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Efficient Extraction of Flavonoids from Lotus Leaves by Ultrasonic-Assisted Deep Eutectic Solvent Extraction and Its Evaluation on Antioxidant Activities

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Abstract: The discovery of a green extraction solvent for natural plants could promote related research. In this study, deep eutectic solvents (DES) were used as green solvents coupled with an ultrasound-assisted extraction method (UAE) to extract flavonoids from lotus leaves. Thirty-four different DES were performed and choline chloride/urea with 40% water was chosen as the most promising one, and the related parameters in the procedures were optimized, resulting in the highest extraction amount of flavonoids in lotus leaves. D-101 was selected from four macroporous resins to separate the flavonoids from DES. Moreover, DES could be recycled and efficiently reused four times with satisfactory performances. In addition, the lotus leaf flavonoids from the DES extract exhibited antioxidant activities in five kinds of assays including DPPH, ABTS, Fe^{3+} reducing, FRAP, and Fe^{2+} chelating. It also showed antibacterial activities on *Staphylococcus aureus* and *Escherichia coli* bacterial strains with minimal inhibitory concentrations at 1666 μg/mL and 208 μg/mL, respectively. In the HPLC analysis, the three main components in the DES extract were identified as astragalin, hyperoside, and isoquercitrin. In conclusion, the developed UAE-DES followed by macroporous resin treatment could become an efficient and environmentally friendly extraction and enrichment method for flavonoids from lotus leaves and other natural products.

Keywords: antioxidant; deep eutectic solvents; flavonoids; lotus leaves; ultrasound-assisted

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1. Introduction

Lotus leaves are the leaves of *Nelumbo nucifera* Gaertn., which are considered to be both foods and traditional medicines in China [1]. Lotus leaves have had a great vogue as drinks such as tea or in herbal formulations and have had a lot of recognition [2]. As reported by many papers, they contain a variety of alkaloids and flavonoids, which made them possess antioxidant, antiviral, anti-obesity, and bacteriostatic functions [3,4]. To develop better lotus-leaf-based products, extraction of lotus leaves could provide more specific materials (such as flavonoids) for research and industrial purposes [5]. Hence, it is necessary to apply a novel extraction method to promote research on lotus leaves.

As in Abbott's first report, transparent and uniform liquids called deep eutectic solvents (DES) could be formed by mixing ammonium salts and amides at low temperatures [6]. After a series of research, DES are typically composed of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) by intermolecular hydrogen bonding [7]. The resulting DES showed a low melting point, which was lower than that of each pure component. Compared with traditional organic solvents, DES exhibited various characteristics such as low cost, simple preparation, less volatility, and non-toxicity, which made it widely used in the extraction of natural, biologically active components in recent years [8,9]. Choline chloride has often been applied as an HBA in DES for the extraction of flavonoids [10]. In Jalel Labidi's report, choline chloride/1,4-butanediol (75 wt%) showed the highest extraction ability for flavonoids (383 mg CE/g dried bark extract) and the highest antioxidant capacity [11]. In addition, a study optimized a ternary combination

in DES with choline chloride/betaine hydrochloride/ethylene glycol with the mole ratio of 1:1:2 and 20% water content in a negative pressure cavitation-assisted extraction method for flavonoids from *Equisetum palustre* L. [12]. In Weng's research, the flavonoids in *Spirodela polyrrhiza* were rapidly extracted by a DES-based microwave-assisted extraction method using choline chloride/levulinic acid with the mole ratio of 1:2 as the DES [13].

The DES could be combined with some assisted extraction technology such as microwave, ultrasonic, and vortex technology [14,15]. These combination methods could improve extraction efficiency and capacity. Among them, ultrasound-assisted extraction (UAE) could promote the solvent to quickly penetrate the plant material and enhance the diffusion of compounds [16]. The instrument condition of UAE was relatively easy to control in the lab compared to the other assisted methods. In recent years, UAE-DES has been applied in the extraction of many natural plants [17–19]. However, these methods still needed selection and optimization for different plant samples due to their complexity and composition. Considering the many benefits of flavonoids and their abundance in lotus leaves and the fact that very little research has been devoted to the utilization of UAE-DES in lotus leaves, the present research aimed to extract flavonoids by an ultrasonic-assisted DES method.

For this purpose, choline chloride, benzyltriethylammonium chloride, betaine, and guanidine-hydrochloride-based DES were prepared to extract flavonoids from lotus leaves to the maximum extent with the assistance of ultrasonic technology (UAE-DES). Then, the flavonoid extract was separated by macroporous resin from the DES, and the antioxidant capacities and antibacterial activities of the extracted flavonoids were systematically studied with beneficial results. Through this study, an effective strategy was provided to extract flavonoids in lotus leaves, which had green and convenient advantages compared to traditional methods. The lotus leaf flavonoids also exhibited antioxidant activities, making them promising in the healthcare area.

