International Journal of Public Health and Medical Research
ISSN: 3006-7707 (Print), ISSN: 3006-3752 (Online) | Volume 5, Number 2, Year 2025
DOI: https://doi.org/10.62051/ijphmr.v5n2.06
Journal homepage: https://wepub.org/index.php/IJPHMR/index

Optimization of Ultrasound-Assisted Deep Eutectic Solvent (DES) Extraction Process for Total Flavonoids from Robinia pseudoacacia Flowers and Evaluation of Their Antioxidant Activity

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ABSTRACT

Objective: To establish a green and efficient extraction process for total flavonoids from Robinia pseudoacacia L. flos using ultrasound-assisted deep eutectic solvents (DESs), optimize the process using response surface methodology, and systematically evaluate their in vitro antioxidant activities, providing experimental evidence for the development and utilization of active components in Robinia pseudoacacia flowers. Methods: Choline chloride was used as the hydrogen bond acceptor (HBA), and 1,2-propanediol, ethylene glycol, and lactic acid were used as hydrogen bond donors (HBD) to prepare three DES systems. Single-factor experiments were conducted to investigate the effects of HBA: HBD molar ratio, water content, liquid-to-solid ratio, ultrasonic time, and temperature on the extraction rate of total flavonoids. The extraction conditions were then optimized using Box-Behnken response surface design. High-performance liquid chromatography (HPLC) was used to determine the content of major flavonoid components (rutin, quercetin, and luteolin). The antioxidant capacity was evaluated by DPPH· and ABTS⁺ free radical scavenging rates. Results: Single-factor analysis showed that the highest extraction rate was achieved when the DES was choline chloride-1,2-propanediol (molar ratio 1:3), water-to-DES volume ratio was 3:1, liquid-to-solid ratio was 35:1 (mL/g), ultrasonic temperature was 65 ℃, and ultrasonic time was 40 min. Response surface optimization indicated that the theoretical extraction rate under these conditions was 4.26%, and the average value of the validation experiment was 4.22%, with an error of less than 1%. HPLC analysis revealed that the contents of rutin, quercetin, and luteolin were 2.06, 1.32, and 0.84 mg/g, respectively. Antioxidant experiments showed that when the extract concentration was 0.4 mg/mL, the scavenging rates of DPPH· and ABTS⁺ were 81.45% and 92.30%, respectively, with no significant difference compared to the vitamin C group ($P>0.05$). Conclusion: The established DES-ultrasound-assisted extraction method is green, environmentally friendly, and reproducible, significantly improving the extraction rate of flavonoids from Robinia pseudoacacia flowers. The extract has strong antioxidant capacity, providing a scientific basis for its application in the development of natural antioxidants.

KEYWORDS

Robinia pseudoacacia flowers; Total flavonoids; Deep eutectic solvents; Ultrasound extraction; Antioxidant activity

1. INTRODUCTION

Robinia pseudoacacia L., a member of the Fabaceae family, is known for its fragrant and nutritious inflorescences. Robiniae Pseudacaciae Flos (flowers of Robinia pseudoacacia) has been traditionally used in medicine and dietary therapy for moistening the lungs, relieving cough, clearing heat, detoxifying, activating blood circulation, and cooling the blood. It has also been utilized in the development of cosmetics and food additives. Modern research has shown that Robinia pseudoacacia flowers contain a variety of flavonoid compounds, such as rutin, quercetin, luteolin, and their glycoside derivatives, as well as phenolic acids, sterols, and essential oils, which exhibit significant antioxidant, anti-inflammatory, and antimicrobial activities [1, 2, 3].

Flavonoids are widely occurring polyphenolic secondary metabolites in plants, whose bioactivities mainly originate from their phenolic hydroxyl groups, enabling them to scavenge free radicals, chelate metal ions, and inhibit lipid peroxidation. Traditional extraction methods for flavonoids often employ organic solvents like ethanol and methanol, but these methods have drawbacks such as solvent residues, environmental pollution, and relatively low extraction yields. In recent years, deep eutectic solvents (DESs) have garnered attention due to their designability, low volatility, biodegradability, and high solubility, making them green alternative solvents for applications in the extraction of natural products, catalysis, and drug delivery. In flavonoid extraction, DESs can alter the permeability of plant cell walls through hydrogen bonding networks, thereby facilitating the release of active components. The cavitation effect of ultrasound can further enhance mass transfer processes and improve extraction efficiency. Therefore, ultrasound-assisted DES extraction (UAE-DES) is considered one of the most promising green extraction technologies at present.

