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Green Extraction of Phenolic Compounds from Lotus (*Nelumbo nucifera* Gaertn) Leaf Using Deep Eutectic Solvents: Process Optimization and Antioxidant Activity

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Abstract: Natural deep eutectic solvents (NDESs) were used to extract flavonoids and polyphenols from lotus (*Nelumbo nucifera* Gaertn.) leaves at the same time, and the extraction process was optimized to provide reference for the effective development and utilization of lotus leaves. The deep eutectic solvents (DESs) with the highest yield of flavonoids and polyphenols were screened out from 19 different NDES combinations. The response surface method was employed to optimize the extraction process. After a rational design, a lactic acid/glycerol (molar ratio 1:2) DES was chosen as the optimal extraction solvent, and the optimum extraction parameters were as follow: water content (29%), liquid–solid ratio (37:1 mL/g), extraction time (61 min), and extraction temperature (53 °C). Compared with traditional water extraction or ethanol extraction, it improved the yield of flavonoids (126.10 mg/g) and polyphenols (126.10 mg/g). By LC–MS analysis, 19 flavonoids or organic acid compounds with known compound structural formulae were identified in the DES extract of lotus leaves. By comparing the free radical scavenging ability and total reducing ability, the extraction of lotus leaves using the NDES method was superior to both ethanol extraction and water extraction. It is a green, environmentally friendly, and efficient extraction method for antioxidants from leaves of *Nelumbo nucifera* Gaertn.

Keywords: deep eutectic solvents; lotus (*Nelumbo nucifera* Gaertn.) leaves; extraction; response surface methodology; antioxidant; flavonoids; polyphenols

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1. Introduction

Lotus (*Nelumbo nucifera* Gaertn.) is a common aquatic perennial herbaceous plant that is cultivated in many regions of China. The lotus leaf was listed as a food and medicine resource by the Chinese Ministry of Health in 1991 [1,2]. In recent years, lotus leaves have attracted more and more attention due to their antioxidant, anti-inflammatory, antiobesity, and lipid-lowering activities [2]. The chemical constituents of lotus leave mainly include alkaloids, flavonoids, polyphenolic acids, volatile oil, and so on [3]. The flavonoids from lotus leaves have been reported to regulate blood lipids [4], have antibacterial [5] and antioxidant [6] properties, inhibit atherosclerosis [7], and improve liver damage [8].

The active ingredients in lotus leaves are flavonoids and phenolic acids; the identified phenolic compounds are reported to be catechin, myricetin, isoquercetin, hyperin, and kaempferol [9,10]. Solvent extraction is the most used method to extract flavonoids and polyphenolic compounds. Different types of solvent extraction methods are used, among which hot water bath extraction and soxhlet extraction are the most commonly used methods to extract the bioactive compounds of flavonoids [11]. Ultrasound-assisted extraction is

a green technique for the extraction of polyphenolic compounds, and this method improves the extraction efficiency and shortens the extraction time [12]. The disadvantage of solvent extraction is the long process time, which eventually leads to thermal degradation of the compounds and reduces environmental sustainability [11].

Deep eutectic solvents (DESs) are a new type of ionic liquid analog, which are eutectics formed by two or more components with a certain ratio of hydrogen bonds [13]. DESs have the advantages of simple preparation, good stability, high chemical purity, low cost, degradability, low viscosity, etc.; moreover, the solvent polarity can be adjusted by adjusting the substance ratio of deep eutectic solvents components. At present, deep eutectic solvents have been widely used in the extraction of active ingredients in traditional Chinese medicine [14] and active substances in food, the preparation of test solutions for quality testing, electrochemistry, and materials science [15]. Leyre Sillero et al. [16]. has successfully synthesized two DESs as green solvents for the extraction of bioactive compounds. They were used as additives to aqueous mixtures to improve the selective extraction of flavonoids from pine bark. Dai et al. [17]. effectively extracted 24 phenolic compounds from safflower, including hydroxy saffron yellow A (HSYA), using the DESs system (proline: malic acid). The use of deep eutectic solvents for natural product extraction has the advantage of simplicity and efficiency. Ali et al. [18] consider the use of DESs as a medium to extract active compounds from medicinal plants as a green method superior to the use of traditional solvents. Several authors have established that DESs have low toxicity properties for human life and the living environment [19,20].

This study focused on extracting flavonoids and polyphenols from lotus leaves with green eutectic solvent, optimizing the extraction process using the response surface method, and evaluating their antioxidant activities in vitro. Flavonoids and polyphenols both have a high antioxidant capacity, and antioxidant indicators such as DPPH (1,1-Diphenyl-2-picrylhydrazyl), ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (Ferric reducing ability of plasma) used on the extracts verified this through antioxidant experiments. This topic provides some scientific directions for the extraction research of lotus leaves and provides an effective theoretical basis for the development and utilization of lotus leaves.

2. Materials and Methods

2.1. Materials

DPPH (1898-66-4, 99%), rutin (153-18-4, ≥95%), gallic acid (149-91-7, 99%), ABTS (28752-68-3, 99%), Folin phenol (12111-13-6, Biological reagent), D(+)-Glucose (50-99-7, Analytical purity; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), betaine (107-43-7, >99%; Refrigerant Cool Chemical Co., Ltd., Jinan, China), lactic acid (50-21-5, >90.0%), ferric trichloride (7705-08-0, Analytical purity), potassium hexacyanoferrate (13746-66-2, Analytical purity), trifluoroacetic acid (76-05-1, Analytical purity), sodium dihydrogen phosphate (89140-32-9, Analytical purity), disodium hydrogen phosphate (10039-32-4, Analytical purity), sodium chloride (7647-14-5, Analytical purity; Tianjin Beichen Founder Reagent Factory, Tianjin, China), Citric acid (77-92-9, >99.5%; Tianjin Aupu Kai Chemical Co., Ltd., Tianjin, China), malic acid (6915-15-7, >99%; Hefei Bomei Biotechnology Co., Ltd., Hefei, China), crystalline aluminum chloride (7784-13-6, >97%; Shenyang City Reagent Five Factory, Shenyang, China), glycerol (56-81-5, >99%), urea (57-13-6, >99%), propylene glycol (57-55-6, Analytical purity; Tianjin Beilian Fine Chemicals Development Co., Ltd., Tianjin, China); ethanol (64-17-5, Analytical purity), sodium hydroxide (1310-73-2, Analytical purity), Sodium nitrite (7632-00-0, Analytical purity), and sodium carbonate (497-19-8, Analytical purity; Yantai Sanhe Chemical Reagent Co., Ltd., Yantai, China) were used.

2.2. Samples

The lotus leaves (Origin: Taiyuan, China; Production date: August 2021) provided by Hebei Renxin Pharmaceutical Co., Ltd., were dried, crumbled, sieved (using a 60-mesh sieve), and then stored at 4 °C for later use.

2.3. Preparation of Deep Eutectic Solvents

A hydrogen bond acceptor and hydrogen bond donor were selected. After mixing, they were then stirred in a water bath at 80 °C with a magnetic agitator until the liquid became uniform and transparent [21]. A synopsis of the DESs prepared is shown in Table 1. The viscosity, pH, and visible properties were observed according to the literature [22,23].