2. Materials and Methods

2.1. Materials

Dried lotus leaves were bought from Mingxing Biotechnology Co., LTD. in Bozhou, Anhui. The dried leaves were pulverized and sieved in 40 mesh sieves before use. Choline chloride, benzyltriethylammonium chloride, betaine, guanidine hydrochloride, ethylene glycol, triethylene glycol, glycerol, 2-chloropropionic acid, malonic acid, lactic acid, formic acid, acetic acid, and urea were purchased from Macklin Chemical Reagent Co. (Shanghai, China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), tri-2-pyridyl-s-triazine (TPTZ), and Folin–Ciocalteu were acquired from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The *Staphylococcus aureus* and *Escherichia coli* bacterial strains were provided by Professor Qi Yang from the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences (Changsha, China). All of the other chemicals were of analytical grade.

2.2. Preparation of DES

Choline chloride, benzyltriethylammonium chloride, betaine, and guanidine hydrochloride were selected as the HBA to synthesize DES with different HBDs (ethylene glycol, triethylene glycol, glycerol, 2-chloropropionic acid, malonic acid, lactic acid, formic acid, acetic acid, urea, oxalic acid, and acetamide). The reagents were weighed with the molar ratio at 1:2 (0.2 mol:0.4 mol) and manually stirred at 80 °C for 2 h until a homogeneous liquid formed. When the DES cooled to room temperature, they were stored at ambient temperature.

2.3. Extraction of Flavonoids by the UAE-DES Method

A dried lotus leaf (1.0 g) was powdered and added to the DES (20.0 mL) in a centrifuge tube. The sample was ultrasonically treated by an ultrasonic cleaner (Kunshan Ultrasonic Instrument, Suzhou, China) for 5 min and then extracted in an oscillator at 60 °C under

shaking for 60 min. After extraction, the mixture was centrifuged at 4000 r/min for 15 min. The supernatant was taken for further analysis.

To investigate the effect of factors on the extraction efficiency of the flavonoids, the detailed conditions including the ultrasonic time (0 to 30 min), extraction time (10 to 140 min), solid–liquid ratio (1:20 to 1:80), and extraction temperature (40 to 120 °C) were optimized in single-factor experiments. All of the experiments were carried out in triplicate and the values were expressed as means ± SD.

2.4. Determination of the Total Flavonoids Content

The total flavonoid content of the extract was determined by a colorimetric method with some modifications [20]. Briefly, 10 µL of DES extract was mixed with 25 µL of sodium acetate solution (1.0 mol/L), 25 µL of aluminum chloride solution (10%, wt%), and 1940 µL of ethanol solution (50%, *v*:*v*). The mixture was thoroughly mixed and incubated at 25 °C for 30 min. After that, the absorbance of the mixture was measured at 415 nm by a UV-2700 UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan). Rutin was used as the standard in this analysis and the results were expressed as milligrams of rutin equivalent per gram of extract.

2.5. Recovery of Flavonoids from the DES Extract

The recovery of flavonoids from the DES extract was carried out by adsorption and desorption by macroporous resins. The pretreatment of macroporous resin was performed before usage as in Li's report [21]. Ten mL of DES extracts were put into a flask containing 2.0 g D101 macroporous resin. The adsorption was conducted in an oscillator under shaking (200 rpm) at 25 °C for 6 h. Then, the resin remained, was washed with water, and then desorbed with 10 mL of ethanol at 25 °C and 200 rpm for 2 h in an oscillator under shaking.

2.6. Evaluation of Antioxidant Activities

2.6.1. DPPH Assay

In brief, fresh DPPH solution (0.1 mmol/L) was prepared in ethanol. Two mL of sample solution (1–20 mg/mL) was mixed with DPPH solution and incubated in a 25 °C constant temperature water bath for 30 min. Then, the absorbance of the mixture at 517 nm was measured with a spectrophotometer. The scavenging rate of DPPH radicals was calculated as in the previously reported equation [22].

2.6.2. ABTS Assay

The ABTS assay was measured as previously described [22]. ABTS stock solution (7 mmol/L of ABTS solution mixed in 2.45 mmol/L of $K_2S_2O_8$ solution with equal volume) was incubated for 12 h in the dark and diluted for use. One mL of sample solution (1–20 mg/mL) was mixed with 2 mL of ABTS working solution and incubated in the dark for 6 min. After that, the absorbance of the mixture at 734 nm was measured. The scavenging rate of ABTS radicals was calculated as the equation reported by the reference.

2.6.3. Fe^{3+} Reducing Assay

The Fe^{3+} reducing ability of the extract was investigated by the reduction of $K_3[Fe(CN)_6]$ method [23]. One mL of the sample solution (1–20 mg/mL) was added into 2 mL of phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferricyanide solution (1%, *w*/*v*) and then incubated at 50 °C for 20 min. Then, 2 mL of water, 2 mL of trichloroacetic acid (10%, *w*/*v*), and 0.1 mL of $FeCl_3$ (0.1%, *w*/*v*) were respectively added into the mixture. The absorbance of the final solution was recorded at 700 nm in a spectrophotometer. Rutin was used as standard (0.1–0.5 mg/mL) and the results were expressed as milligrams of rutin equivalents concentration (mg RE/mL).