Literature has shown that the use of a choline chloride-1, 2-propanediol system can significantly increase the extraction efficiency of flavonoids from Albizia julibrissin, with a total flavonoid yield reaching 4.15%, and its antioxidant capacity is superior to that of ethanol extracts [4]. On the other hand, ethanol extracts of Robinia pseudoacacia flowers are rich in quercetin, rutin, and luteolin, exhibiting strong antioxidant properties [5]. However, there is currently no systematic study on the extraction and antioxidant performance evaluation of Robinia pseudoacacia flowers using DES systems.

Based on this, the present study aims to use choline chloride-1, 2-propanediol as the main DES system to construct an ultrasound-assisted extraction process. Key parameters will be optimized using single-factor and response surface analyses, and the antioxidant properties of the extracts will be evaluated through HPLC quantification and free radical scavenging experiments. The study is intended to provide a green and feasible technological approach for the high-value development of Robinia pseudoacacia flowers.

2. MATERIALS AND METHODS

2.1. Materials

Robinia pseudoacacia flowers were collected from the campus of Jianghan University and authenticated by Deputy Director Bao Xuemei, a traditional Chinese medicine pharmacist from the Authentication Center of Anhui University of Chinese Medicine, as the dried inflorescences of Robinia pseudoacacia L.

Chemical standards: rutin, quercetin, luteolin (purity $\geq$ 98%); Solvents and reagents: choline chloride, 1,2-propanediol, ethylene glycol, lactic acid (analytical grade, Shanghai Macklin); DPPH, ABTS, vitamin C. All solvents were of analytical or chromatographic grade. All chemical reagents were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

2.2. Instruments

Ultrasound cleaner (KQ-500B, Kunshan Ultrasonic Instrument Co., Ltd.), UV-2600 UV-Vis spectrophotometer (Shimadzu, Japan), LC-20A high-performance liquid chromatography system (Shimadzu), constant temperature water bath, electric hot air oven, and precision analytical balance, etc.

2.3. Preparation of DESs

Choline chloride and 1, 2-propanediol were weighed and mixed at a molar ratio of 1:3. The mixture was stirred magnetically in a water bath at 80 ℃ for 30 minutes until a transparent homogeneous liquid was formed. The resulting DES was cooled and stored in a sealed container. DESs of choline chloride-ethylene glycol (1:1) and choline chloride-lactic acid (1:2) were prepared using the same method.

2.4. Determination of Total Flavonoid Content

The determination was based on the rutin standard curve method. A rutin standard solution (0.01 mg/mL) was used, and the absorbance was measured at 510 nm to establish the standard curve: $A = 12.633C + 0.00076$ ($R^2 = 0.9999$). For the sample, 2 mL of the extract was taken, followed by the addition of 0.3 mL of 10% $Al(NO_3)_3$ and 0.3 mL of 5% $NaNO_2$. After standing for 6 minutes, 4 mL of 1 mol/L NaOH was added and reacted for 10 minutes. The absorbance was then measured at 510 nm.

2.5. Single-Factor Experimental Design

To determine the effects of various process parameters on the extraction yield of total flavonoids from Robinia pseudoacacia flowers and to provide a basis for response surface optimization, this study employed a single-factor variable control method under the ultrasound-assisted deep eutectic solvent extraction system. The extraction yield of total flavonoids was used as the evaluation index, and the effects of solvent type, molar ratio of hydrogen bond acceptor (HBA) to hydrogen bond donor (HBD), water content in the solvent, liquid-to-solid ratio, ultrasound extraction time, and ultrasound temperature on the extraction efficiency were systematically investigated.

2.5.1. Selection of Deep Eutectic Solvent Types

Three deep eutectic solvent systems were prepared: choline chloride-ethylene glycol, choline chloride-lactic acid, and choline chloride-1, 2-propanediol. 1.00 g of Robinia pseudoacacia flower samples, which had been dried, ground, and passed through a 60-mesh sieve, were weighed and mixed with 30 mL of each of the different DES systems. The mixtures were then subjected to ultrasonic extraction at 60 ℃ for 30 minutes. After extraction, the mixtures were centrifuged, and the supernatants were collected to determine the content of total flavonoids. The extraction efficiency of different solvent systems was compared to select the appropriate extraction solvent system for subsequent experiments.