Table 1. Composition and characteristics of DESs.

Number	Hydrogen Bond Receptor (HBA)	Hydrogen Bonded Donor (HBD)	Mole Ratio	Water Content (%)	Viscosity (mpa s)	PH Value	Character
DES-1	Choline chloride	Glycerol	1:2	20%	67.03	5.75	Transparent liquid
DES-2		Propylene glycol	1:2		39.1	5.88	Transparent liquid
DES-3		Lactic acid	1:2		31.3	1.22	Transparent liquid
DES-4		Citric acid	1:2		213	0.07	Transparent liquid
DES-5		Malic acid	1:2		138.8	0.11	Transparent liquid
DES-6		D(+)-Glucose	1:2		222.8	3.6	Transparent liquid
DES-7		Urea	1:2		26.4	9.28	Transparent liquid
DES-8	Betaine	Glycerol	1:2		147.3	6.82	Transparent liquid
DES-9		Propylene glycol	1:2		131.3	7.37	Transparent liquid
DES-10		Lactic acid	1:2		66.5	3.57	Transparent liquid
DES-11		Citric acid	1:2		711	2.27	Transparent liquid
DES-12		Malic acid	1:2		424.33	2.57	Transparent liquid
DES-13		D(+)-Glucose	1:2		470	5.37	Transparent liquid
DES-14	Lactic acid	Glycerol	1:2		66.03	2.11	Transparent yellow liquid
DES-15		Propylene glycol	1:2		33	2.24	Transparent yellow liquid
DES-16		D(+)-Glucose	1:2		351.17	1.85	Transparent yellow liquid
DES-17	Citric acid	Glycerol	1:2		326.67	1.75	Transparent liquid
DES-18		Propylene glycol	1:2		106.67	1.67	Transparent liquid
DES-19		D(+)-Glucose	1:2		627	1.65	Transparent liquid

2.4. Extraction of Flavonoid and Polyphenol Components

The extraction process was carried out according to previously reported methods with some modifications [24]. A sample (0.10 g) was added to the DES solution (4 mL, containing 20% water), extracted at 140 r/min for 60 min (50 °C). The extract was centrifuged at 8000 r/min for 10 min, and the supernatant was collected for later use.

2.5. Optimization of Extraction Process

The effect of extraction process parameters on the yield of flavonoids and polyphenols in lotus leaves extract was investigated by a single-factor test. Experiments were performed using a variety of different conditions: 19 different deep eutectic solvents, different molar ratios (3:1, 2:1, 1:1, 1:2, 1:3, 1:4), water contents (0%, 10%, 20%, 30%, 40%, 50%, 60%), material–liquid ratios (1:10, 1:20, 1:30, 1:40, 1:50, 1:60), extraction times (15, 30, 45, 60, 75, 90 min), and extraction temperatures (30, 40, 50, 60, 70 °C).

The Box–Behnken design (BBD) was adopted to optimize the extraction methods. Based on the above single-factor analysis of variance, four variables, namely (A) water content, (B)material-to-liquid ratio, (C)extraction temperature, (D) and extraction time, were selected as independent variables of the BBD, and the response values of the flavonoid yield (Y_1) and polyphenol yield (Y_2) were used to design a four-factor three-level response surface. A total of 29 sets of experiments were designed to optimize the four-factor three-level response surface test, as shown in Table 2.

Table 2. Response surface factor level design.

Factor	Level		
	−1	**0**	**1**
Moisture content (%)	20	30	40
Extraction temperature (°C)	40	50	60
Extraction time (min)	45	60	75
Liquid–solid ratio (mL/g)	30	40	50

2.6. Traditional Extraction Method Comparison

Compared with traditional extraction methods, 75% ethanol and water were used for the water bath (140 r/min, 50 °C, 60 min).

2.7. Determination of Total Flavonoids

The content of flavonoids was calculated using the modified method described by Ji et al. [25]. A standard solution was prepared by dissolving rutin (82.2 mg) in 60% ethanol to give a total volume of 100 mL. Rutin dilutions were prepared with mass concentrations ranging from 82.2 to 822 μg/mL. The total flavonoid content was determined by the color development method of sodium nitrite-aluminum nitrate-sodium hydroxide, and the absorbance was measured at 510 nm by UV spectrophotometer. In addition, the regression equation of total flavonoid was Y = 0.0059 X + 0.0021 (R^2 = 0.9999), where X is the mass concentration of rutin (μg/mL) and Y is the absorbance value. The concentration of rutin showed a good linear relationship with the absorbance.

2.8. Determination of Polyphenols

The total phenolic content was determined using the Folin–Ciocalteu method. The method was in reference to Sutivisedsak et al. [26] and modified appropriately. The gallic acid was accurately weighed (80.2 mg), dissolved in double-pure water, fixed into a 100 mL volumetric flask, and mixed as the mother liquor. Preparation of gallic acid dilutions with mass concentrations of 80.2–802 μg/mL. In total, 50 μL was taken from the above standard solution with different concentrations, 125 μL from Folin-Ciocalteu reagent, and 1250 μL from a 7% $NaCO_3$ solution and then added together, and the reaction was carried out at 40 °C for 90 min under light protection. After the reaction, measuring the absorbance at 760 nm using UV spectrophotometer and yielded a regression equation of polyphenols of Y = 0.0602 X + 0.0104 (R^2 = 0.9991), where X is the mass concentration of gallic acid (μg/mL) and Y is the absorbance value. The concentration of gallic acid showed a good linear relationship with the absorbance.

2.9. Antioxidant Activity

2.9.1. DPPH Radical Scavenging Rate Measurement

To evaluate the ability of extracts to eliminate DPPH free radicals, the previously reported methods were modified and used [27]. The supernatant was diluted 10 times, mixed with 9 mL of DPPH solution (200 μmol/L, dissolved in anhydrous ethanol), and allowed to react in the dark for 30 min. The standard curve was plotted with the concentration of different standards (X) as the horizontal coordinate and the scavenging rate of DPPH radicals (Y) as the vertical coordinate (Y = 0.7758 X − 4.0584 (R^2 = 0.9994)). The absorbance values were then measured at 517 nm and used to calculate the scavenging activity according to the formula:

$$\text{DPPH inhibition } (\%) = (1 - \frac{A_i - A_j}{A_0}) \times 100\%$$

where A_i is the absorbance value of the sample group, A_j is the absorbance value of the blank reagent, and A_0 is the absorbance value of the blank control sample.

2.9.2. ABTS Cation Radical Rate Determination

The supernatant (1 mL) was mixed with 10 mL of $ABTS^+$ solution (7 mmol/L ABTS aqueous solution and 2.45 mmol/L potassium persulfate aqueous solution were mixed and protected from light for 12–16 h), and the reaction was carried out for 10 min in the dark. The absorbance values were then measured at 734 nm. The standard curve was drawn with the concentration of different standards (X) as the horizontal coordinate, the scavenging rate of ABTS cationic radicals (Y) as the vertical coordinate, and the regression equation being Y = 0.113 X + 2.0628 (R^2 = 0.9934). The ABTS cationic radical scavenging rate was then calculated using the equation:

$$\text{ABTS inhibition } (\%) = (1 - \frac{A_i - A_j}{A_0}) \times 100\%$$

where A_i, is the absorbance value of the sample group, A_j is the absorbance value of the blank reagent, A_0 is the absorbance value of the blank control sample group.