2.6.4. Ferric Ion Reducing Antioxidant Power (FRAP) Assay

According to Tungjai's report, TPTZ solution (10 mmol/L), sodium acetate solution (300 mmol/L), and $FeCl_3$ solution (20 mmol/L) were mixed in the proportion of 10:1:1 (*v*:*v*:*v*) as the working solution. Then, 0.1 mL of the sample solution (1–20 mg/mL) was mixed in 2 mL of water and 3 mL of the FRAP working solution and incubated at 37 °C for 50 min. After that, the absorbance of the mixture was measured at 596 nm. $FeSO_4$ solution was used for the calibration curve and the results were expressed as the FRAP value (mmol/L Fe^{2+}) [24].

2.6.5. Fe^{2+} Chelating Assay

The metal chelating ability of the sample was conducted according to Buravlev et al. with some modifications [25]. A measurement of 0.1 mL of $FeSO_4$ solution (2 mmol/L) and 1 mL of sample solution (1–20 mg/mL) were added to 4 mL of water. Two hundred μL of ferrozine solution (5 mmol/L) was added to start the reaction. The mixture was incubated at room temperature for 10 min and monitored the absorption at 562 nm. The results were given as the percentage of inhibition.

2.7. Antibacterial Activity

The diameter of the inhibition zone (DIZ) was determined by the paper disc diffusion method as previous reports have described [26]. Bacteria suspensions were cultured in a nutrient broth at 37 °C for 48 h and diluted to about 1×10^{-6} CFU. One hundred μL of bacterial suspensions was evenly spread on an agar plate. Then, sterilized filter paper discs (Φ = 6 mm) were soaked in samples (50 mg/mL and 100 mg/mL) and then carefully placed on the plates. The plates were incubated at 37 °C for 24 h, and the DIZ was measured by a vernier caliper. Rutin was used as a positive control, and a solvent without samples was used as a negative control. The DIZ values were expressed in millimeters and values < 6.00 mm were considered as no inhibition zone (NIZ).

The minimal inhibitory concentration (MIC) was determined by the micro-dilution broth method with some modification [26]. five hundred μL of nutrient broth and 50 μL of samples were mixed in a tube. Then, the bacterial solution (1×10^{-6} CFU) was added to the tube and incubated at 37 °C for 12 h. The absorbance at 600 nm of the bacterial suspensions was monitored by a spectrophotometer at predetermined time intervals. Rutin was used as the positive control and the nutrient broth was used as a negative control. The lowest concentration of the sample inhibiting the growth of bacteria was defined as the MIC value.

2.8. HPLC Analysis

The components analysis of the DES extract was carried out on an Agilent 1260 HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). The chromatographic separation was performed on an Agilent ZORBAX C18 column (150 mm × 4.6 mm i.d.; 5 μm, Santa Clara, CA, USA). The mobile phase consists of solvent A (0.1% *v*/*v* acetic acid solution) and solvent B (acetonitrile containing 0.1% *v*/*v* acetic acid) with gradient elution mode: 0–4 min, 10% B; 4–25 min, 10–20% B; and 25–30 min, 20–20% B. The flow rate, temperature, injection volume, and detection wavelength were controlled at 1.0 mL/min, 25 °C, 5 μL, and 254 nm, respectively.

2.9. Statistical Analysis

The statistical analysis was performed by SPSS V20 software (IBM SPSS Statistics 20, New York, NY, USA). The data were then compared using Duncan's multiple range tests at 5% significance levels, and one-way ANOVA with Tukey's post-hoc test [27,28]. Values followed by different letters in the same column are significantly different according to Duncan's test ($p < 0.05$).

3. Results and Discussion

3.1. Selection of DES

The extraction of active compounds using DES was easily influenced by many conditions related to the target compounds and the composition of DES. A lot of research used one or two HBAs for selection [29,30]. While to select appropriate DES on a larger scale for the full extraction and dissolution of flavonoids in lotus leaves, the extraction efficiencies of 34 DES belonging to four kinds of HBA were investigated. The extraction was completed in the same ultrasonic condition and molar ratio of HBA/HBD, and the results are recorded in Table 1. The highest flavonoid yield by DES was achieved when the DES was prepared by choline chloride and urea as in HBA and HBD. The DES system using choline chloride and urea often exhibited the best extraction capacity for flavonoids and polyphenols in natural products. Choline chloride–lactic acid had the best extraction effect among forty groups of DES in the extraction of flavonoids from *Dendrobium officinale* [31]. Choline chloride/levulinic acid (1:2, mol/mol) could be applied as the solvent for extracting the flavonoids in *Spirodela polyrrhiza* [13]. Choline chloride/1,4-butanediol exhibited the best extraction efficiency from seven different DES for extracting valuable bioactive compounds from Chinese wild rice [32]. The finding in this study was also in accordance with several reports [11,33,34]. Therefore, the optimal DES type for the extraction of flavonoids in lotus leaves was set as choline chloride and urea.

Table 1. The extraction efficiencies of flavonoids with different DES types.