2.5.2. Influence of the Molar Ratio of Hydrogen Bond Acceptor to Donor

Based on the choline chloride-1, 2-propanediol system, different solvent systems with molar ratios of 1:2, 1:3, 1:4, 1:5, and 1:6 were prepared. 1.00 g of Robinia pseudoacacia flower powder was weighed and mixed with 30 mL of the corresponding DES solution. The mixtures were subjected to ultrasonic extraction at 60 ℃ for 30 minutes. The total flavonoid content in the supernatants of the extracted samples was determined after centrifugation to compare the extraction efficiency under different HBA: HBD ratios and to determine the optimal ratio.

2.5.3. Effect of Water Content

Under the condition of HBA:HBD = 1:3, DES was mixed with deionized water at volume ratios of 1:2, 1:3, 1:4, and 1:5 to prepare the extraction solvents. 1.00 g of Robinia pseudoacacia flower powder was weighed and mixed with 30 mL of the corresponding mixed solution. The mixtures were subjected to ultrasonic extraction at 60 °C for 30 minutes. The extraction yield of total flavonoids under different water contents was determined to identify the appropriate water:DES ratio for optimization experiments.

2.5.4. Influence of Liquid-to-Solid Ratio

The liquid-to-solid ratio (i.e., the ratio of solvent volume to the mass of herbal material) is an important factor affecting extraction efficiency. Under the conditions of ultrasonic temperature at 65 °C and ultrasonic time of 40 minutes, the liquid-to-solid ratios were set at 15, 25, 35, and 45 mL/g. Different amounts of Robinia pseudoacacia flower powder were weighed and mixed with the choline chloride-1,2-propanediol (1:3, water:DES = 3:1) system solvent according to the corresponding liquid-to-solid ratios. After ultrasonic extraction, the yield of total flavonoids was determined, and the differences among the groups were compared to select the appropriate liquid-to-solid ratio.

2.5.5. Effect of Ultrasound Extraction Time

Under consistent solvent system and other conditions, the ultrasound extraction time was set at 15, 25, 35, 40, 50, and 55 minutes. 1.00 g of Robinia pseudoacacia flower powder was weighed and mixed with 30 mL of the choline chloride-1,2-propanediol (1:3) system (water content 3:1). The mixture was subjected to ultrasonic extraction at 60 °C. The supernatant was collected after centrifugation at each time point to determine the content of total flavonoids, in order to investigate the effect of ultrasound exposure time on extraction efficiency and to identify the appropriate extraction time.

2.5.6. Effect of Ultrasound Temperature

Temperature significantly affects both extraction efficiency and chemical stability. Under the fixed conditions of a liquid-to-solid ratio of 35 mL/g and ultrasound time of 40 minutes, the temperature was set at 40, 50, 55, 60, 65, and 70 °C. 1.00 g of Robinia pseudoacacia flower powder was weighed and mixed with 30 mL of the choline chloride-1,2-propanediol (1:3, water:DES = 3:1) system solvent. The mixture was subjected to ultrasonic extraction at different temperatures, and the content of total flavonoids was determined. The appropriate ultrasound temperature was selected by comparing the extraction results under different temperature conditions.

2.6. Response Surface Optimization Design

Based on the results of the single-factor experiments, ultrasound time (A), temperature (B), and liquid-to-solid ratio (C) were selected as independent variables, with the yield of total flavonoids (Y) as the response value. A three-factor, three-level Box-Behnken design (BBD) was employed, consisting of 17 experimental data points. The data were analyzed using Design-Expert 10.0 to establish a quadratic regression model and to plot the response surfaces.

2.7. HPLC Analysis

Chromatographic conditions: C18 column (4.6 mm × 250 mm, 5 μm); mobile phase: methanol (A)–0.1% phosphoric acid aqueous solution (B) with gradient elution (0–5 min, 60% A; 10 min, 50% A; 20 min, 40% A; 30 min, 35% A; 40 min, 90% A); flow rate 1 mL/min; detection wavelength 360 nm; column temperature 35 °C; injection volume 10 μL. The contents of rutin, quercetin, and luteolin were calculated using the external standard method.

2.8. Antioxidant Activity Assays

2.8.1. DPPH Radical Scavenging Assay

100 μL of sample solution at different concentrations (0.2–1.0 mg/mL) was mixed with 100 μL of DPPH ethanol solution. The mixture was reacted in the dark for 30 minutes, and the absorbance was measured at 517 nm.