2.9.3. FRAP Total Reduction Capacity Measurement

The sample solution (5 μL) was mixed with 200 μL FRAP working solution (Equal volume of phosphate buffer (pH = 6.6), 1 mg/mL ferric chloride solution, 10 mg/mL potassium ferricyanide, and 100 mg/mL trifluoroacetic acid were mixed), and the reaction was carried out at 37 °C for 5 min under light protection. The absorbance values were measured at 593 nm. The standard curve was plotted with different concentrations of Fe^{2+} (X) as the horizontal coordinate, the corresponding absorbance (Y) as the vertical coordinate, and the regression equation being Y = 0.0003 X + 0.0032 (R^2 = 0.9997), which indicated that the model fitted well.

2.10. LC–MS Structural Analysis

Chromatographic conditions: ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm); column temperature 30 °C; flow rate 0.3 mL/min; injection volume 10 μL; mobile phase A: 0.1% formic acid aqueous solution, *v*/*v*; mobile phase B: 0.1% formic acid acetonitrile solution, *v*/*v*. Gradient elution conditions: 0–2 min, 30% B; 2–20 min, 30–100% B; 20–26 min, 100% B; 26–26.1 min, 100–10% B; 26.1–30 min, 10% B.

Mass spectrometry conditions: The LC–MS/MS system consists of the Waters ACQUITY UPLC system connected to the Waters Xevo TQ-S triple quadrupole time of flight mass spectrometer (Waters Corp., Milford, MA, USA). Detection was performed using MSE mode and processed using the Masslynx 4.1 software (Waters Corp.). The UPLC effluent was introduced into the mass spectrometer by positive mode electrospray ionization. The capillary voltage was 3.00 kV (ESI+), extractor voltage was 5 V, desolvation nitrogen flow rate was 800 L/h (N_2, purity 99.9%), desolvation gas temperature was 400 °C, and source temperature was 150 °C. Data were collected in centroid mode from 100 to 1500 *m*/*z*.

2.11. Statistical Analysis

The response surface design experiments and data statistics were performed with Design Expert 12.0 (Minneapolis, Minnesota, MN, USA). Analysis of variance (ANOVA) and correlation analysis were performed using SPSS 20.0 software (Amenk, New York, NY, USA) and different lowercase letters were used to indicate significant differences ($p < 0.05$). Calculation of IC_{50} using GraphPad prism8.0 (San Diego, CA, USA); Origin 2018 software (Northampton, MA, USA) was used for plotting.

3. Results and Discussion

3.1. Single-Factor Test

3.1.1. Selection of the Optimal DES

The deep eutectic solvents consist of two parts: a hydrogen bond donor and a hydrogen bond acceptor. Different combinations form hydrogen bonding forces of different strengths.

The extraction rates of different deep eutectic solvents on the flavonoid and polyphenol components of lotus leaves were investigated. Four substances were selected as hydrogen bond acceptors in the experiment, namely choline chloride, betaine, lactic acid, and citric acid, where the selection of hydrogen bond donors included four donors, carboxylic acids, polyols, sugars, and amides. The yields of polyphenols and flavonoids measured under the same extraction conditions, i.e., 20% water content, 1:20 ratio, and 60 min at 50 °C, were compared with the traditional extraction method of water and 70% ethanol (Figure 1). The yield of flavonoids was slightly lower than that of the combination of betaine and propylene glycol. Considering the yield of lotus flavonoids and polyphenols and the low price of lactic acid and propanetriol, they are pure natural solvents that are green, pollution-free, and easily degradable; therefore, the combination of lactic acid and propanetriol was chosen.

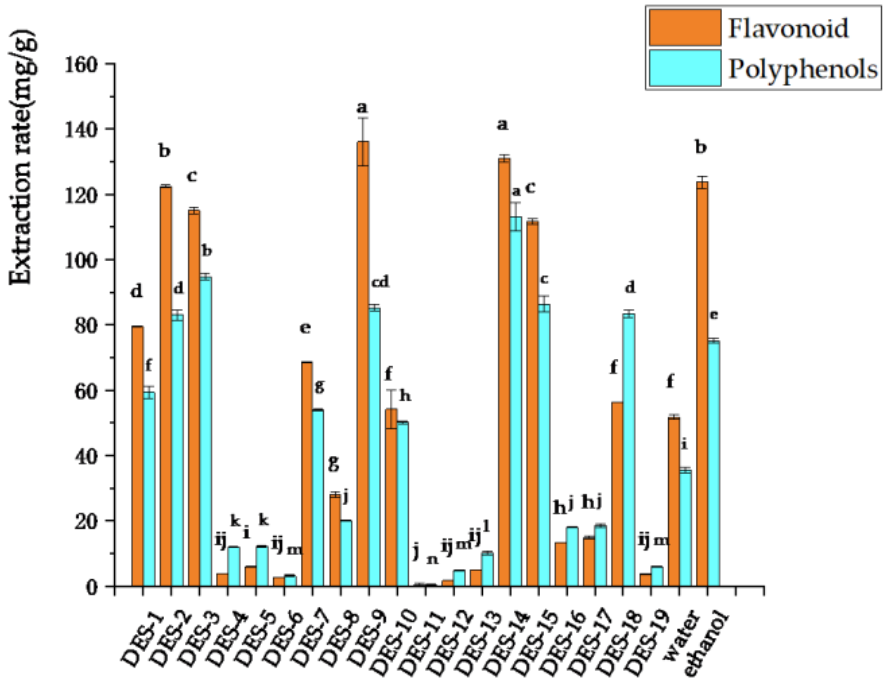

Figure 1. Effect of different solvents on the yield of flavonoids and polyphenols from lotus leaves. Different letters indicate significant differences, $p < 0.05$.

3.1.2. Selection of the Optimal DES Molar Ratio

The effect of the molar ratio of hydrogen bond acceptor to hydrogen bond donor of DESs solvent on the extraction of lotus flavonoids and polyphenols was investigated (Figure 2). The yield tended to decrease when the molar ratio gradually changed from 1:2 to 1:4. This indicated that too many hydrogen bond donors or hydrogen bond acceptors can affect the yield of phenolic compounds. This is because when the content of propanetriol increased, this significantly promoted the diffusion and mass transfer of the reaction system, which led to a higher extraction rate, whereas, when the viscosity of propanetriol is high, excess propanetriol leads to stronger spatial site resistance, which results in a lower extraction rate [28]. Therefore, the highest yield of flavonoids and polyphenols was obtained with a molar ratio of 1:2.