No.	HBA	HBD	Molar Ratio	Extraction Efficiency (mg/g)
1	Choline chloride	Ethylene glycol	1:2	114.9 ± 2.5^{d}
2		Triethylene glycol	1:2	91.5 ± 1.2^{h}
3		Glycerol	1:2	49.6 ± 1.2^{p}
4		2-Chloropropionic acid	1:2	33.7 ± 0.1^{rs}
5		Malonic acid	1:2	36.3 ± 0.4^{r}
6		Lactic acid	1:2	54.1 ± 1.5^{o}
7		Formic acid	1:2	81.4 ± 0.7^{l}
8		Acetic acid	1:2	88.3 ± 0.8^{ij}
9		Urea	1:2	178.9 ± 2.8^{a}
10	Benzyltriethylammonium chloride	Ethylene glycol	1:2	82.1 ± 0.9^{kl}
11		Triethylene glycol	1:2	100.9 ± 1.7^{g}
12		Glycerol	1:2	92.2 ± 1.3^{h}
13		2-Chloropropionic acid	1:2	49.6 ± 0.4^{p}
14		Malonic acid	1:2	49.3 ± 0.5^{p}
15		Lactic acid	1:2	83.4 ± 1.4^{kl}
16		Formic acid	1:2	154.9 ± 2.7^{b}
17		Acetic acid	1:2	48.0 ± 0.9^{p}
18		Oxalic acid	1:2	107.4 ± 0.8^{f}
19		Acetamide	1:2	30.7 ± 0.2^{st}
20	Betaine	Glycerol	1:2	87.6 ± 0.6^{ij}
21		Malonic acid	1:2	73.3 ± 0.4^{m}
22		Lactic acid	1:2	97.7 ± 1.0^{g}
23		Formic acid	1:2	84.4 ± 0.5^{g}
24		Acetic acid	1:2	113.3 ± 1.1^{d}
25		Urea	1:2	59.3 ± 0.4^{n}
26		Oxalic acid	1:2	39.8 ± 0.1^{q}

Table 1. *Cont.*

No.	HBA	HBD	Molar Ratio	Extraction Efficiency (mg/g)
27	Guanidine hydrochloride	Ethylene glycol	1:2	99.3 ± 0.9^{g}
28		Triethylene glycol	1:2	105.2 ± 1.1^{f}
29		Glycerol	1:2	85.3 ± 0.7^{jk}
30		2-Chloropropionic acid	1:2	83.7 ± 0.4^{kl}
31		Lactic acid	1:2	111.4 ± 3.0^{e}
32		Formic acid	1:2	98.7 ± 1.0^{g}
33		Acetic acid	1:2	29.1 ± 0.8^{t}
34		Acetamide	1:2	138.9 ± 2.5^{c}

Values followed by different letters ($^{a–t}$) in the same column are significantly different according to Duncan's test ($p < 0.05$).

3.2. Optimization of Extraction Parameters

3.2.1. Effect of the HBA/HDB Ratio

The variation of the HBA/HBD ratio would affect the viscosity and surface tension of the DES solution and the resulting different extraction capacities of flavonoids. To improve the extraction ability of DES, the extraction efficiencies of flavonoids with DES in different HBA/HBD ratios were investigated (Figure 1a). With the increasing HBA/HBD ratio, the extraction efficiencies of flavonoids first increased and then decreased. The highest extraction efficiency of 195.2 mg/g was obtained when the ratio was 1:2, which was then set as the optimal HBA/HBD ratio for preparing DES. This molar ratio (1:2) of HBA/HBD has often been selected in a lot of research and can be supported [29,35,36].

3.2.2. Effect of Water Content in DES

The water content in DES could change the viscosity and polarity of DES. The appropriate water content of DES would decrease the solvent viscosity, improve mass transfer efficiency, and increase the extraction yield of polyphenol and flavonoids [37]. In this study, the extraction efficiencies of various DES with water contents from 10% to 50% were conducted. With the results shown in Figure 1b, when the water content increased up to 40%, the extracted flavonoid amount improved, which was because of the reduction of the viscosity of solvents and the weakening of the hydrogen bonds [38]. When the water content surpassed this value, the extracted flavonoid amount started to drop off. Hence, 40% water content in DES was chosen since this was the highest extraction efficiency for the flavonoids. In Gan's research, 40% water content in DES was chosen for the extraction of polyphenols from green tea as well [39].

3.2.3. Effect of Ultrasonic Time

The influence of ultrasonic time ranged from 0 to 30 min on the extraction efficiency was monitored. It was observed in Figure 1c that the extraction amount of flavonoids increased with the increasing of the ultrasonic time before 10 min and then was not improved with longer ultrasonic time, which indicated that a dynamic balance was reached during the ultrasonic-assisted extraction. Thus, 10 min was chosen for the extraction in further experiments taking into account the extraction efficiency and energy saving.

3.2.4. Effect of the Solid–Liquid Ratio

The effect of the solid–liquid ratio (g/mL) on the extraction efficiency of total flavonoids was monitored. As indicated in Figure 1d, the extraction efficiency of flavonoids increased with an increasing solid–liquid ratio from 1:20 to 1:80 and then decreased slightly. As a result, a 1:80 solid–liquid ratio was selected for later study.

