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Enhanced Antioxidant Extraction from *Lonicerae japonicae Flos* Based on a Novel Optimization Strategy with Tailored Deep Eutectic Solvents

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Abstract: *Lonicerae japonicae Flos* (LJF) is a natural plant containing abundant antioxidant ingredients. In order to extract more antioxidants from LJF, in this study, a novel strategy was proposed for optimizing the extraction factor level by response surface methodology with a tailored deep eutectic solvent (DES) as the extraction solvent and antioxidant ability as the evaluation index. After optimizing the composition of DES and the extraction condition, the extracts obtained by our proposed method yielded better antioxidant ability (229.1–249.1 μmol TE/g DW) and higher antioxidant contents (34.2–36.5 mg GAE/g DW for total phenolics and 119.6–123.0 mg RE/g DW for total flavonoids) from LJF in 5 min without organic solvent consumption that were significantly superior to the Chinese Pharmacopoeia extraction method. The K-T solvation parameter and a scanning electron microscope were adopted to explore the extraction mechanism, and the results showed that the polarity and damage effect on plant cells of DES were crucial for the extraction of antioxidants. In addition, after combining the HPLC fingerprint and partial least squares model, chlorogenic acid, rutin, and 3,5-O-Dicaffeoylquinic acid were screened as the antioxidant Q-markers of LJF. This work demonstrates that an optimization strategy based on antioxidant ability and tailored DES has the potential to extract more antioxidants from natural plants.

Keywords: deep eutectic solvents; extraction of antioxidants; *Lonicerae japonicae Flos*; sample pretreatment; quality evaluation

Citation: Deng, W.-W.; Sun, B.; Yang, H.; Hou, X.-J.; Zhang, Y.-J.; Gan, T.-X.; Cheng, X.-Y.; Yuan, A.; Dong, X.-Y.; Zhou, C.-Y.; et al. Enhanced Antioxidant Extraction from *Lonicerae japonicae Flos* Based on a Novel Optimization Strategy with Tailored Deep Eutectic Solvents. *Separations* **2024**, *11*, 189. https://doi.org/10.3390/separations11060189

Academic Editor: Xingchu Gong

Received: 16 May 2024
Revised: 5 June 2024
Accepted: 13 June 2024
Published: 17 June 2024

1. Introduction

Lonicerae japonicae Flos (LJF), known as Jinyinhua in China, is the dried flower bud of *Lonicera japonica* Thunb, which is pervasively cultivated in eastern Asia, including China, Korea, Japan, etc. [1,2]. LJF is a natural plant with food and medicinal properties and is often used in the production of various teas, such as scented tea, medicinal tea, etc. [1,3]. As a widely used traditional Chinese medicine (TCM), LJF has main active constituents, including phenolic acids, flavonoids, saponins, iridoids, etc., which have many health benefits including antibacterial, anti-inflammatory, antioxidant, hepatoprotective, anticancer, antidiabetic, etc. [4,5]. Because of its diverse health benefits, LJF has been widely used in medicine, health care products, healthy food, cosmetics, etc., and more

than 150,000 products containing LJF (or LJF extracts) can be found on the online market (https://www.jd.com, accessed on 27 November 2023).

Antioxidants are the key components of LJF's medicinal and edible value [2,6], which mainly include phenolic acids, flavonoids, etc. This has motivated many researchers to explore the methods used to extract antioxidant components from LJF. In conventional methods, the extraction of chlorogenic acid was predominantly achieved through water extraction [7] or reflux ethanol extraction [8]. However, these extraction methods consume a lot of time and organic solvents. Nowadays, microwave-assisted extraction [9,10], ultrasound-assisted extraction [11,12], and enzyme-assisted extraction [13] have been developed as feasible extraction methods to reduce extraction time. Additionally, Liu et al. adopted an alcohol/salt aqueous two-phase system (ATPS) to extract and purify flavonoids from LJF with less ethanol, which achieved a higher purity of flavonoids [14]. Duan et al. utilized a homogenate-assisted high-pressure disruption extraction (HHPDE) method to extract phenolic acids from LJF [15]. Although these extraction methods greatly lessen the extraction time, they still adopted volatile organic solvents as extraction solvents, which might cause damage to the environment. What is more, it is difficult to extract antioxidants with different physicochemical properties by methanol and ethanol because of the limited performance of a single solvent. Therefore, it is of great significance to explore a green and multi-solvent solvent to achieve a higher extraction yield for different antioxidants from LJF.

As a new generation of green solvents, deep eutectic solvents (DESs) are composed of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) by the hydrogen bond interaction, which form a stable eutectic system with a eutectic point temperature significantly lower than that of an ideal liquid mixture [16]. Compared with traditional organic solvents, DESs have the advantages of high safety and greenness, and they are easy to prepare [17,18] because of their non-toxicity, non-flammability, non-volatility, and biodegradability [19]. In addition, DESs can be tailored by optimizing their composition and proportion to improve the extraction performance of target compounds [20]. Nowadays, DESs have been widely used to extract active ingredients from natural plants, such as quercetin from tomatoes [21], anthocyanins from Brazilian berries [22], resveratrol from grapevine canes [23], κ-carrageenan from *Kappaphycus alvarezii* [24], and flavonoids from *Flos sophorae* [25]. In addition, Lin et al. found the performance of DESs in extracting antioxidants from *Aronia melanocarpa* was obviously better than that of traditional solvents [26]. These studies indicated that DESs could be adopted as a green medium for extracting antioxidants efficiently from LJF. Recently, Peng et al. adopted a series of DESs for extracting phenolic acids from LJF by optimizing the extraction factor level with response surface methodology (RSM) [27]. However, their optimization strategy ignored the extraction of other antioxidants (such as flavonoids and polyphenols) from LJF. In fact, LJF contains a variety of antioxidant active ingredients, and it is difficult to completely extract the antioxidant components with the content ingredients as the evaluation index. Thus, it is of great significance to explore a novel optimization strategy using a reasonable evaluation index with DESs as the extraction solvent for extracting a higher content of antioxidants from LJF.

In this study, a novel optimization strategy was proposed with the antioxidant ability of the extracts as the evaluation index and a tailored DES as the extraction solvent to obtain more antioxidants from LJF. First, a series of DESs were synthesized and characterized, and different antioxidant evaluation methods were developed, including DPPH·, $ABTS^{+}$·, and ·OH scavenging efficiency and FRAP investigation. Then, the antioxidant properties of the LJF extracts using different solvents were evaluated and an accurate evaluation of the antioxidant ability was completed. Finally, the detailed extraction factor and level were screened through One-Variable-at-a-Time (OVAT) and RSM-based Box–Behnken Design (BBD) with antioxidant ability as the evaluation index, and the extraction mechanism was also investigated. In addition, the HPLC fingerprint of LJF obtained using our proposed extraction method was constructed and successfully used for quality evaluation

of LJF based on antioxidant activity studies. As far as we know, this study is the first to adopt antioxidant ability as the evaluation index for optimizing the extraction process of antioxidants from natural plants.

2. Materials and Methods

2.1. Materials

Dried LJF and *Lonicerae Flos* (LF) from different regions (including Shandong, Hebei, Henan, Hunan, and Guangxi Provinces in China) were obtained through a local pharmacy in Wuhan, China (detailed information on the collected LJF and LF samples is shown in Table S1) and stored tightly in a desiccator. The obtained LJF samples were identified based on external morphological and microscopic characteristics according to the Chinese Pharmacopoeia (2020 Edition, Volume I) [28], and the identification results are shown in Figure S1 of the Supplementary Materials. The dried LJF and LF samples were pulverized and passed through a 65-mesh sieve, and the resulting LJF and LF powders were sealed in a desiccator and stored at room temperature. The water content of the powders was determined by a halogenated moisture tester (XY-105W, TUOKE Instrument, Qingdao, China) according to the Chinese Pharmacopoeia method (2020 Edition). A standard procedure was carried out by putting more than 1 g of powder on a sample tray, heating it at 105 °C, and maintaining the mass at a constant weight; the loss of mass was considered the water content of the powder. As shown in Table S1 of the Supplementary Materials, the water content of the LJF powder was between 8.90 and 9.77% (m/m), and the LF powder was stable between 9.02 and 10.00%, both meeting the requirements of the Chinese Pharmacopoeia (not exceed 12.0% for LJF and 15.0% for LF).