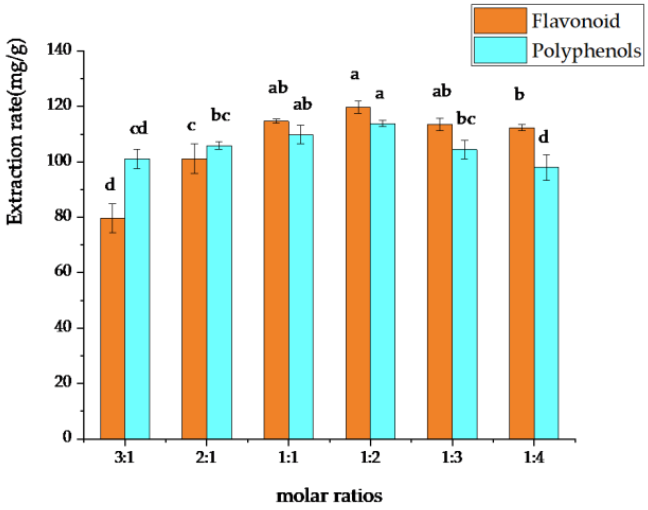

Figure 2. Effect of different molar ratios of DESs on the yields of lotus flavonoids and polyphenols. Different letters indicate significant differences, $p < 0.05$.

3.1.3. Selection of Optimal Water Content

The presence of water in deep eutectic solvents affects the physicochemical properties of the solvent [29], while the right amount of water has no significant effect on the structure of the solvent components [30]. The addition of water reduces the viscosity and surface tension of the system, increases the osmotic pressure, and enhances mass transfer, thus positively influencing the extraction [31]. The effect of increasing the water content from 0% to 10% and to 60% on the yield of lotus flavonoids and polyphenols was investigated, and the results are shown in Figure 3. The yield of flavonoids and polyphenols increased gradually with the increase in water content (0% to 30%) in the solvent system. When the water content in the solvent increased from 30% to 60%, the extraction rate decreased with the increase in water content. This may be related to the fact that the excess water in the DESs broke the structure of the DES system as well as weakened the hydrogen bonds between the constituents of the lotus leaves and DESs, which led to a decrease in the extraction rate of the target compounds [32,33]. The experimental results showed that the extraction rate of flavonoids and polyphenols of DESs reached the maximum when the water content of DESs was 30%; therefore, the optimal water content choice for the DES system was 30%.

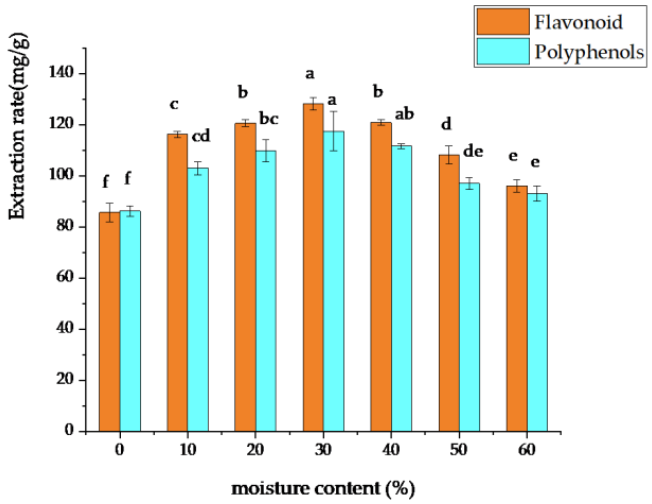

Figure 3. Effect of different water content on the yield of flavonoids and polyphenols of lotus leaves. Different letters indicate significant differences, $p < 0.05$.

3.1.4. Selection of the Optimum DESs Liquid–Solid Ratios

Considering the economics, excessive use of DES solutions for extraction can result in large losses; moreover, extractions with too little solvent will lead to incomplete extraction, so finding the right ratio of solution to material has a greater impact on extraction [28]. The effect of liquid–solid ratio of 10 mL/g, 20 mL/g, 30 mL/g, 40 mL/g, 50 mL/g and 60 mL/g on the yield of lotus flavonoids and polyphenols was investigated, and the results are shown in Figure 4. When the liquid–solid ratio was below 40 mL/g, the yield of flavonoids and phenolic compounds increased with the increase in the liquid–solid ratio and with the increase in the solvent amount, the reason being that the increase in the solvent helped more flavonoids and polyphenolic compounds to diffuse into the solvent. With the further increase in the feed–liquid ratio to 40 mL/g, the extraction rate showed a decreasing trend. The reason may be since the concentration of the solution becomes smaller with the increase in the stock–liquid ratio, and the percentage of lotus leaves per ml DES solution becomes less; therefore, a liquid–solid ratio of 40 mL/g was chosen.

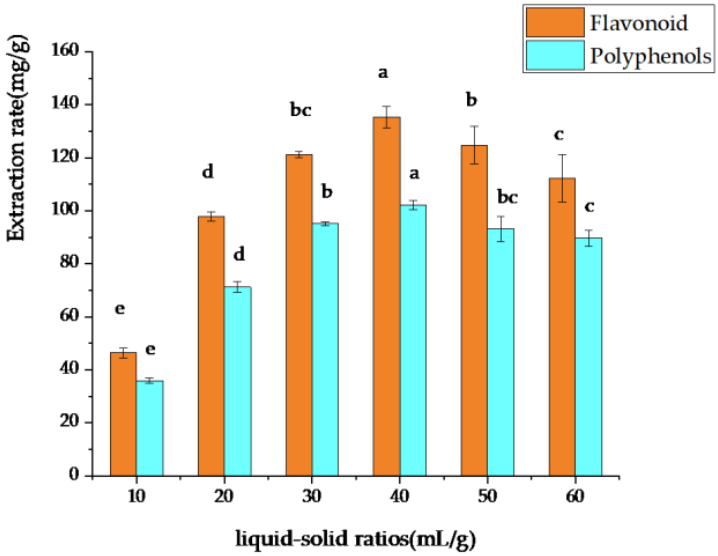

Figure 4. Effect of different liquid–solid ratios on the yields of flavonoids and polyphenols of lotus leaves. Different letters indicate significant differences, $p < 0.05$.

3.1.5. Selection of Optimum DES Extraction Time

During the extraction of lotus leaves, the yield of flavonoids and polyphenols is maximized after a certain period, and when the extraction time is extended, the active ingredients in lotus leaves will decompose and the yield will be reduced. A short extraction time will lead to the loss of raw materials, so the optimal extraction time should be selected. From 15 to 60 min, the yields of flavonoids and polyphenols in lotus leaves showed an increasing trend, and then, with the increase in extraction time, the extraction amount decreased slightly, but the difference was not significant, probably because the extraction process was very close to the solid–liquid equilibrium and the extraction reached the maximum (Figure 5). However, the flavonoids and polyphenols were decomposed in the process of continued extraction, so the extraction rate decreased slightly; therefore, 60 min was chosen as the most appropriate extraction time for the experiment.

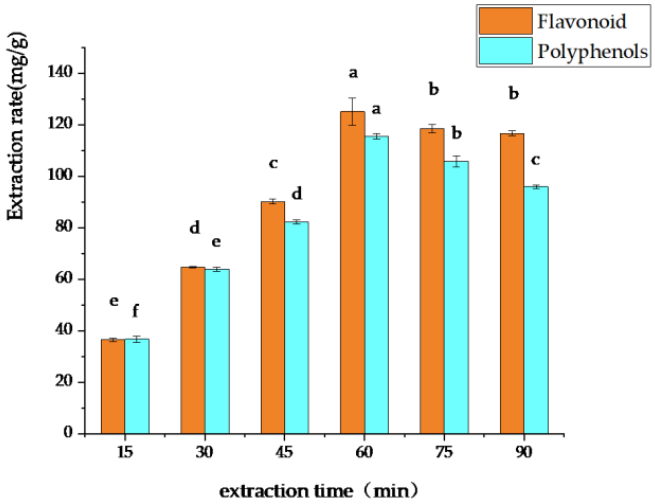

Figure 5. Effect of different extraction time on the yield of lotus leaf flavonoids and polyphenols. Different letters indicate significant differences, $p < 0.05$.