2.8.2. ABTS$^+$ Radical Scavenging Assay

The ABTS$^+$ solution was prepared by mixing 7 mM ABTS with 2.45 mM $K_2S_2O_8$. After reacting in the dark for 12 hours, the solution was diluted with PBS to achieve an absorbance (A_{734}) of 0.70 ± 0.02. 100 μL of sample solution was mixed with 150 μL of ABTS$^+$ solution. The mixture was reacted at room temperature for 30 minutes, and the absorbance was measured at 734 nm. The scavenging rate was then calculated.

3. RESULTS AND DISCUSSION

3.1. Selection of DES Systems

Among the three DESs, the choline chloride-1, 2-propanediol system exhibited the highest extraction yield (3.86%), which was significantly better than the ethylene glycol (3.42%) and lactic acid systems (3.33%) (Table 1). The 1, 2-propanediol system has moderate viscosity and higher polarity, which allows it to form stable hydrogen bonds with flavonoid molecules, thereby enhancing solubility.

Table 1. Effects of Different Deep Eutectic Solvent Systems on the Extraction Yield of Total Flavonoids from Robinia pseudoacacia Flowers (n=3, ± s)

DES System	HBA:HBD Molar Ratio	Extraction Yield (%)
Choline chloride-ethylene glycol	1:1	3.42 ± 0.06
Choline chloride-lactic acid	1:2	3.33 ± 0.04
Choline chloride-1, 2-propanediol	1:3	3.86 ± 0.05
Choline chloride-1, 2-propanediol	1:4	3.64 ± 0.03
Choline chloride-1, 2-propanediol	1:5	3.21 ± 0.07

3.2. Single-Factor Results

When the HBA:HBD ratio increased from 1:2 to 1:3, the extraction yield increased from 3.55% to 3.92%. A higher ratio led to increased solution viscosity, which was detrimental to diffusion. The extraction yield was highest (4.03%) when the water-to-DES volume ratio was 3:1. Excess water weakened the hydrogen bond network structure. Both ultrasonic temperature and time significantly affected the extraction yield, with a peak at 65 ℃ and 40 minutes. Further increasing the temperature might lead to partial degradation of flavonoids. The extraction was sufficient at a liquid-to-solid ratio of 35 mL/g, and further increase had limited effect.