Chlorogenic acid, cynaroside, rutin, and 3,5-O-Dicaffeoylquinic acid standards were purchased from Push Bio-technology Co., Ltd. (Chengdu, China). Choline chloride (ChCl, 98%), betaine (Be, 98%), tetramethylammonium chloride (TMAC, 99.7%), tetraethylammonium chloride (TEAC, 98%), tetrabutylammonium chloride (TBAC, 99.5%), 2,2-Diphenyl-1-picrylhydrazyl (DPPH, 96%), potassium persulfate (99.5%), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 99%), and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, 99%) were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). DL-Malic acid (MA, 99%), acetic acid (Aa, 99%), and oxalic acid (OA, 98%) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Glucose (Glu, 99.5%), ethylene glycol (EG, 99%), 1,2-Propanediol (Pro, 99.5%), 1,4-Butylene glycol (But, 99%), glycerol (Gly, 99%), urea (99%), and lactic acid (LA, 99.7%) were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox, 98%) was purchased from Tocris Bioscience (Ellisville, MO, USA). Deionized water was obtained from an ultrapure water machine (UPT-I-60L, ULUPURE, Wuhan, China). All other reagents used were of analytical grade.

2.2. Synthesis and Characterization of DESs

DESs were prepared by the heating method with ChCl, Be, TMAC, TEAC, TBAC as HBA, and EG, Gly, Pro, But, Aa, urea, MA, OA, LA, Glu, and EGas HBD. HBA and HBD were mixed and heated under magnetic stirring (500 rpm) at 80 °C until a clear, homogeneous, and stabilized liquid formed at room temperature [29]. The viscosity of the DESs was measured at 25 °C with a rheometer (HAAKE RheoStress 6000, Thermo Scientific, Waltham, MA, USA). The pH of the DESs was determined by an acidimeter (Five Easy Plus FE28, Mettler Toledo, Columbus, OH, USA). The densities of the DESs were determined by aspirating 100 μL of liquid at room temperature utilizing a micro-syringe and weighing the change in syringe mass using an analytical balance (ME204E, Mettler Toledo, Columbus, OH, USA). Density was calculated utilizing the equation $\rho = \Delta m/V$ (the detailed physicochemical properties of the prepared DESs are shown in Table 1). An FT-IR spectrometer (Nicolet iS10, Thermo Scientific, Waltham, MA, USA) was adopted to explore the interaction of the components of DESs with TMAC-EG-1 as a representative (Figure S2).

Table 1. Compositions and physicochemical properties of the studied DESs.

No.	Abb.	HBA	HBD	Molar Ratio	Viscosity (mPa·s)	Density (g/mL)	pH
1	ChCl-EG-1	ChCl	EG	1:4	19.60	1.13	7.34
2	ChCl-EG-2	ChCl	EG	1:2	38.58	1.11	4.21
3	ChCl-Pro-1	ChCl	Pro	1:4	49.91	1.04	6.52
4	ChCl-Pro-2	ChCl	Pro	1:2	84.19	1.03	3.43
5	ChCl-But-1	ChCl	But	1:4	65.31	1.01	5.41
6	ChCl-But-2	ChCl	But	1:2	41.36	1.06	4.42
7	ChCl-Gly	ChCl	Gly	1:2	288.03	1.19	6.02
8	ChCl-Aa-1	ChCl	Aa	1:1.5	68.96	1.11	1.53
9	ChCl-Aa-2	ChCl	Aa	1:2	47.01	1.08	1.26
10	ChCl-Urea	ChCl	Urea	1:2	449.00	1.25	8.37
11	ChCl-MA	ChCl	MA	1:2	N.C.	1.34	0.26
12	ChCl-OA	ChCl	OA	1:2	N.C.	1.35	0.71
13	ChCl-LA	ChCl	LA	1:2	163.21	1.14	0.52
14	ChCl-Glu	ChCl	Glu	1:2	N.C.	1.27	3.57
15	Be-EG	Be	EG	1:2	N.C.	1.26	7.00
16	TMAC-EG-1	TMAC	EG	1:2	N.C.	1.08	4.00
17	TMAC-EG-2	TMAC	EG	1:3	19.92	1.08	6.03
18	TEAC-EG	TEAC	EG	1:2	43.10	1.05	5.75
19	TBAC-EG	TBAC	EG	1:2	15.82	1.08	4.18

N.C. indicates that the data could not be measured.

2.3. Extraction of LJF

The extraction of LJF was conducted using a heating and stirring method based on DESs, which is an easy-to-operate method suitable for industrial amplification that was inspired by our previous studies [29,30]. In a test tube with a magnetic rotor, 0.1 g of dried LJF powder was mixed with a given volume of DES with different water contents. The obtained tube was then placed in a water bath on a hot plate magnetic stirrer (MS-H-Pro+, DLAB, Beijing, China). After being extracted by heating and stirring under certain conditions (DES content: 30–70% *v*/*v*; extraction time: 1–30 min; extraction temperature: 40–80 °C; liquid–solid ratio: 10/1–50/1 mL/g). In addition, extraction with traditional solvents (water, methanol, and ethanol) was also studied. The resulting sample was centrifuged at 10,000 rpm for 10 min, and the supernatant was collected for antioxidant activity evaluation (diluted 10-fold with deionized water) and chromatographic analysis. All magnetic speeds were set to 500 rpm. The loss mass of the mixture was supplemented with the appropriate extraction solvent.

2.4. Antioxidant Activities of LJF Extracts

Scavenging of DPPH Free Radicals: The antioxidant ability of the LJF extracts was investigated by the DPPH free radical (DPPH·) scavenging method according to the literature precedent with ethanol as the solvent for DPPH· [31–33]. DPPH· was dissolved in anhydrous ethanol to a final concentration of 0.13 mM. Then, 600 µL of DPPH· solution was homogeneously mixed with 20 µL of an LJF extract. The obtained mixtures were incubated for 30 min in the dark, and the absorbance of DPPH· at 517 nm was determined using a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, CA, USA). The DPPH· scavenging activity was evaluated as follows:

$$\text{DPPH·scavenging activity (\%)} = \frac{A_0 - A_1}{A_0}, \quad (1)$$

where A_0 and A_1 are the absorbance of DPPH· at 517 nm in the absence and presence of the LJF extracts, respectively.

Scavenging of ABTS Free Radicals: The ABTS free radical ($ABTS^{+}$·) scavenging capacity of the LJF extracts was determined using a method previously described in the

literature [34]. The $ABTS^{+}\cdot$ work solution was prepared by mixing 14 mM ABTS aqueous solution and 4.9 mM $K_2S_2O_8$ solution in equal volumes for 12–16 h at room temperature without light. The obtained $ABTS^{+}\cdot$ solution was then diluted with deionized water until the absorbance value achieved 0.7 ± 0.02 at 734 nm. Next, 20 µL of an LJF extract was mixed with 1 mL of diluted $ABTS^{+}\cdot$ solution and reacted for 6 min. The $ABTS^{+}\cdot$ scavenging efficiency was measured as follows:

$$ABTS^{+}\cdot \text{ scavenging activity } (\%) = \frac{A_0 - A_1}{A_0}, \quad (2)$$

where the A_0 and A_1 refer to the absorbance of the $ABTS^{+}\cdot$ at 734 nm in the absence and presence of the LJF extracts, respectively.

