Response Surface Experiment and Analysis of Variance (ANOVA) of Lotus Leaf. Response surface regression analysis of Table 2 was performed using Design-Expert 13 software, and the following response surface regression model was obtained:

$$Y=-938.143+2.11395*A+20.8206*B+0.530815*C+2.46439*D+0.00378523*AB+0.000957977*AC+-0.00267741*AD+0.0131636*BC+-0.00527353*BD+-0.00487175*CD+-0.00515856*A^2+-0.152866*B^2+-0.0180143*C^2+-0.0164138*D^2.$$

Table 3. Analysis of Variance (ANOVA) for Each Term of the Regression Equation

Source	SumofSquares	df	MeanSquare	F-value	p-value	
Model	19578.68	14	1398.48	50.36	<0.0001	significant
A-Power	853.19	1	853.19	30.72	0.0002	
Time	298.18	1	298.18	10.74	0.0083	
C-Solid-liquid Ratio	204.62	1	204.62	7.37	0.0218	
D-Ethanol Concentration	320.57	1	320.57	11.54	0.0068	
AB	63.25	1	63.25	2.28	0.1622	
AC	22.9	1	22.9	0.8248	0.3852	
AD	652.42	1	652.42	23.49	0.0007	
BC	110.79	1	110.79	3.99	0.0737	
BD	63.64	1	63.64	2.29	0.161	
CD	292.31	1	292.31	10.53	0.0088	
A^2	1217.43	1	1217.43	43.84	<0.0001	
B^2	839.7	1	839.7	30.24	0.0003	
C^2	356.02	1	356.02	12.82	0.005	
D^2	3374.59	1	3374.59	121.52	<0.0001	
Residual	277.7	10	27.77			
Lack of Fit	191.65	5	38.33	2.23	0.2	Not significant
Pure Error	86.05	5	17.21			
Cor Total	19856.38	24				

It can be concluded from the analysis of variance in Table 3 that the linear terms and quadratic terms of the flavonoid extraction rate equation are extremely significant, indicating that the influence of each specific factor on the response value is not a simple linear relationship. The interaction terms are extremely significant, thus indicating that the interaction between various factors is good, and the entire response surface is composed based on the interaction between various factors. In addition, the extremely significant total regression term proves that the fitting degree of the regression equation is good, indicating that the experiment has high reliability and accuracy; the extremely insignificant lack-of-fit term indicates that the experimental error is very small.

Response Surface Analysis. Response surfaces were plotted to analyze the effects of power (A), extraction time (B), solid-liquid ratio (C), and ethanol concentration (D) on the flavonoid extraction rate. The results are shown in **Figures 7 to 12.**

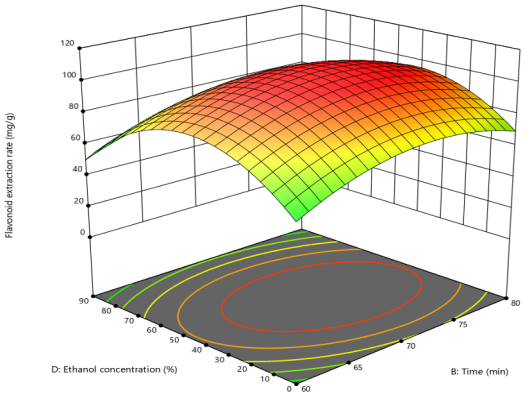

Fig. 7. Response surface for the effects of B and D on flavonoid extraction yield

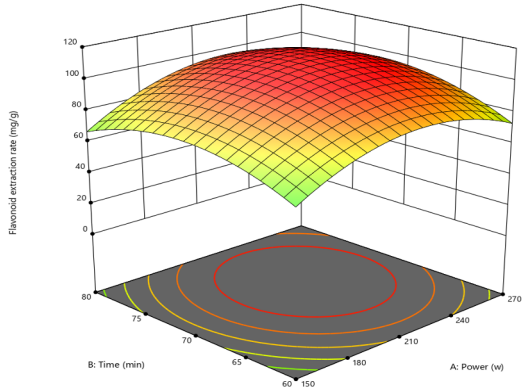

Fig. 8. Response surface for the effects of B and A on flavonoid extraction yield