3.2.5. Effect of Temperature

As the literature has reported, high temperatures could reduce the viscosity and surface tension of DES as well, and then improve the mobility and solubility of compounds [40]. To obtain the optimal extraction ability, the effect of different temperatures in the range of 40–120 °C on the extraction efficiency of flavonoids was studied (Figure 1e). As the temperature increased from 40 °C to 60 °C, the extraction amount of flavonoids increased up to a maximum value, but a decrease was then observed at temperatures higher than 60 °C. The decrease in high temperature might be attributed to the elevated temperature increasing the oxidation of flavonoid compounds. This trend can be observed in Wang's and Weng's reports as well [13,41]. Accordingly, a moderate temperature (60 °C) was set for the extraction.

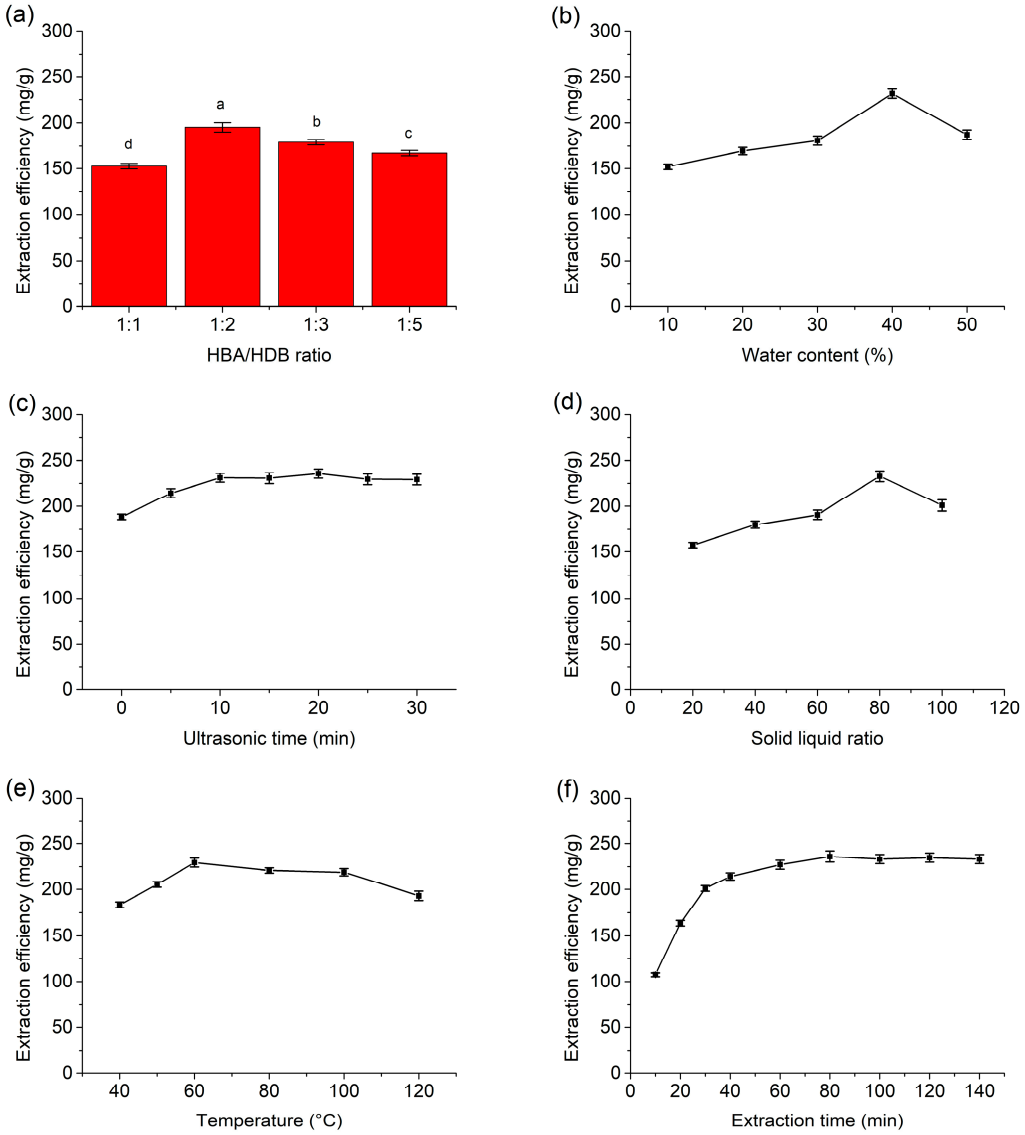


Figure 1. Optimization of extraction parameters in the UAE-DES method. (**a**) Effect of the HBA/HBD ratio on extraction efficiency; (**b**) effect of water content on extraction efficiency; (**c**) effect of ultrasonic time on extraction efficiency; (**d**) effect of the solid–liquid ratio on extraction efficiency; (**e**) effect of temperature on extraction efficiency; (**f**) effect of extraction time on extraction efficiency. Values followed by different letters (a–d) are significantly different according to Duncan's test ($p < 0.05$).