Scavenging of Hydroxyl Radicals: The hydroxyl radical (·OH) was produced by the classical Fenton reaction with salicylic acid according to the literature precedent [35]. First, 100 µL of $FeSO_4$ (9 mM) was mixed with 100 µL of salicylic acid (9 mM) and 200 µL of an LJF extract. Then, 100 µL of H_2O_2 (9 mM) was added, and the reaction occurred at room temperature for 30 min in the dark. After being centrifuged at 12,000 rpm for 5 min, the supernatant was collected, and its absorbance was measured at 510 nm. The ·OH scavenging capacity was calculated using the following formula:

$$\cdot OH \text{scavenging activity } (\%) = \frac{A_0 - A_1}{A_0}, \quad (3)$$

where A_0 and A_1 represent the absorbance in the absence and presence of the LJF extracts, respectively.

Ferric Reducing Antioxidant Power (FRAP): The FRAP method was adopted to explore the antioxidant ability according to the literature precedent with $FeSO_4$ as a standard [36,37]. First, the FRAP working solution was formed by mixing tripyridyltriazine solution (TPTZ, 10 mM) with acetate buffer (300 mM) and ferric chloride solution (20 mM) with a volume ratio of 1:10:1. Then, 1.2 mL of the obtained FRAP working solution was reacted with 20 µL of an LJF extract for 5 min at 37 °C in the dark, and the absorbance at 593 nm was measured. The antioxidant ability of the LJF extracts was expressed as mM ferrous sulfate equivalents (SEs) per gram of the dried weight of LJF powder (mM Fe(II)SE/g DW) according to the constructed standard curve as follows: $y = 0.3855x - 0.0417$ (0.6–4.0 mM, $R^2 = 0.9998$).

Antioxidant Ability: In order to accurately evaluate the antioxidant level of the LJF extracts, the antioxidant ability was adopted with Trolox (a known antioxidant) as the reference with the DPPH· assay, based on the previous studies [35]. The resulting antioxidant ability of the LJF extracts was expressed as micromole Trolox equivalents (TEs) per gram of dried weight of the sample (µmol TE/g DW), which was calculated by the constructed standard curve as follows: $y = -2.8267x + 1.3618$, (0.1–0.45 mM, $R^2 = 0.9996$, Figure S3).

2.5. HPLC Analysis

The chromatographic conditions were optimized with reference to the work of Liu et al. [38] and Zhang et al. [39]. Chromatographic analysis was performed on a high-performance liquid chromatography system (Prominence LC-20A, Shimadzu Corporation, Kyoto, Japan) equipped with a UV detector and a quadruple pump. Chromatographic detection was performed on an Amethyst C18-H column (4.6 × 250 mm, 5 µm, Sepax Technologies, Newark, DE, USA) at 38 °C. The mobile phases were composed of acetonitrile (eluent A) and 0.2% formic acid aqueous solution (eluent B) with gradient elution, the gradient program was set as follows: 0–10 min 8–9% A; 10–25 min 9–11% A; 25–35 min 11–15% A; 35–50 min 15–16% A; 50–65 min 16–20% A; 65–75 min 20–24% A; and 75–80 min 24–38% A. The injection volume was 20 µL. The mobile phase flowed at 0.5 mL/min. The detection wavelength of the UV detector was set to 245 nm (the set wavelength was according to the number of chromatographic peaks by optimization).

2.6. Experimental Design Strategy

The extraction condition was first optimized by the tailored DES strategy and the OVAT method to obtain the optimized DES and extraction conditions of factors for designing the high and low levels of subsequent RSM experiments. Subsequently, RSM combined with a three-level (−1, 0, +1) three-factor BBD was adopted to optimize the three major influence factors, namely, DES content (A, 60–75–90%), extraction temperature (B, 60–70–80 °C), and liquid–solid ratio (C, 20:1–30:1–40:1, mL/g). The 17 runs conducted with five central points BBD project are shown in Table S2 with antioxidant ability (μmol TE/g DW) as the response. The significance of the coefficients and the interaction among the independent variables were analyzed using Design Expert 10.0.1 Trial software.

2.7. UPLC-MS Analysis

The antioxidants were identified by liquid chromatography–mass spectrometry (U3000, Q-Exactive, Thermo Scientific, Waltham, MA, USA). The chromatographic detection was performed on an Amethyst C18-H column (4.6 × 250 mm, 5 μm, Sepax Technologies, Newark, DE, USA) at 38 °C. The detection wavelength of the UV detector was set to 245 nm. The injection volume was 20 μL. The mobile phase flowed at 0.5 mL/min. The gradient elution program used was the same as that described in Section 2.5. Positive and negative ion modes were used to analyze the samples. The spray voltage was set at 3.5 kV, and the sheath gas pressure and auxiliary gas pressure were at 40 Arb and 10 Arb, respectively. The ion transfer tube temperature and heated evaporation temperature were set at 350 °C and 250 °C; respectively. The normalized collision energy was set to 30 NCE. Full MS—dd MS2 mode was adopted with a scan range from 80 to 1200 m/z. Data processing was performed using Xcalibur^{TM} 2.2 software (Thermo Scientific, Waltham, MA, USA).

2.8. Statistical Analysis

All data are shown as the mean of three replicates ± standard deviation (SD). IBM SPSS Statistics 26.0 software (IBM SPSS Inc., Armonk, NY, USA) was used for statistical analysis. The hypothesis of normality was assessed using the Shapiro–Wilk test ($p \geq 0.05$). One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed to evaluate the significance of the optimization level and the accuracy of the RSM model. A p-value less than 0.05 was considered statistically significant

3. Results and Discussion

3.1. Solvent Screening and DES Tailoring

Extraction solvents impact the mass transfer efficiency of active ingredients and rupture of plant cell walls, which greatly affects the extraction yield of target analytes [40]. Here, five types of natural DESs were prepared with ChCl as HBA for extraction antioxidants from LJF, and the antioxidant ability of extracts was explored by evaluating the DPPH·, $ABTS^{+}$· and ·OH scavenging efficiency and FRAP investigation, in comparison to traditional solvents (methanol, ethanol, and water). The results (Figure 1A–D) showed the free radical scavenging efficiency (44.94 to 56.76% for DPPH·, 70.11 to 78.70% for $ABTS^{+}$·, and 26.17 to 30.43% for ·OH) and FRAP performance (500.92 to 572.05 mM Fe(Π)SE/g DW) of all the five DESs were significantly higher than that of water (27.25% for DPPH·, 47.19% for $ABTS^{+}$·, 22.17% for ·OH, and 263.04 mM Fe(Π)SE/g DW for FRAP). In particular, ChCl-EG-1 had a significantly higher free radical cleansing effect and FRAP property than those of traditional organic solvents (methanol and ethanol). The above results indicated that ChCl-based DES has the capacity to extract antioxidants from LJF with a higher content compared with conventional solvents.