3.1.6. Selection of Optimum DES Extraction Temperature

Extraction temperature is also one of the conditions affecting the extraction of active substances extracted from lotus leaves, as increasing the temperature helps to increase the solubility of lotus active substances in DESs, decreases the viscosity, density, and surface tension of the DES solution, and increases the diffusion coefficient, thus increasing the extraction rate [15,34]. The effects of different extraction temperatures on the yield of flavonoids and polyphenols at a molar ratio of lactic acid–propanetriol of 1:2, a water content of 30%, a liquid-to-solid ratio of 40 mL/g, and an extraction time of 60 min are shown in Figure 6. Below 50 °C, the yield of lotus leaf flavonoids and polyphenols improved with the increase in temperature and attained the maximum yield at 50 °C. When the temperature exceeded 50 °C, the yields of lotus flavonoids and polyphenols decreased slightly with the increasing temperature. The reason may be that when the temperature exceeded 50 °C, the active ingredients in lotus leaves would decompose due to the increase in temperature as the extraction progressed, so the yield decreased slightly with the increase in temperature; therefore, the optimum extraction temperature was 50 °C.

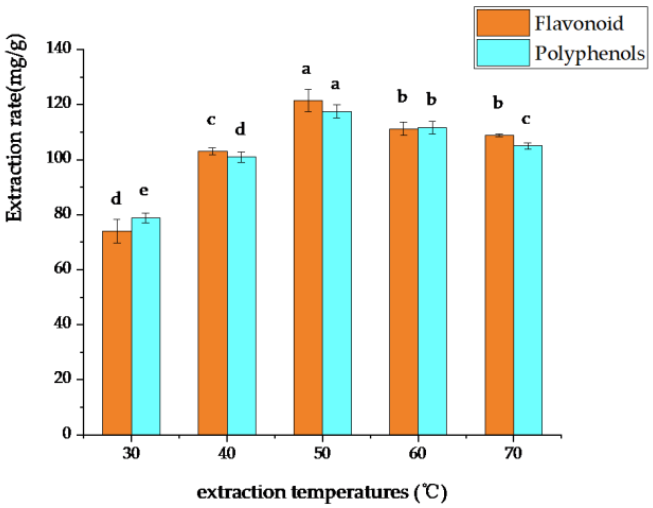

Figure 6. Effect of different extraction temperatures on the yield of lotus leaf flavonoids and polyphenols. Different letters indicate significant differences, $p < 0.05$.

3.2. Results of Response Surface Experiments

3.2.1. Response Surface Experimental Design and Analysis of Variance

The results of the response surface experimental design are shown in Table 3, the regression equations were fitted to the data in Table 3 using Design-Expert12 analysis software, and the quadratic multiple regression equations for each factor (A: water content, B: liquid-solid ratio, C: extraction temperature, D: extraction time) on the yield of flavonoids (Y_1) and polyphenols (Y_2) were obtained as follows:

$$Y_1 = 128.29 - 3.11\,A - 14.66\,B + 4.15\,C + 2.14\,D + 0.2420\,AB - 3.70\,AC + 2.08\,AD + 3.09\,BC - 0.7930\,BD + 1.23\,CD - 10.69\,A^2 - 14.67\,B^2 - 9.19\,C^2 - 13.06\,D^2$$

$$Y_2 = 113.04 - 1.45\,A + 2.40\,B + 6.21\,C - 0.7261\,D + 1.91\,AB - 0.2530\,AC - 0.8942\,AD - 2.05\,BC + 1.50\,BD + 5.08\,CD - 7.51\,A^2 - 3.89\,B^2 - 8.27\,C^2 - 10.24\,D^2$$

Table 3. Response surface test design and results.

Number	Factors				Total Flavonoid Yield mg/g	Polyphenol Yield mg/g
	A: Water Content %	B: Liquid-Solid Ratio mL/g	C: Extraction Temperature °C	D: Extraction Time min		
1	−1	−1	0	0	121.24 ± 3.22	103.93 ± 3.37
2	0	−1	1	0	117.06 ± 1.48	109.63 ± 2.15
3	0	0	−1	−1	101.06 ± 2.76	94.59 ± 1.71
4	1	1	0	0	82.37 ± 2.02	102.45 ± 4.90
5	0	0	0	0	130.83 ± 3.33	111.56 ± 3.35
6	1	0	0	1	110.49 ± 0.91	94.52 ± 1.48
7	1	0	0	−1	96.59 ± 2.41	95.95 ± 0.58
8	1	−1	0	0	116.34 ± 3.50	96.03 ± 3.99
9	0	−1	0	1	117.10 ± 4.75	91.34 ± 6.33
10	0	0	0	0	132.91 ± 4.11	114.91 ± 5.97
11	−1	0	0	1	109.32 ± 3.04	98.88 ± 4.61
12	0	1	0	−1	87.33 ± 1.96	101.01 ± 2.85
13	1	0	1	0	108.77 ± 3.31	100.35 ± 1.69
14	0	0	1	1	110.73 ± 0.75	103.57 ± 0.56
15	−1	0	1	0	121.43 ± 8.10	102.88 ± 1.45
16	−1	1	0	0	86.30 ± 0.29	102.70 ± 1.63
17	0	1	−1	0	86.63 ± 3.84	99.04 ± 4.28
18	0	0	−1	1	99.66 ± 4.36	81.17 ± 1.40
19	0	1	0	1	87.79 ± 2.19	102.53 ± 3.49
20	0	0	0	0	127.36 ± 4.29	110.26 ± 5.26
21	0	1	1	0	97.48 ± 0.80	109.17 ± 4.67
22	0	−1	−1	0	118.59 ± 2.94	91.33 ± 5.58
23	−1	0	0	−1	103.76 ± 2.94	96.72 ± 3.83
24	0	0	0	0	129.23 ± 15.84	112.54 ± 1.64
25	0	−1	0	−1	113.48 ± 3.53	95.82 ± 1.76
26	0	0	0	0	121.12 ± 3.07	115.92 ± 5.67
27	1	0	−1	0	104.50 ± 5.71	90.03 ± 1.87
28	−1	0	−1	0	102.38 ± 0.39	91.56 ± 0.31
29	0	0	1	−1	107.21 ± 1.65	96.65 ± 1.18

The ANOVA (analysis of variance) of total flavonoid yield is shown in Table 4. According to the analysis in Table 4, the model $p < 0.01$ is significant, indicating that the model was successfully established, the misfit term $p > 0.05$ is not significant, demonstrating that the model fit is good, the coefficient of determination $R^2 = 0.9619$ and the model adjustment coefficient $R_{adj}^2 = 0.9238$ both exceed 0.9 and are close to each other, indicating that the experimental values are strongly correlated with the predicted values and that the model correlation is good. From the response surface test results, the factor of liquid-to-solid ratio (B) is the most influential factor on the total flavonoid yield, followed by the extraction temperature (C). The *p*-values of extraction time (D) and water content (A) were not significant, which indicated that these two factors had a little effect on the total flavonoid yield.