3.2.6. Effect of Extraction Time

To optimize the extraction equilibrium, the extraction of flavonoids in lotus leaves was performed at different times from 10 min to 140 min using the UAE-DES method. As indicated in Figure 1f, the extraction efficiency increased as the extraction time increased from 10 to 60 min. However, the value kept stable after 60 min, which could be considered as the extraction having achieved balance. Typically, long-term extraction was not suitable for natural active components, which would affect the chemical structures and activities [13]. Accordingly, the DES extraction time was confirmed at 60 min.

3.2.7. Comparison to Conventional Solvents

To evaluate the extraction efficiency of UAE-DES, conventional solvents were used for comparison in the same extraction condition and procedure [42]. The used conventional solvents included water, 50% methanol, 95% methanol, and 95% ethanol. Under the same treatment, 50% methanol solvent exhibited the highest extraction efficiency (246.9 mg/g), which was higher than that using DES (236.6 mg/g) by 4% (Figure 2), while water demonstrated the worst extraction efficiency for target compounds. Methanol was a good organic solvent in the extraction of natural products including flavonoids. However, its toxicity to the human body and harm to the environment could not be ignored. The extraction efficiency of DES is the second-best solvent in this comparison, which was slightly lower than that of 50% methanol. Considering the ease of recycling, low cost in preparation, and environmentally friendliness, DES were selected as the extraction solvent because of its satisfactory extraction ability and sustainable properties [43].

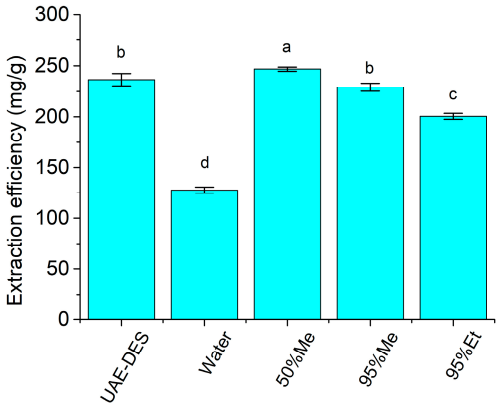


Figure 2. Extraction efficiencies of prepared DES and conventional solvents (50%me-50% methanol, 95%me-95% methanol, and 95%et-95% ethanol). Values followed by different letters (a–d) are significantly different according to Duncan's test ($p < 0.05$).

3.3. Recovery of Flavonoids and Reusability of DES

As studied in many pieces of research, macroporous resin has often been used to enrich natural active compounds, especially for flavonoids, and then target compounds could be eluted using organic solutions [44]. In this study, four different resins (HPD-500, AB-8, D-101, and NKA-9) were selected to investigate. Through the comparison, D-101 showed the best adsorption ability for flavonoids with 90% during the same conditions (Figure 3a). In many pieces of research, D-101 has also been selected for the collection of coumarins and flavonoids [45,46]. However, AB-8 and other kinds of resin have been used for the recovery of target compounds, which exhibited the importance of selection [29,47]. Then, different concentrations of ethanol solutions have been used for desorption. As shown in Figure 3b, the desorption ratio reached 95% when ethanol concentrations were higher than 60%, and the optimal desorption ratio (99.5%) was obtained by 100% ethanol. Moreover, 100% ethanol solution was easier for removing solvent. Therefore, D-101 was used for the recovery of flavonoids, and 100% ethanol was chosen as the optimal desorption solvent.

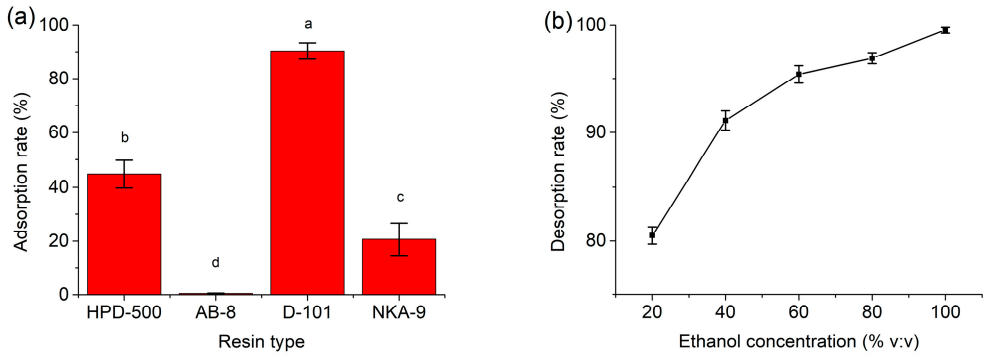


Figure 3. (**a**) The adsorption rate of four different macroporous resins (HPD-500, AB-8, D-101, and NKA-9); (**b**) The desorption rate obtained by different ethanol concentrations. Values followed by different letters (a–d) are significantly different according to Duncan's test ($p < 0.05$).