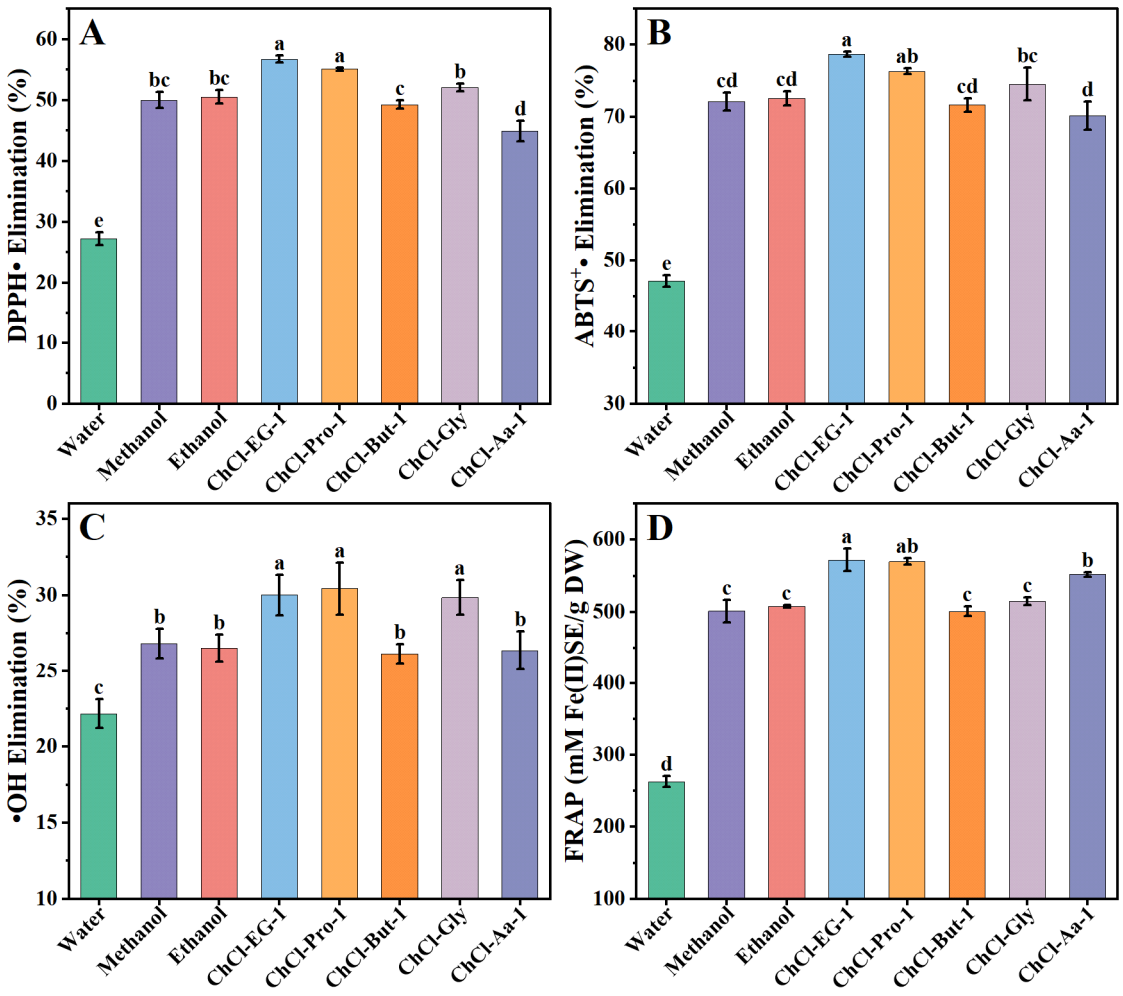

Figure 1. Antioxidant ability of LJF extracts with different solvents. DPPH· (**A**), $ABTS^{+}$· (**B**), ·OH (**C**), and FRAP (**D**). Different letters in the same series indicate significant differences at the $p < 0.05$ level.

To achieve the optimized extraction capacity for various antioxidants, the composition and proportion of DES were tailored according to the literature precedent [41]. Initially, a series of DESs was prepared based on ChCl and different alcohol-based compounds (EG, Pro, But, and Gly), acid-based compounds (Aa, MA, OA, and LA), sugar-based compounds (Glu), and amine-based compounds (Urea) with a 1:2 mole ratio for screening the HBD. As shown in Figure 2A, ChCl-EG-2 exhibited the highest DPPH· scavenging activity (63.89%) among the 10 DESs. This phenomenon may be due to the low viscosity of ChCl-EG-2 (Table 1), which improved the mass transfer efficiency of the target component. Subsequently, five kinds of DESs were synthesized with EG and different quaternary ammonium salts (ChCl, Be, TMAC, TEAC, and TBAC), and the antioxidant ability of extracts by DESs was investigated for selecting HBA. The results (Figure 2B) demonstrated that the TMAC-EG-1 extract displayed the highest DPPH· scavenging activity (66.96%). This might be attributed to the shorter chain length of TMAC, which decreased the steric hindrance between DESs and antioxidants and was conducive to the formation of hydrogen bonds between DESs and antioxidants [41]. Lastly, TMAC-EG DESs with different molar ratios (HBA/HBD, ranging from 1:2 to 1:5) were tested, and the results (Figure 2C) showed that the scavenging activity of DPPH· slightly increased initially and then decreased slightly with elevated HBA/HBD values. This phenomenon was consistent with our previous work on optimizing the mole ratio for the extraction of sulforaphane [30]. The increased antioxidant ability was attributed to the strengthened hydrogen bond interaction between DES and antioxidants with the enhanced HBD content. When HBA/HBD exceeds 1:3, the

viscosity of DES might increase with the increasing ratio of HBD in DES [42], which will hinder the diffusion of the antioxidants in the DES. Considering the high antioxidant ability of the LJF extracts, TMAC: EG with a 1:3 molar ratio (HBA/HBD) was selected for the subsequent investigation.