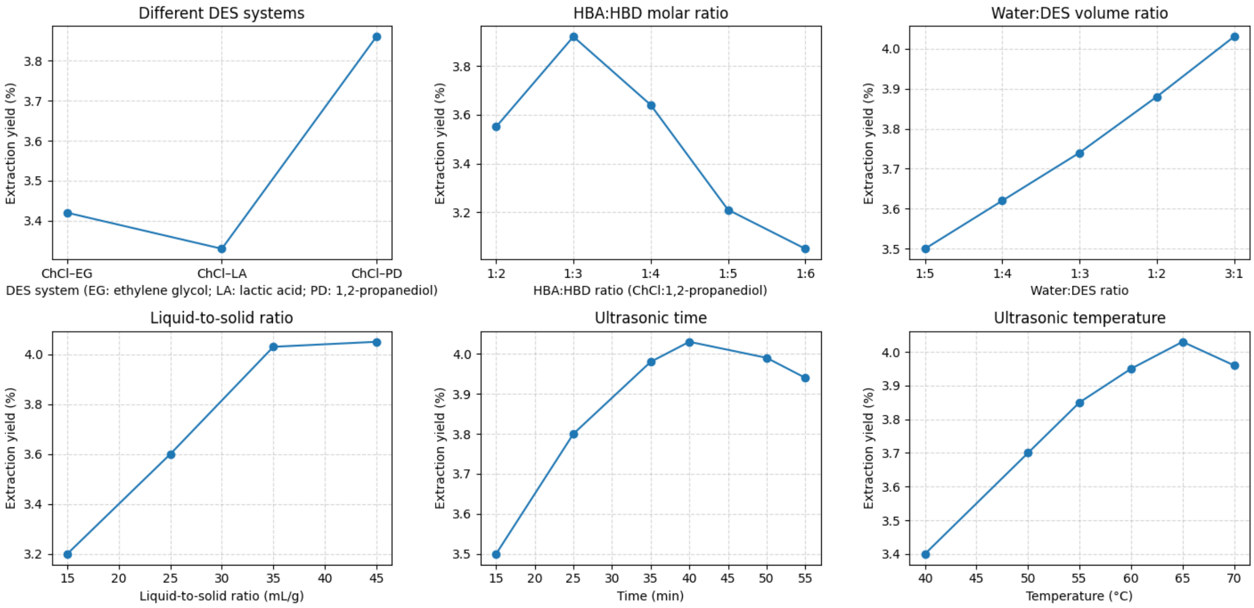

Figure 1. Effects of single extraction factors on the yield of total flavonoids from Robinia pseudoacacia flowers using ultrasonic-assisted deep eutectic solvent (DES) extraction

Each point represents the mean of three independent experiments (mean ± SD). The optimal extraction conditions determined from single-factor tests were: ChCl–1, 2-propanediol (1:3) as the DES, water: DES = 3:1, liquid-to-solid ratio = 35 mL g^{-1}, ultrasonic time = 40 min, and temperature = 65 °C [4].

3.3. Response Surface Model Analysis

The response surface experimental design and results were based on the Box-Behnken experimental scheme, and the results are shown in Table 2.

Table 2. Response Surface Design and Results (Box-Behnken Design, n=17)

Run No.	A: Time (min)	B: Temperature (°C)	C: Liquid-to-Solid Ratio (mL·g^{-1})	Measured Extraction Yield (%)
1	35	60	30	3.80
2	40	60	35	3.85
3	35	65	25	3.97
4	30	60	25	3.76
5	40	65	30	4.07
6	30	55	30	3.54
7	35	55	35	3.58
8	40	55	25	3.75
9	30	65	30	3.92
10	35	60	35	3.84
11	40	60	25	3.90
12	35	65	35	4.05
13	35	55	25	3.74
14	40	65	35	4.06
15	30	60	35	3.83
16	35	60	30	3.85
17	35	60	30	3.84

The analysis of variance (ANOVA) showed that the model was highly significant ($P < 0.0001$), while the lack-of-fit term was not significant ($P = 0.23$), with $R^2 = 0.970$, indicating a good fit of the model. The regression equation is:

$$Y=4.03+0.072A+0.16B-0.031C-0.025AB+0.058BCY = 4.03 + 0.072A + 0.16B - 0.031C - 0.025AB + 0.058BCY=4.03+0.072A+0.16B-0.031C-0.025AB+0.058BC$$

The order of significance for the three factors is temperature (B) > time (A) > liquid-to-solid ratio (C). The response surface showed a significant interaction effect between temperature and time, while the effect of liquid-to-solid ratio was relatively weaker. The optimized theoretical conditions were as follows: temperature 65 ℃, time 40 min, and liquid-to-solid ratio 35 mL/g. The predicted yield was 4.26%, and the validated measured value was 4.22%, with a relative error of less than 1%, indicating that the model is feasible.

3.4. HPLC Results

At a wavelength of 360 nm, the retention times of rutin, quercetin, and luteolin were 17.2, 23.5, and 28.7 minutes, respectively. The peaks were symmetrical and well-separated. Their contents were 2.06, 1.32, and 0.84 mg/g, respectively, which were consistent with the total flavonoid content determined by the colorimetric method. Compared with traditional ethanol extraction, the average content of the main flavonoids was increased by 27.5%, indicating that the DES system has a significant advantage in the extraction of polyphenolic compounds [6].

3.5. Antioxidant Activity

The DES extract of Robinia pseudoacacia flowers exhibited strong free radical scavenging ability in both DPPH· and $ABTS^+$ systems, with a concentration-dependent relationship. At a concentration of 0.4 mg/mL, the scavenging rate of DPPH· was 81.45%, and the scavenging rate of $ABTS^+$ reached 92.30%, which was not significantly different from the vitamin C group (95.2%) ($P>0.05$). The results indicate that the extract has strong antioxidant capacity, which is likely attributed to the ortho-dihydroxy substitutions in the flavonoid structure (such as the 3′,4′-OH in the B ring of quercetin) and the stable free radical-scavenging ability of the glycosylated rutin.