Since the flavonoids in the solvent were adsorbed by macroporous resin, the DES could be reused for the next round of extraction [48]. After the evaporation of water from DES through rotary evaporators, a new solvent was prepared based on the appropriate water content and applied for the next cycle of extraction. As shown in Figure 4, the extraction efficiency of DES for flavonoids was decreased with increasing reuse of recovered DES. The extraction efficiency of the fifth cycle was only 78% of the initial cycle. The results demonstrated that DES could be reused four times leading to cost savings.

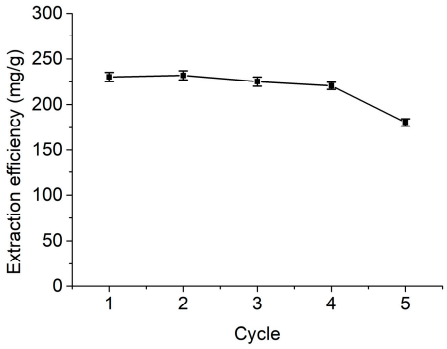


Figure 4. The recycling of recovered DES. One g of powdered dried lotus leaf was added to 20.0 mL of the DES. After extraction by the UAE-DES method, the DES extracts were adsorbed with 4.0 g of D101. After the adsorption procedure, the resin was desorbed and the DES were reused. The DES were evaporated to remove water and an appropriate amount of water was added for the next cycle of extraction.

3.4. Antioxidant Activities of the DES Extract

Antioxidant activity was an important bioactivity for natural components, and five assays were introduced to multi-aspect evaluate the antioxidant capacity [49]. The antioxidant activities including DPPH, ABTS, Fe^{3+} reducing, FRAP, and Fe^{2+} chelating of the DES extracts are presented in Figure 5. As a result, the DES extract ranged from 1 mg/mL to 20 mg/mL and expressed gradually increased antioxidant activities in different degrees. For the DPPH and ABTS radical scavenging abilities (Figure 5a), the DES extract showed the highest inhibition at 10 mg/mL (94.1% and 94.7%). These results showed that the DES extract had strong free radical scavenging activities, although it was lower than that of rutin. The iron chelating activity was significant of active compounds in the food and cosmetic industries, which meant the protection of the metabolism, skin, and environment against heavy metals [50]. The DES extract exhibited an increasing inhibition ability with increasing concentrations and reached 41.3% at 20 mg/mL (Figure 5a), while rutin showed no activity and only 0.11% at 0.5 mg/mL. In the Fe^{3+} reduction and FRAP assays, the values were expressed as the equivalent concentration of standards. The higher the values,

the better the antioxidant activities the sample exhibited. The measured Fe^{3+} reduction and FRAP values reached 0.73 mg RE /mL and 0.71 mmol/L Fe^{2+} (Figure 5b), while the FRAP value of 0.1 mg/mL rutin was 0.74 mmol/L Fe^{2+}, which could be referred to as a reference. As some reports have shown, the ethanol extract of lotus leaves exhibited antioxidant activities in DPPH scavenging activity, ABTS scavenging activity, and the FRAP assay [51]. Compared to water extraction, methanol extraction had higher antioxidant activities [52]. In conclusion, the DES extract showed dose-dependent antioxidant activities in these five assays, and it could be further developed as a crude antioxidant in many areas.

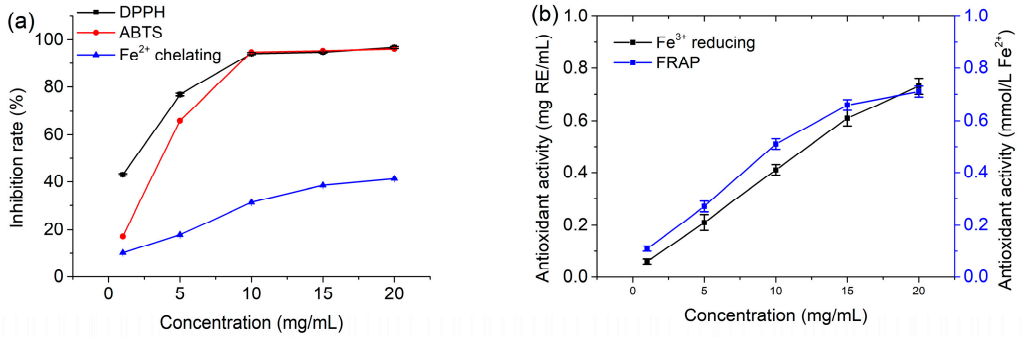


Figure 5. The antioxidant activities of DES extract in different concentrations. (**a**) DPPH, ABTS, and Fe^{2+} chelating assay. (**b**) Fe^{3+} reducing and FRAP assay.