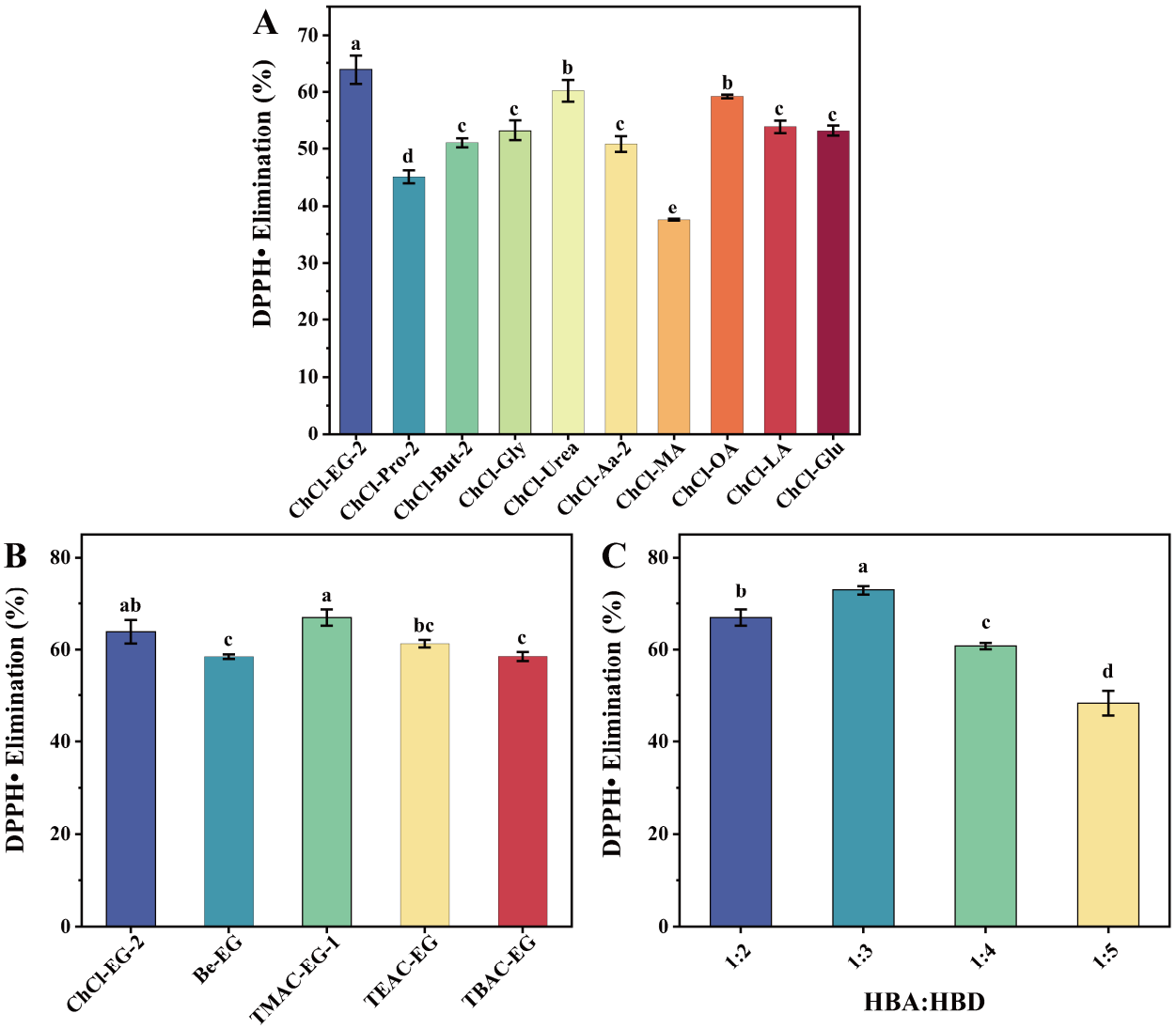

Figure 2. Antioxidant ability of LJF extracts with tailored DES. The influence of HBD type (**A**), HBA type (**B**), molar ratio of TMAC, and EG (**C**) of DES on the DPPH· scavenging of LJF extracts. Different letters in the same series indicate significant differences at the $p < 0.05$ level.

3.2. Optimization of Extraction Conditions by OVAT

To achieve the optimal extraction conditions, different factors including the content of DES (30%, 45%, 60%, 75%, and 90%, *v*/*v*), extraction time (1, 3, 5, 10, 15, and 30 min), extraction temperature (40, 50, 60, 70, and 80 °C), and liquid–solid ratios (L/S, 10:1, 20:1, 30:1, 40:1, and 50:1 mL/g) were optimized via OVAT experiments with the antioxidant ability of extracts as the evaluation index.

3.2.1. Content of DES

Generally, DES is often mixed with water to reduce viscosity when extracting active compounds from natural plants [29]. However, the addition of water could decrease the solubility of antioxidants in DES because of the existing hydrophobicity of isochlorogenic acid A (log*P* of 2.46) [43] in LJF. In this work, different contents of aqueous TMAC-EG-2

(30–90%, *v*/*v*) were adopted to explore the extraction performance of antioxidants from LJF with heating at 60 °C for 90 min and a liquid–solid ratio of 20:1. The results (Figure 3A) showed that the antioxidant ability of LJF extracts increased with increasing DES content in the range of 30–75%. The maximum antioxidant ability (241.06 μmol TE/g DW) was reached when the DES content reached 75%. However, as the DES content continued to increase up to 90%, the antioxidant ability decreased. This phenomenon may be attributed to the higher viscosity of higher concentrations of DES, which impedes the mass transfer of active ingredients in solution, thereby reducing the extraction of antioxidants [40]. Therefore, the 75% (*v*/*v*) TMAC-EG-2 DES content was selected for the subsequent investigation.

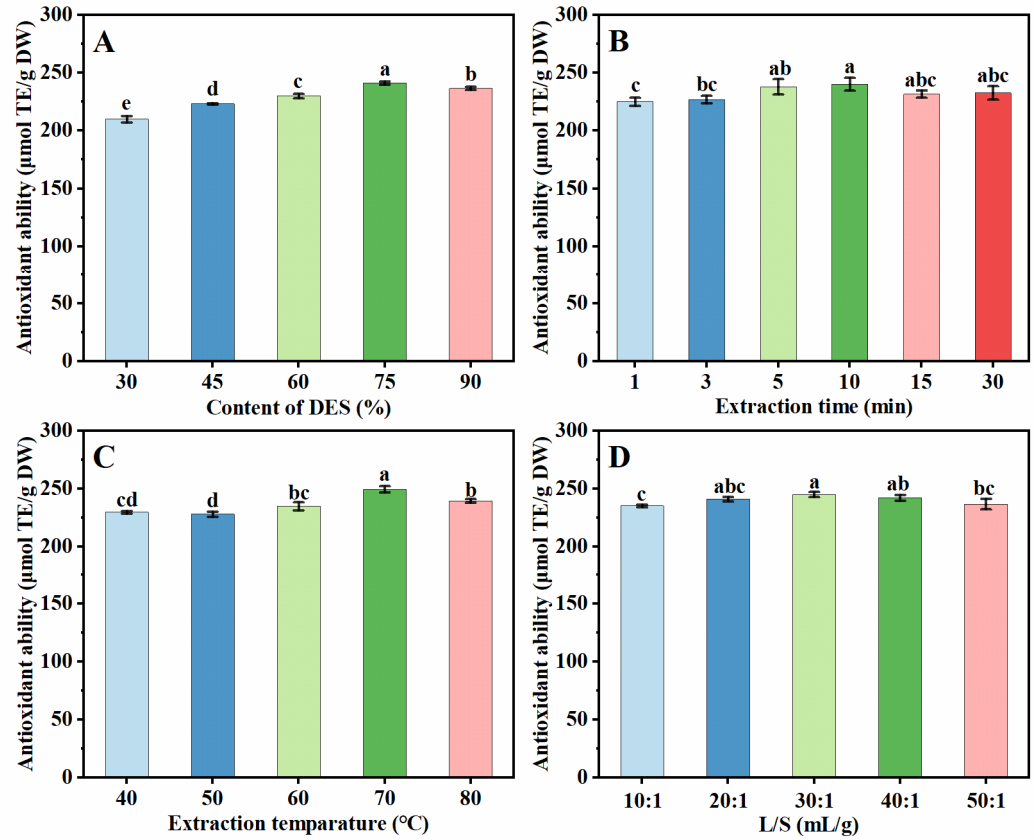

Figure 3. Effect of DES content (**A**), extraction time (**B**), extraction temperature (**C**), and liquid–solid ratio (**D**) on the antioxidant ability of LJF extracts. Different letters in the same series indicate significant differences at the $p < 0.05$ level.

3.2.2. Extraction Time

The impact of extraction time on the antioxidant ability of extracts was conducted with 75% of TMAC-EG-2 DES as the extraction solvent at 80 °C with a liquid–solid ratio of 20:1. As depicted in Figure 3B, the antioxidant ability of the LJF extracts increased significantly at first and then reached a maximum (240.05 μmol TE/g DW) and remained constant with extraction time longer than 5 min, which indicated 5 min of stirring could extract the main antioxidants. Thus, a 5 min extraction time was selected for the subsequent optimization.

3.2.3. Extraction Temperature

Extraction temperature is crucial for extracting active ingredients from plants through DES because of variable viscosity [44]. To explore the influence of extraction temperature (40–80 °C) on the extraction yield of antioxidants, different LJF extracts were prepared with TMAC-EG-2 DES (75%) extraction at various temperatures (40–80 °C) for 5 min with a liquid–solid ratio of 20:1. The results (Figure 3C) indicated the antioxidant ability increased with increasing temperature and then decreased when the temperature was

higher than 70 °C. The increased temperature could decrease the viscosity of DESs, thus enhancing the contact of LJF powder with the extraction solvent and enhancing the extraction yield of antioxidants. With a temperature of more than 70 °C, the antioxidants might have been oxidized or decomposed, leading to the observed decrease in antioxidant ability [45]. Considering the extraction yield and energy consumption, 70 °C was adopted for further studies.