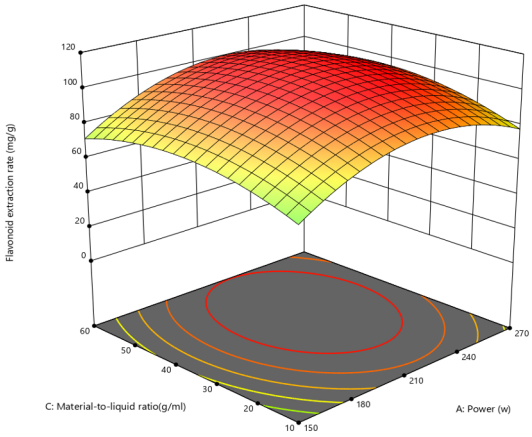

Fig.9. Response surface for the effects of C and A on flavonoid extraction yield

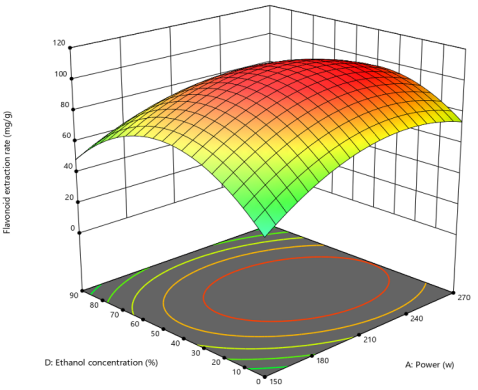

Fig. 10. Response surface for the effects of D and A on flavonoid extraction yield

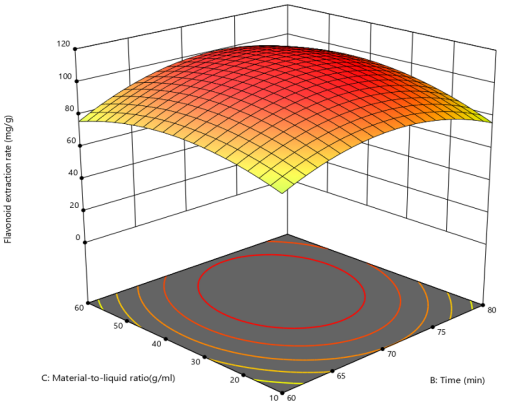

Fig. 11 Response surface for the effects of B and C on flavonoid extraction yield

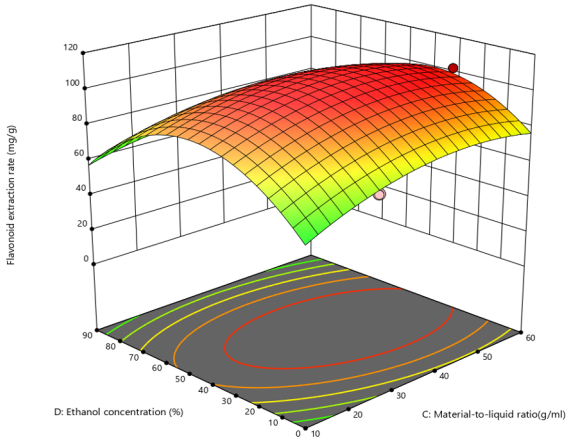

Fig. 12. Response surface for the effects of C and D on flavonoid extraction yield

According to the analysis by Design-Expert 13 software, combined with the response surface plots, contour plots and regression equation, the optimal preparation conditions for flavonoid extraction were determined as follows: ethanol concentration 38%, solid-liquid ratio 1:41, extraction temperature 50℃, extraction time 72min, and ultrasonic power 225W. Verification experiments were carried out under the above optimized conditions, and the total flavonoid extraction yield could reach 108.532mg/g.

4.3 Study on Antioxidant Activity of Lotus Leaf Extract

As shown in Fig 13, the lotus leaf extract exhibits strong inhibitory activity against hydroxyl radicals. At a concentration of 4.69mg/mL, its inhibitory capacity was 41.844U/mL, which was comparable to 45.613U/mL for vitamin C under the same conditions, indicating that lotus leaf extract can be used as a raw material for the development of natural antioxidant active ingredients. As shown in Fig 14, the DPPH radical scavenging capacity of lotus leaf petiole extract decreased with decreasing concentration. At a concentration of 37.50 mg/mL, the scavenging rate was approximately 29%, much lower than the nearly 94% for vitamin C. Even at the highest concentration, its scavenging capacity was still significantly weaker than that of vitamin C. It only showed certain antioxidant activity at high concentrations and has the potential for further study as a natural antioxidant material.