Table 4. ANOVA of total flavonoid yield.

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-Value	
Model	5500.78	14	392.91	25.26	<0.0001	**
A	53.6	1	53.6	3.45	0.0846	
B	2578.55	1	2578.55	165.76	<0.0001	**
C	207.17	1	207.17	13.32	0.0026	**
D	54.88	1	54.88	3.53	0.0813	
AB	0.2343	1	0.2343	0.0151	0.9041	
AC	54.62	1	54.62	3.51	0.082	
AD	17.36	1	17.36	1.12	0.3087	
BC	38.31	1	38.31	2.46	0.1389	
BD	2.52	1	2.52	0.1617	0.6937	
CD	6.06	1	6.06	0.3897	0.5425	
A^2	741.76	1	741.76	47.68	<0.0001	**
B^2	1395.06	1	1395.06	89.68	<0.0001	**
C^2	548.11	1	548.11	35.23	<0.0001	**
D^2	1106.71	1	1106.71	71.14	<0.0001	**
Residual	217.78	14	15.56			
Lack of Fit	136.88	10	13.69	0.6767	0.7198	
Pure Error	80.91	4	20.23			
Cor Total	5718.57	28				

$p < 0.01$ ** means significant statistical difference; A: water content, B: liquid–solid ratio, C: extraction temperature, D: extraction time.

Figure 7 shows the response surface diagram of the interaction between the factors of total flavonoid yield, and the F-value analysis of the interaction between the factors in Table 4 shows that the interaction between water content and extraction temperature has the greatest effect on the yield of total flavonoid, and the interaction between water content and liquid–solid ratio has the least effect.

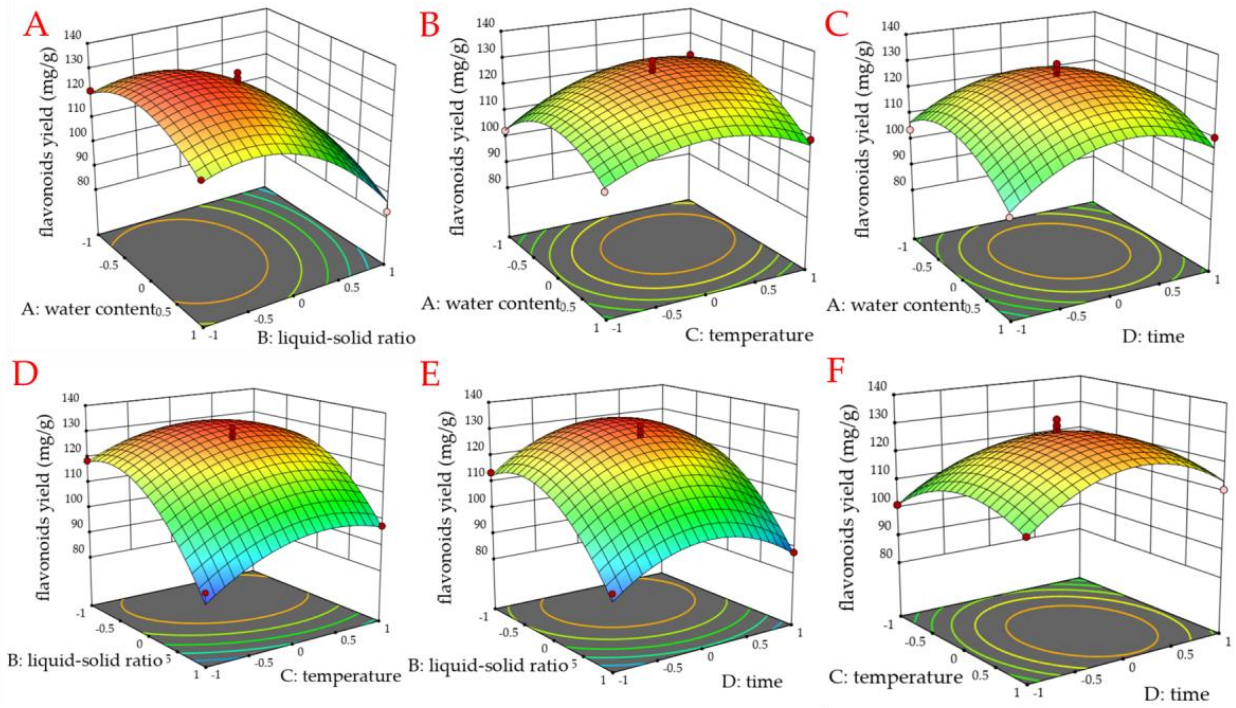

Figure 7. Response surface diagram of the interaction of different factors of total flavonoid yield. (**A**) Water content and liquid–solid ratio; (**B**) water content and temperature; (**C**) water content and time; (**D**) liquid–solid ratio and temperature; (**E**) liquid–solid ratio and time; (**F**) temperature and time.

The ANOVA of polyphenol yield is shown in Table 5, based on which we know that the experimental model $p < 0.01$ is significant, indicating successful model building. The misfit term $p > 0.05$ was not significant, indicating that the model has good fit. The coefficient of determination $R^2 = 0.9579$ and the model adjustment coefficient $R_{adj}^2 = 0.9159$ both exceed 0.9 and were close to each other, which shows that the experimental values were strongly correlated with the predicted values and the model correlation was good. From the results of the response surface test, the relationship between the four factors set on the polyphenol yield is as follows: C (extraction temperature) > B (liquid to solid ratio) > A (water content) > D (extraction time). The extraction temperature had the greatest effect on the yield of polyphenols, probably because of the poor stability of polyphenols, and the change of temperature had a greater effect on polyphenols.

Table 5. ANOVA of polyphenol yield.

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-Value	
Model	1824.14	14	130.3	22.77	<0.0001	**
A	25.07	1	25.07	4.38	0.055	
B	69.22	1	69.22	12.1	0.0037	**
C	462.89	1	462.89	80.9	<0.0001	**
D	6.33	1	6.33	1.11	0.3108	
AB	14.59	1	14.59	2.55	0.1326	
AC	0.256	1	0.256	0.0447	0.8355	
AD	3.2	1	3.2	0.559	0.467	
BC	16.75	1	16.75	2.93	0.1091	
BD	8.99	1	8.99	1.57	0.2305	
CD	103.37	1	103.37	18.07	0.0008	**
A^2	366.1	1	366.1	63.98	<0.0001	**
B^2	98.12	1	98.12	17.15	0.001	**
C^2	443.17	1	443.17	77.45	<0.0001	**
D^2	704.02	1	704.02	123.04	<0.0001	**
Residual	80.1	14	5.72			
Lack of Fit	58.14	10	5.81	1.06	0.5224	
Pure Error	21.96	4	5.49			
Cor Total	1904.24	28				

$p < 0.01$ ** means significant statistical difference; A: water content, B: liquid–solid ratio, C: extraction temperature, D: extraction time.