3.2.4. Liquid–Solid Ratio

An elevated liquid–solid ratio increases the concentration difference in targets in the solvent and plant powder, which enhances the driving force of mass transfer for targets [46]. To optimize this factor, five liquid–solid ratios (from 10:1 to 50:1, mL/g) were evaluated at 70° for 5 min with 75% of TMAC-EG-2 DES as the extraction solvent. The results (Figure 3D) showed that the antioxidant ability of LJF extracts first increased significantly with an increasing liquid–solid ratio from 10:1 to 30:1 and then reached a maximum value of 244.82 μmol TE/g DW. After a further increase in the liquid–solid ratio, the antioxidant ability of the LJF extracts showed a decreasing trend. Considering the excellent antioxidant ability, we selected the liquid–solid ratio of 30:1 mL/g for the extraction of antioxidants from LJF.

3.3. Optimization of the Extraction Condition Using RSM

RSM has been widely adopted for the optimization of extraction conditions for natural ingredients from TCM because of its ability to evaluate interaction effects among different variables using fewer experiments [47]. In this study, the BBD model was adopted for the optimization of three factors (A: DES content; B: extraction temperature; C: liquid–solid ratio) according to the results of OVAT. Considering that 5 min of extraction time is short enough for extraction from natural plants, we did not optimize the extraction time in the RSM experiments but fixed the extraction time at 5 min. The antioxidant ability of LJF extracts was adopted as the response (R). The results of the 17 experimental runs of BBD are shown in Table S2 with the antioxidant ability of the extracts in the range of 210.22 to 247.84 μmol TE/g DW. Then, these data were fitted to a second-order polynomial model by multiple regression analysis, and the obtained model regression equations for the response values (R) and variables were as follows:

$$R = 243.68 - 6.79\,A - 1.86\,B + 8.82\,C + 3.90\,AB - 3.34\,AC + 6.11\,BC - 13.23\,A^2 - 5.67\,B^2 - 9.43\,C^2 \quad (4)$$

ANOVA was used to evaluate the statistical significance of the obtained BBD model. As shown in Table S3, the model owned a high F-value of 59.63 and a low *p*-value ($p < 0.0001$), which indicated the model was significant and reliable for analyzing experimental data [44]. The *p*-value of the lack of fit was 0.6107 ($p > 0.05$), indicating the reliable predictive ability of this model. The fitting constants (R^2), adjusted fitting constants (adjusted R^2), and predicted fitting constants (predicted R^2) were 0.9871, 0.9706, and 0.9174, respectively, indicating that the model had satisfactory accuracy, reliability, and predictive capability. In addition, the *p*-values of factors A, B, and C were all less than 0.05, indicating that all the factors selected had a significant effect on the antioxidant ability of the LJF extracts. Among the interactional factors, AB, AC, and BC were highly significant ($p < 0.05$). This phenomenon indicated that the extraction performance for antioxidants depends on the DES content, DES dosage (affected by the liquid–solid ratio), and DES viscosity (affected by the extraction temperature).

To achieve optimized antioxidant performance, 3D response surface curves were plotted. As shown in Figure 4A–C, all three response surfaces were upward convex with the maximum point within the experimental range, indicating the selected level ranges of the factors were reasonable. In addition, the trends in these factors were similar to the results of the OVAT experiment. Based on the RSM model, the predicted strongest antioxidant ability of the LJF extract was achieved at an extraction temperature of 70.09 °C, a DES content of 70.18% (*v*/*v*), and a liquid–solid ratio of 35.27 mL/g, and the predicted

R value was 247.09 μmol TE/g DW. For easy operation, the conditions were slightly adjusted to the extraction temperature of 70 °C, DES content of 70%, and liquid–solid ratio of 35 mL/g to verify the predicted result. Under these conditions, the experimental value (249.12 ± 3.71 μmol TE/g DW) was consistent ($p > 0.05$) with the predicted results (247.09 μmol TE/g DW) with an error of 0.82%, confirming the accuracy and reliability of the fitted model.

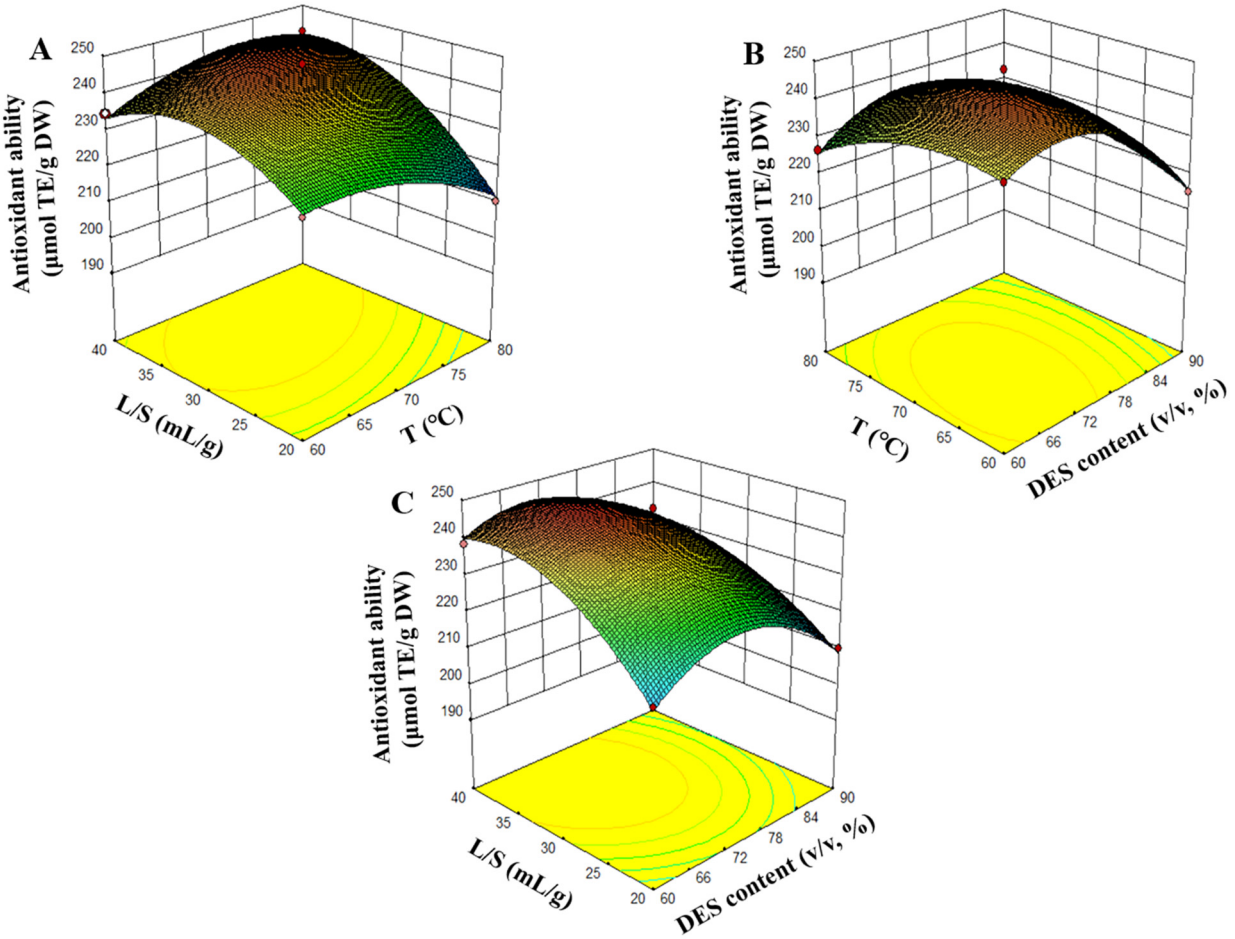

Figure 4. Response surface plots for the antioxidant ability of LJF extracts using BBD. (**A**): Mutual effects of the liquid–solid ratio and extraction temperature on the antioxidant ability of LJF extracts; (**B**): mutual effects of extraction temperature and DES content on the antioxidant ability of LJF extracts; and (**C**): mutual effects of the liquid–solid ratio and DES content on the antioxidant ability of LJF extracts.