3.5. Antibacterial Activity of the DES Extract

Antibacterial evaluation is a rapid, simple, and effective method to determine antimicrobial activity [53]. The antibacterial potentials of the DES extract against *Staphylococcus aureus* and *Escherichia coli* bacterial strains were investigated and are shown in Table 2. The DIZ of the DES extract at the concentration of 100 mg/mL was 8.49 ± 0.01 mm and 12.29 ± 0.01 mm for *S. aureus* and *E. coli*. It could be found that the antibacterial effect of the DES extract on two bacteria was confirmed, although the activities were lower than rutin. The antibacterial ability of the DES extract was further verified by the determination of the MIC value. As revealed in Table 2, the MIC values of the DES extract to *S. aureus* and *E. coli* were 1666 and 208 μg/mL, respectively, while the MIC values of rutin were 100 and 400 μg/mL, which was consistent with the results of DIZ. Antibacterial activity tests showed that the DES extract had a good inhibitory effect on *E. coli* at a low concentration, which made the potential usage of the DES extract an antibacterial agent [54]. The antibacterial testing of lotus leaf extracts is rare. Abderrahim Benslama reported that methanol extract has a moderate antibacterial activity against *Staphylococcus aureus*, *Micrococcus luteus*, and *Bacillus subtilis* [55]. The antibacterial ability of the DES extract in this study could expand the application range of lotus leaves.

Table 2. The antibacterial activities of DES extracts.

Antibacterial Properties	Samples	*S. aureus*	*E. coli*
DIZ (mm)	DES extract (50 mg/mL)	7.29 ± 0.01	9.02 ± 0.02
	DES extract (100 mg/mL)	8.49 ± 0.01	12.29 ± 0.01
	Rutin (1 mg/mL)	7.21 ± 0.01	7.11 ± 0.01
MIC (μg/mL)	DES extract	1666	208
	Rutin	100	400

3.6. HPLC Analysis of the DES Extract

For a better understanding of the extraction of DES, the composition of the DES extract was analyzed by HPLC. The typical chromatogram of the DES extract is shown in Figure 6. As shown in the Figure, the peaks were well separated, and three peaks at 17.3 min, 20.1 min, and 21.6 min could be found. In the previous study in our group, many

flavonoid standards were acquired and analyzed [56]. Through the comparison of the UV spectra and retention time in the same HPLC analysis conditions between the DES extract and the standards, these three main peaks in the DES extract were identified as astragalin, hyperoside, and isoquercitrin. The structures and UV spectra of them are shown in the Supplementary File. The chromatograms of the three standards are also illustrated in Figure 6 (colorful lines). It could be found that the retention times of these standards were the same as those peaks in the DES extract. Astragalin was identified from the receptacles of N. nucifera as the main component in the inhibition of tyrosinase activity [57]. In other groups' reports, hyperoside and isoquercitrin were also identified from lotus leaves, which could confirm their existence in lotus leaves [2,58]. Based on these data and analyses, three peaks in the DES extract were identified as astragalin, hyperoside, and isoquercitrin, which made further utilization of the DES extract easier.

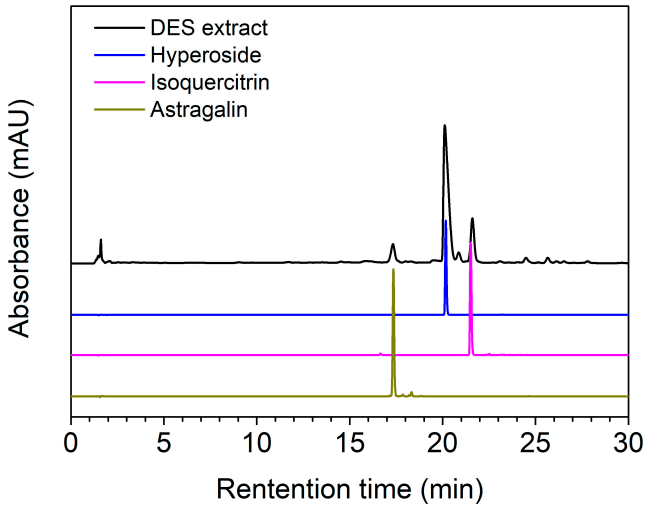


Figure 6. The chromatograms of the DES extract (black line), hyperoside (blue line), isoquercitrin (violet line), and astragalin (dark yellow line).

4. Conclusions

In this study, the flavonoids from lotus leaves were extracted using UAE-DES. The choline chloride/urea system was selected from 34 kinds of DES compositions, and the factors related to the preparation of the DES and the extraction process were optimized. Through the following purification by D-101 macroporous resin, the flavonoid extract was concentrated and the solvent could be recycled. The antioxidant and antibacterial tests confirmed that the extracted lotus leaf flavonoids had good activities in dose-dependent manners, which has promising applications in food additives and food preservations. Through the HPLC analysis of the DES extract, three peaks were positively identified as astragalin, hyperoside, and isoquercitrin by comparing the UV and retention time data. Based on the findings in this study, a proper deep eutectic solvent was optimized for the extraction of flavonoids from lotus leaves using UAE-DES, which gave a more eco-friendly method for the development of lotus-based products. To meet the challenge, further research in the future could be focused on research of specific flavonoids and the industrial application of DES in the separation of natural compounds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10020065/s1, Figure S1: The DIZ of DES extracts (w: Control, x: 50mg/mL DES extract, y: 100mg/mL DES extract, z: 1mg/mL Rutin); Figure S2: The structures of astragalin, hyperoside and isoquercitrin; Figure S3: The UV spectra of three peaks in DES extract, astragalin, hyperoside and isoquercitrin.

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