Figure 8 shows the response surface diagram of the interaction between the factors of polyphenol yield, and from the results of the analysis in Table 5, we can conclude that the relationship of the interaction between the factors is CD > BC > AB > BD > AD > AC, in which the interaction between extraction temperature and extraction time has a significant effect on the yield of polyphenol. In the extraction process, with the increase in temperature and time, the yield of polyphenols showed a tendency to increase before decreasing, because in a certain range, the increase intemperature and longer extraction time were beneficial to the extraction of polyphenols. When the temperature and time continued to increase, the yield of polyphenols showed a slightly decreasing trend, indicating that the high temperature and long extraction time had a negative effect on the yield of polyphenols, which might be caused by the decomposition of the structure of polyphenols.

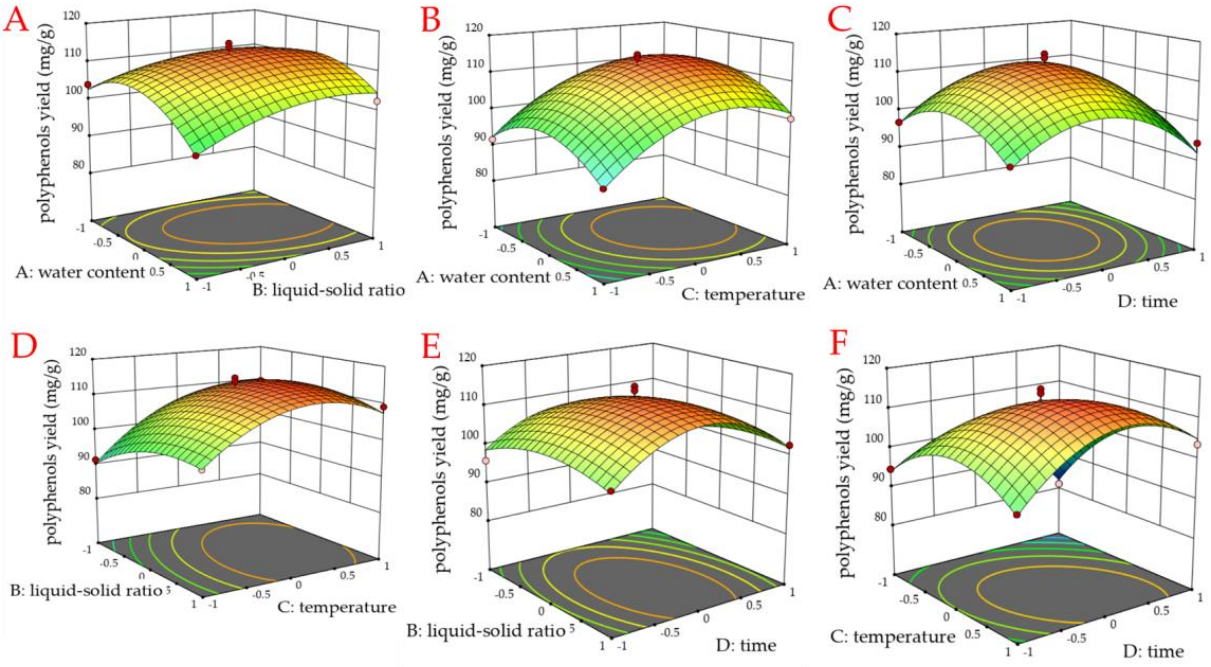

Figure 8. Response surface diagram of the interaction of different factors of polyphenols yield. (**A**)Water content and liquid–solid ratio; (**B**) water content and temperature; (**C**) water content and time; (**D**) liquid–solid ratio and temperature; (**E**) liquid–solid ratio and time; (**F**) temperature and time.

3.2.2. Analysis of Validation Test Results

A high extraction rate is the major objective of this research on the extraction of lotus leaves. The optimal extraction process was optimized by Design Expert 12.0 with the following four factors: water content 28.53%, liquid–solid ratio 37.23:1 (mL/g), extraction temperature 53.39 °C, and extraction time 60.915 min. Under the conditions of these four factors, the theoretically estimated number of flavonoids was 131.661 mg/g and the yield of polyphenols was 113.567 mg/g with 95.4% confidence. In the validation experiments, the operating parameters were adjusted to 29% water content, 37:1 liquid–solid ratio (mL/g), 53 °C extraction temperature, and 61 min extraction time, and three sets of parallel experiments were conducted, considering the actual conditions. The yield of lotus leaf flavonoids was 126.10 ± 3.64 mg/g and the yield of polyphenols was 113.12 ± 4.28 mg/g, which were 4.23% and 0.40% different from the theoretical values, indicating that the model fit well with the actual one and proved the feasibility of the model. In respect to polyphenol extraction, Viktoria Vorobyova et al. [35] investigated the extraction of polyphenols from tomato pomace with the help of ultrasound in a deep eutectic solvent based on choline chloride, and the total phenolic content of the extract was (51.75 ± 1.15 mg GAE (gallic acid equivalent)/g extract dry fraction), which was much lower than the polyphenol extraction rate of the present study.

3.2.3. Comparison of the Effects of Different Extraction Methods on Antioxidant Activity

The extracts of lotus leaves were performed with water, 70% ethanol, and a DES (lactic acid-propanetriol) to compare the antioxidant activity of the 3 extracts in terms of FRAP total reducing capacity, DPPH radicals, and ABTS cationic radical scavenging capacity, respectively. The FRAP total reducing capacity was reflected by comparing the ability of the extracts to convert Fe^{3+} to Fe^{2+}, i.e., the amount of Fe^{2+} production; therefore, a higher FRAP value in Table 6 indicates a higher total reducing capacity using this extraction method of lotus leaves. In both DPPH and ABTS free radical scavenging ability measurements, the smaller the value, the stronger the scavenging ability it represents. By comparing the values in Table 6, DES extraction has the smallest values for both DPPH radical scavenging capacity and ABTS scavenging capacity compared to the traditional water extraction and

70% ethanol extraction, while it has the largest values for total reducing capacity; therefore, a comprehensive comparison of the 3 antioxidant activities showed that DES extraction was superior to the conventional water and 70% ethanol extracts. Both the total flavonoids and the total phenolic acids showed better performance in terms of antioxidants; therefore, DES extraction has more obvious advantages in maintaining the activity of flavonoids and polyphenols. It shows that the extraction method used in this study is suitable for the extraction of lotus leaf flavonoids, as it improves the yield of active ingredients while ensuring the antioxidant activity.

Table 6. Antioxidant activity of different extracts.

	Water	Ethanol	DESs
FRAP (mmolFe^{2+}/g)	0.40	0.66	0.76
DPPH-IC50 (mg/g)	3.12	1.29	0.25
ABTS-IC50 (mg/g)	13.38	9.26	6.48

3.3. Correlation Comparison of Different Extraction Methods

The results of the correlation analysis are given in Table 7. Analysis of the data in Table 7 shows that the values of total polyphenol content (TPC), total flavonoid content (TFC), ABTS, DPPH, and FRAP are positive, indicating that the five factors are positively correlated with each other, and in terms of significance, the values of both comparisons are highly significant, indicating that the five factors have a strong positive correlation with each other.

Table 7. Analysis of correlation.