3.4. Comparison of the Antioxidant Activity of LJF Extracts with the Chinese Pharmacopoeia Method

Traditionally, the antioxidants (including phenolic acids, flavonoids, etc.) of LJF were extracted by organic solvents such as methanol and ethanol [15,48]. To explore the extraction performance of our proposed method based on a tailored DES, three types of LJF samples from different origins (Henan Province, Shandong Province, and Hebei Province) were analyzed by our proposed method and the Chinese Pharmacopoeia (2020 Edition) method [28] (extraction with 75% methanol assisted by ultrasound for 30 min), which was the standard extraction method for the quality evaluation of LJF. The results (Table 2) showed that the antioxidant ability of the LJF extracts based on the tailored DES was between 229.1 and 249.1 μmol TE/g DW, which was obviously superior to the method of the Chinese Pharmacopoeia (189.7 to 213.1 μmol TE/g DW). Furthermore, the results of the antioxidant content (total phenolic content and total flavonoid content) showed that our proposed method could extract more antioxidants than the Chinese Pharmacopoeia

extraction method. In addition, as shown in Table 2, our proposed method consumed less organic solvent (without consuming organic solvent) and time (5 min of extraction time) than the Chinese Pharmacopoeia method (30 min of extraction time, 75 mL methanol for 1 g LJF powder). Thus, our proposed tailored TMAC-EG-2 DES-based extraction method is a green, easy-to-operate, fast, and adaptable method for extracting antioxidants from LJF.

Table 2. Comparison of our tailored DES-based extraction method with the Chinese Pharmacopoeia method.

Extraction Method		Tailored DES-Based Extraction Method	Chinese Pharmacopoeia Extraction Method
Extraction yields of antioxidants from different origins (μmol TE/g DW)	Henan	241.6 ± 5.5 [a]	213.1 ± 8.2 [b]
	Shandong	249.1 ± 4.6 [a]	189.7 ± 6.0 [b]
	Hebei	229.1 ± 1.2 [a]	199.3 ± 6.2 [b]
Total phenolic content from different origins (mg GAE/g DW)	Henan	35.7 ± 0.2 [a]	28.4 ± 0.8 [b]
	Shandong	36.5 ± 0.4 [a]	29.5 ± 1.0 [b]
	Hebei	34.2 ± 0.6 [a]	30.0 ± 1.4 [b]
Total flavonoid content from different origins (mg RE/g DW)	Henan	120.5 ± 1.7 [a]	79.6 ± 4.0 [b]
	Shandong	119.6 ± 1.8 [a]	88.0 ± 4.9 [b]
	Hebei	123.0 ± 1.3 [a]	87.6 ± 4.0 [b]
Extraction time (min)	Henan Shandong Hebei	5	30
Organic solvent consumption /1.0 g LJF powder	Henan Shandong Hebei	None	75 mL of methanol

Different letters in the same row indicate significant differences at the $p < 0.05$ level.

3.5. Extraction Mechanism

Generally, the extraction performance of the DESs was mainly related to the interaction between the DESs and targets [30] and the damaging effect of the DESs on the plant cell wall [29]. To explore the extraction mechanism of our proposed method, the solvation characteristics of various DESs were measured using the K-T parameters (including α: hydrogen bond-donating ability, β: hydrogen bond acceptor ability, π*: polarizability) according to our previously reported method [30]. The results (Table S4) showed that the α (0.623–1.203) and β values (0.525–1.360) of most DESs (except for ChCl-Aa DES) were obviously larger than that of water (0.300 for α and 0.209 for β). The π* values (1.001–1.165) of the DESs were close to water (1.270) and more than those of the traditional organic solvents (0.672–0.712). The above results indicated these DESs had strong polarity and hydrogen bonding interactions, which could form stronger H-bonds with antioxidants than water and traditional organic solvents. To explore the key parameters for the extraction of antioxidants, the measured K-T parameters were correlated with the antioxidant ability and the content of antioxidants (flavonoids and polyphenols) of the LJF extracts. The results indicated that there was no obvious correlation between antioxidant ability and α or β (Figure 5A,B), and only π* showed a positive correlation (Figure 5C), which was consistent with the fitting results of flavonoids and polyphenols (Figure S4A–F). The results demonstrated that a higher polarity of the DESs was beneficial for extracting antioxidants from LJF. This might be due to the rich polar functional groups (such as phenolic hydroxyl groups of polyphenols and phenolic acids) that easily formed hydrogen bonds with the polar solvents.

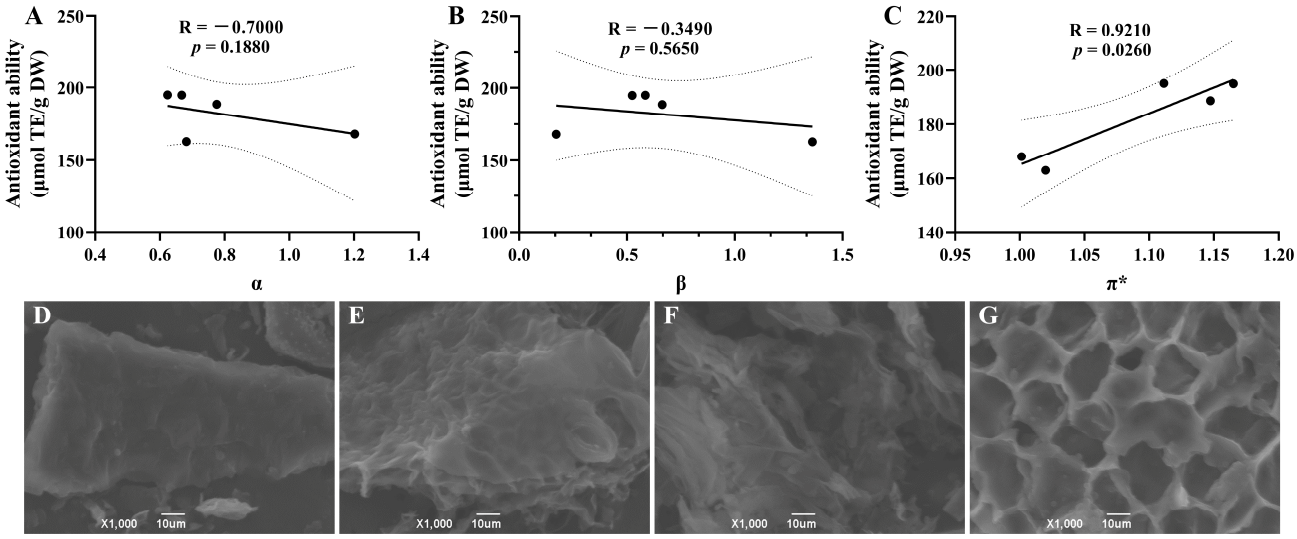

Figure 5. The correlation between the antioxidant ability of LJF extracts and the subtraction value of α (**A**), β (**B**), and π* (**C**) of DESs. SEM images of LJF powder before extraction (**D**) and after extraction by water (**E**), ethanol (**F**), and the DES (**G**) method.