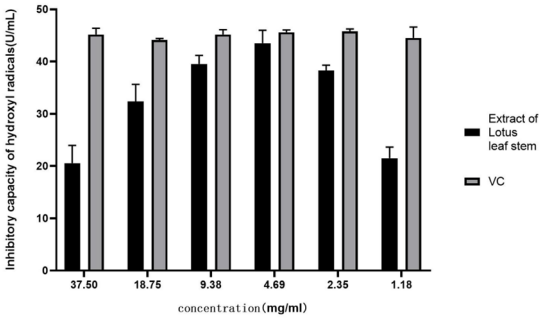

Fig. 13. Ability of Lotus leaf stem extract to inhibit hydroxyl radicals

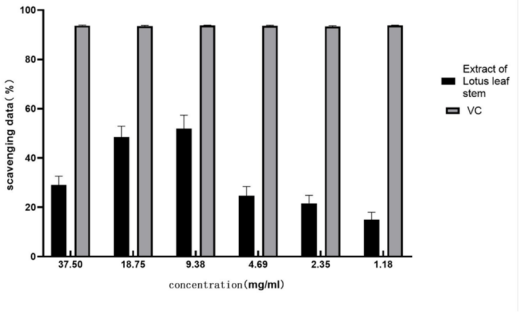

Fig. 14. DPPH Radical Scavenging Activity of Lotus Leaf Petiole Extract

5 Discussion

Oxidative stress refers to a pathological state caused by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system in the body. Excessive ROS, such as hydroxyl radicals (OH·) and DPPH radicals, attack biological macromolecules including proteins, lipids and nucleic acids, causing cell damage, and are closely associated with sub-health, aging, as well as chronic diseases including cardiovascular diseases and tumors [13]. Antioxidants derived from natural plants have become a research hotspot in the field of antioxidation due to their advantages of high safety, wide sources and mild effects. Lotus leaf, a medicinal and edible plant approved by the National Health Commission of China, has been shown by modern research to have antioxidant, anti-inflammatory and cardiovascular protective effects. However, systematic studies on the efficient extraction technology of its constituents and its antioxidant mechanism remain insufficient. Therefore, this study focuses on the optimization of extraction and the evaluation of antioxidant activity of total flavonoids from lotus leaf.

The results of extraction process optimization showed that after ultrasonic-assisted extraction combined with response surface optimization, under the conditions of ethanol concentration 38%, solid-liquid ratio 1:41, extraction temperature 50 ℃, extraction time 72 min and ultrasonic power 225 W, the extraction yield of total flavonoids reached 108.532 mg/g. The advantages of this process are as follows: the cavitation effect generated by ultrasound can rapidly destroy the cell wall structure of lotus leaves, promote solvent penetration, shorten the extraction time and reduce solvent consumption; the mild extraction temperature of 50 ℃ avoids the degradation of thermosensitive flavonoid components, and the polarity of 38% ethanol matches best with flavonoids, which can improve the extraction yield while reducing the dissolution of fat-soluble impurities. Compared with new technologies such as ultra-high pressure extraction and subcritical extraction, this process features low equipment investment and simple operation, making it more suitable for large-scale production and application.

The results of in vitro antioxidant activity assays showed that lotus leaf extract exhibited significant scavenging capacity against hydroxyl radicals (OH·) and DPPH radicals in a concentration-dependent manner. At a concentration of 4.69 mg/mL, its hydroxyl radical inhibition capacity reached 41.844 U/mL, which was comparable to that of the positive control vitamin C (45.613 U/mL), confirming that its antioxidant activity was close to the benchmark of natural antioxidants. Further analysis indicated that the antioxidant mechanism of lotus leaf extract was multi-dimensional: on the one hand, the phenolic hydroxyl groups of flavonoid components could directly bind to free radicals to terminate the oxidative chain reaction; on the other hand, components such as β-sitosterol and oleanolic acid in the extract could chelate metal ions such as Fe^{2+} to reduce the generation of hydroxyl radicals [14][15]; in addition, components such as epicatechin and gallocatechin could enhance the body's own antioxidant defense capacity by regulating the level of intracellular reduced glutathione (GSH) [16]. These mechanisms synergistically constitute the molecular basis for the efficient antioxidant activity of lotus leaf extract.

This study has limitations: First, in vitro antioxidant tests cannot fully mimic the in vivo environment. After digestion, absorption and metabolism, the extract's in vivo antioxidant effect and bioavailability remain unproven, failing to reflect its real activity. Second, chemical characterization of lotus leaf extract is insufficient: only total flavonoids were measured, with no isolation or identification of key flavonoid monomers. In addition, antioxidant evaluation is incomplete, as reducing power was only mentioned in the abstract without detailed data. Future research should focus on in vivo tests, monomer purification and structure-activity relationship analysis to support the high-value utilization of lotus leaves. Future research can focus on in vivo antioxidant experiments, single component separation and purification, and structure-activity relationship analysis to provide a more comprehensive scientific basis for the high-value utilization of lotus leaf resources.

6 Conclusion

In this study, the optimal conditions for extracting total flavonoids from lotus leaves were determined as follows: ethanol concentration 38%, solid–liquid ratio 1:41, extraction temperature 50 °C, extraction time 72 min, and ultrasonic power 225 W. Under these conditions, the extraction yield of total flavonoids reached 108.532 mg/g. In vitro antioxidant experiments confirmed that the lotus leaf extract exhibits significant scavenging capacity against OH^- and DPPH free radicals. This study clarified the efficient extraction process and antioxidant properties of total flavonoids from lotus leaves, providing experimental support for the development and utilization of lotus leaf as a medicinal and edible resource, as well as a reference for high-quality raw materials in the research and development of natural antioxidants.

Acknowledgments

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