	TPC	TFC	ABTS	DPPH	FRAP
TPC	1				
TFC	0.972 **	1			
ABTS	0.971 **	0.941 **	1		
DPPH	0.829 **	0.773 **	0.892 **	1	
FRAP	0.843 **	0.77 **	0.877 **	0.903 **	1

$p < 0.01$ ** means significant statistical difference.

3.4. LC–MS Detection Results

The basal peak ion flow spectrum obtained from DES extract of lotus leaves analyzed by UPLC-QToF-MS is shown in Figure 9, from which the separation on C18 column by 2.9.1 gradient sub is better. Its main peaks were more than 20, and the peak shape was sharper. It proves that the changed conditions are suitable for LC–MS analysis.

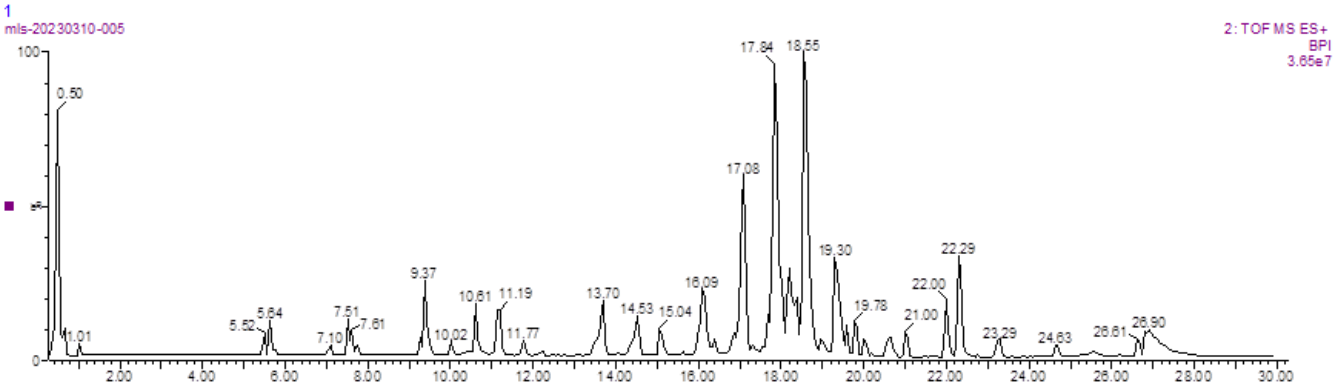

Figure 9. Example of the chromatogram of lotus leaves extract.

The results of compound analysis are shown in Table 8. It was found in the literature that most of the compounds contained in lotus leaves are flavonoids and organic acids [36,37]; therefore, 19 flavonoids or organic acid compounds with known compound structural formula were screened and determined to be contained in the DES extract of lotus leaves by MS profiling. Thus, it was demonstrated that the DES extraction of the constituents in lotus leaves was more complete.

Table 8. Identification of the chemical constituents contained in lotus leaves extract.

NO.	Rt (min)	Formula	Theoretical MASS (Da)	Calculated MASS (Da)	MASS ERROR (ppm)	Component Name
1	0.50	$C_{16}H_{12}O_5$	284.263	284.0685	−1.1	Oroxylin A
2	0.60	$C_{15}H_{10}O_7$	302.236	302.0427	1.6	Quercetin
3	0.67	$C_{15}H_{10}O_6$	286.236	286.0471	1.7	Kaempferol
4	1.14	$C_{16}H_{12}O_7$	316.2623	316.0583	−3.8	Isorhamnetin
5	10.19	$C_7H_6O_4$	154.12	154.0266	0.0	Protocatechuic acid
6	14.53	$C_{20}H_{18}O_{11}$	434.35	434.0849	1.1	Quercetin 3-O-arabinoside
7	14.58	$C_{21}H_{18}O_{13}$	478.36	478.0747	−3.3	Quercetin 3-O-glucuronide
8	14.99	$C_{22}H_{20}O_{11}$	460.4	460.1006	2.6	Oroxindin
9	15.28	$C_{21}H_{18}O_{12}$	462.36	462.0798	0.4	Kaempferol 3-O-glucuronide
10	15.99	$C_9H_8O_4$	180.157	180.0423	3.3	Caffeic acid
11	16.86	$C_{22}H_{22}O_{12}$	478.403	478.1111	−1.9	Isorhamnetin 3-O-hexose
12	16.91	$C_{22}H_{22}O_{11}$	462.41	462.1162	1.7	Diosmetin 7-O-hexose
13	17.96	$C_{21}H_{20}O_{12}$	464.376	464.1033	3.2	Quercetin 3-O-galactoside (hyperoside)
14	18.32	$C_{21}H_{20}O_{11}$	448.38	448.1006	−1.8	Kaempferol 3-O-glucoside (astragalin)
15	18.37	$C_{21}H_{20}O_{12}$	464.376	464.1033	−4.3	Quercetin 3-O-glucoside (isoquercitrin)
16	18.37	$C_{22}H_{20}O_{13}$	492.386	492.0904	−1.0	Isorhamnetin 3-O-glucuronide
17	26.80	$C_{21}H_{20}O_{13}$	480.376	480.0982	0.8	Myricetin 3-O-hexose
18	26.80	$C_{27}H_{30}O_{16}$	610.518	610.1534	4.3	Quercetin 3-O-rhamnopyranosyl-(1→2)-glucopyranoside
19	27.02	$C_{26}H_{28}O_{16}$	596.491	596.1377	2.8	Quercetin 3-O-arabinopyranosyl-(1→2)-galactopyranoside

Rt: retention time on LC–MS.

4. Conclusions

Nature deep eutectic solvents were used for the extraction of flavonoids from lotus leaves. Based on the single-factor assay, the procedure for the extraction of flavonoids and phenolic acids from lotus leaves by NDESs was optimized using the Box–Behnken design method. The optimal extraction process parameters were obtained as a DES system of lactic acid–propanetriol (molar ratio 1:2) with 29% water content, 37:1 solid–liquid ratio (mL/g), extraction temperature 53 °C, and extraction time 61 min. The yield of flavonoids under this condition was 126.0972 mg/g and the yield of polyphenols was 113.1163 mg/g, which were the same as the predicted values of the model. The extraction method used in this study improved the yield of flavonoids while increasing the antioxidant activity compared to the traditional extraction method and is suitable for the research on the extraction and antioxidant activity of lotus leaves. By comparing with the traditional extraction method, we observed that the deep eutectic solvent lactic acid–propanetriol system was superior to the traditional extraction method and that DESs were significantly better than water extraction and ethanol extraction in terms of their clearing ability of DPPH radicals and scavenging ability of ABTS cation radicals. It indicates that the extraction method used in this experiment is efficient and the application of deep eutectic solvent in the extraction of lotus leaf flavonoids is of high practical value. The DES extract of lotus leaves was analyzed by LC–MS and it was determined that 19 compounds in lotus leaves were extracted, indicating a highly efficient DES extraction. As a new extraction system, deep eutectic solvents have the advantages of being green, sustainable, and low-cost, and many studies have shown that its efficiency in extracting active ingredients is higher than that of traditional organic solvents, so deep eutectic solvents can replace traditional organic solvents to a certain extent and can be applied to functional food and pharmaceutical fields, providing a theoretical basis for further development and utilization of lotus leaves.

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Conflicts of Interest: The authors declare no conflict of interest.

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