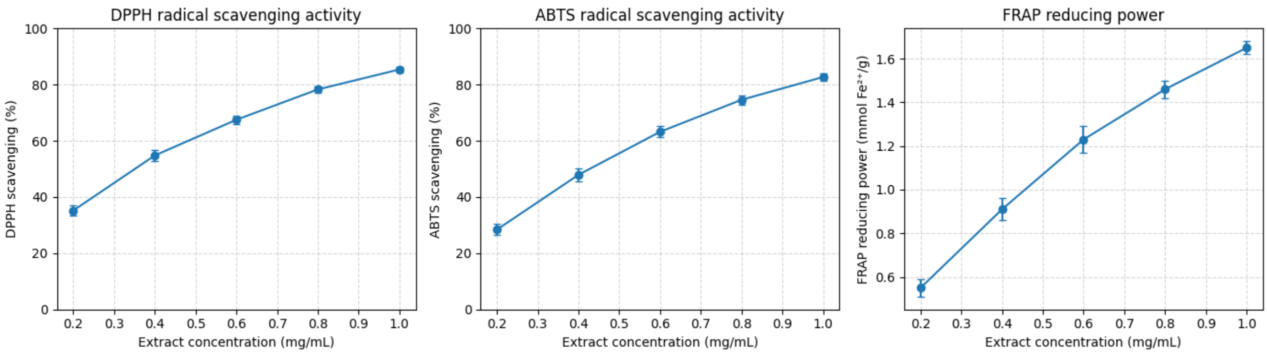

Figure 2. Antioxidant activities of total flavonoid extract from Robinia pseudoacacia flowers obtained under the optimized DES extraction conditions.

Each point represents the mean ± SD (n = 3). The extract exhibited a concentration-dependent increase in free-radical scavenging and reducing capacity, indicating strong antioxidant potential of the DES-extracted flavonoids.

4. DISCUSSION

The green designability of DESs gives them a significant advantage in the extraction of natural products. The results of this study indicate that the choline chloride-1, 2-propanediol system not only increased the dissolution rate of flavonoids but also reduced the use of organic solvents, in line with the principles of green chemistry.

In terms of mechanism, the hydrogen bond network in DESs can disrupt the cellulose structure of plant cell walls, allowing the release of intracellular flavonoids. The low viscosity and polarity matching of 1,2-propanediol enhance the diffusion of target molecules. The micro-jets generated by ultrasonic cavitation can further rupture the cell walls, accelerating the migration of solutes to the solvent, with a significant synergistic effect [7][6].

From a chemical composition perspective, the flavonoids in Robinia pseudoacacia flowers are mainly polyphenolic compounds, with quercetin and rutin having strong reducing capabilities that can directly scavenge DPPH· and $ABTS^{+}$ free radicals. Luteolin, on the other hand, blocks free radical chain reactions through hydrogen donation. The antioxidant effect of the DES extract is slightly lower than that of vitamin C but far higher than that of traditional ethanol extracts, indicating that it effectively retains the structural integrity of the active components.

Furthermore, this study established a reproducible mathematical model through response surface optimization, providing a reference for the extraction of flavonoids from other flower-based medicinal materials (such as Albizia julibrissin flowers and Sophora japonica buds). Future research could further explore the effects of DES composition on the biological activity, toxicological safety, and recyclability of extracts to achieve industrial promotion.

5. CONCLUSIONS

This study established an optimized method for the extraction of total flavonoids from Robinia pseudoacacia flowers using ultrasound-assisted deep eutectic solvents (DESs). Through a combination of single-factor experiments and response surface analysis, the effects of solvent type, molar ratio, water content, liquid-to-solid ratio, ultrasound time, and temperature on the extraction yield were systematically investigated. The optimized results indicated that using choline chloride-1,2-propanediol (molar ratio 1:3) as the extraction system, with a water-to-DES volume ratio of 3:1, liquid-to-solid ratio of 35 mL/g, ultrasound time of 40 minutes, and ultrasound temperature of 65 °C, the yield of total flavonoids could reach 4.06%, which is significantly higher than that obtained by traditional organic solvent extraction. The antioxidant activity test results showed that the extract exhibited a concentration-dependent increase in DPPH and ABTS free radical scavenging rates and FRAP reducing power, indicating that the flavonoid components extracted by DES have strong antioxidant capacity. In summary, the DES system can be used as a green, efficient, and stable medium for the extraction of plant active components, providing scientific evidence and technical references for the industrial utilization of flavonoid compounds from Robinia pseudoacacia flowers and their development and application in the fields of food, medicine, and cosmetics.

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