To examine the damaging effect of DESs on plant powder, the surface characteristics and morphology of the LJF powder after extraction based on the tailored DES were analyzed by Scanning Electron Microscopy (SEM) according to our previously reported method [29]. As shown in Figure 5D–G, the untreated LJF powder contained dense and well-ordered cell walls (Figure 5D). Upon treatment with water (Figure 5E) and ethanol (Figure 5F), the powder experienced more shrinking and slight damage, which might be because the heat conduction damaged the plant cells. After extraction by DES (Figure 5G), a significant amount of porous slice was formed, indicating a more severe cell wall breakage, which could enhance the release of antioxidants into the surroundings (DES). According to the above results, the polarity of the DES and its damaging effect on plant cells were crucial for the extraction of antioxidants from LJF by the tailored DESs.

3.6. Quality Evaluation of LJF Based on Antioxidant Extraction and Chromatographic Fingerprinting

The quality of LJF was affected by many factors such as the origin, cultivation condition, processing methods and conditions, etc. Some counterfeit products often appear on the market, such as *Lonicerae Flos* (LF, called Shanyinhua in China), the dried flower buds of *Lonicera macranthoides* Hand. -Mazz., *Lonicera hypoglauca* Miq., *Lonicera confusa* DC., and *Lonicera fulvotomentosa* Hsu et S. C. Therefore, it is of great practical significance to use the quality evaluation method for authenticity identification and origin distinction of LJF to ensure its quality and health benefits.

First, in order to explore the real antioxidants of LJF, a gray relation analysis (GRA) was performed with antioxidant capacity and extract content (total reducing sugars, flavonoids, and polyphenols) of various LJF samples. The results suggested that flavonoids and polyphenols are the main antioxidant components of LJF (Tables S5 and S6). Then, the HPLC-DAD-based fingerprint was adopted to explore the specific components of flavonoids and polyphenols for screening the antioxidant "quality marker" (Q-marker) by the UV absorption characteristics of flavonoids and polyphenols. In order to detect more compounds, different UV absorption wavelengths (225, 245, 260, 325, and 350 nm) were investigated according to the reported separation method [38]. The results (Figure S5) showed that the HPLC fingerprint at 245 nm had more peaks than those of 225, 260, 325 nm, and 350 nm. Therefore, 245 nm was chosen as the detection wavelength for fingerprinting. After methodological investigation (Table S7), the fingerprints of LJF antioxidants were constructed (Figures 6A and S6A), and nine common peaks were found on the fingerprint

spectrum. Similarity is widely recognized as an index for the legal evaluation of the fingerprint [49]. Subsequently, the similarity in the samples and the corresponding reference chromatogram R were examined. As shown in Table S8, the similarity in the different LJF samples was more than 0.934, suggesting that the overall quality of LJFs from different regions was similar.

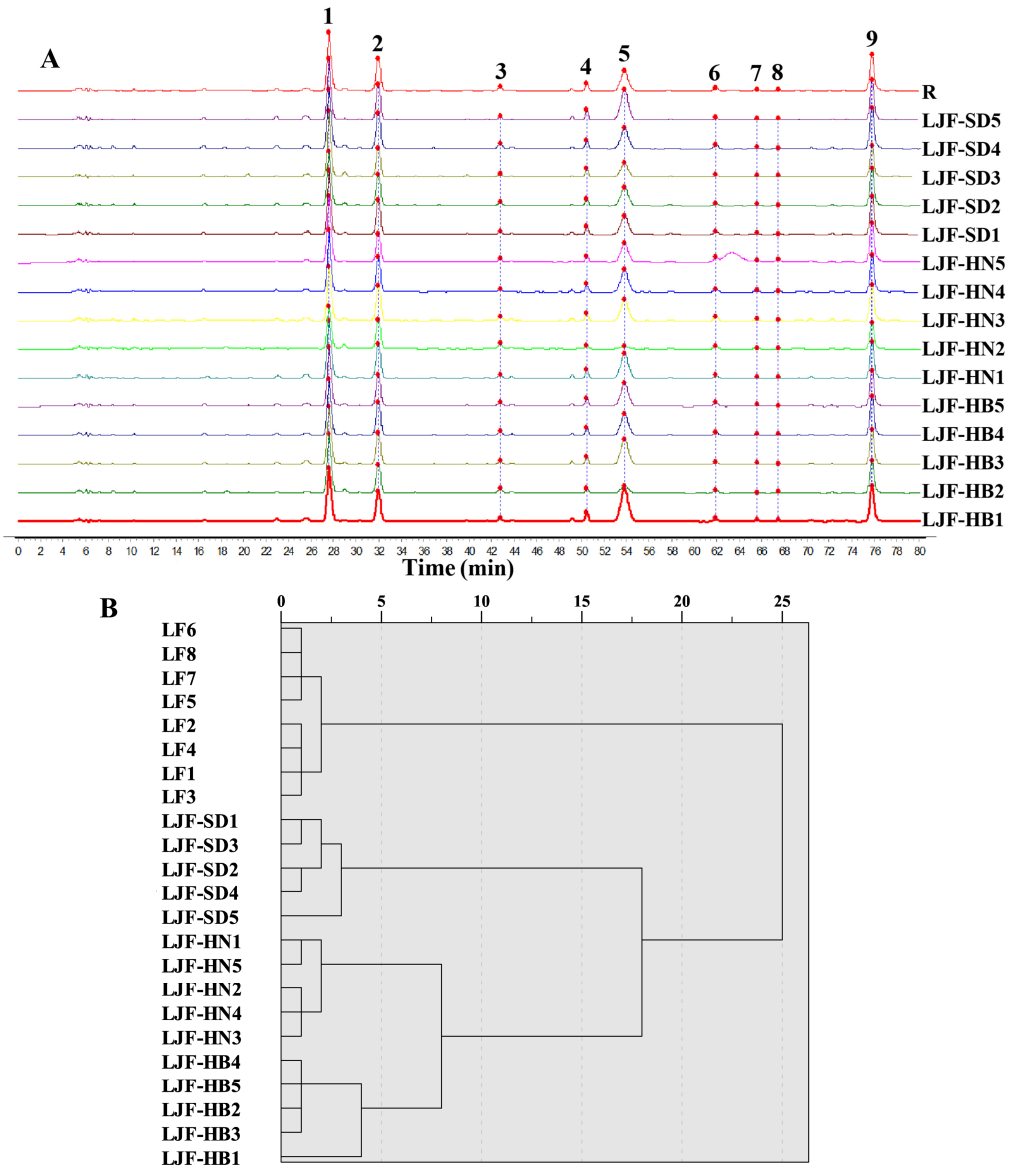

Figure 6. HPLC chromatogram fingerprints (**A**) of 15 batches of LJF. The hierarchical cluster dendrogram of LJF (with different origins) and LF samples (**B**).

To characterize the nine common peaks, UPLC-MS analysis was adopted. By comparing the retention times, the m/z of the characteristic molecular, and fragment ions with those of standards and information reported in the literature, the nine common peaks were identified as chlorogenic acid (peak1), secologanic acid (peak2), sweroside (peak3), unknown (peak4), ferulic acid (peak5), rutin (peak6), isoquercitrin (peak7), cynaroside (peak8), and 3,5-O-Dicaffeoylquinic acid (peak9). The nine common peaks identified and their detailed information are presented in Table S9.

Next, GRA was applied to explore the relationship between the detailed compounds and antioxidant ability. The results (Table S10) demonstrated that the degree of association among the different peaks ranged from 0.698 to 0.894 with the order as follows: peak1

> peak9 > peak6 > peak2 > peak3 > peak8 > peak4 > peak7 > peak5. Considering the high correlation (more than 0.8), peak1, peak9, peak6, peak2, and peak3 might be the potential antioxidant Q-marker (the detailed steps and results are shown in the Results and Discussion Section of Supplementary Material). For a more accurate screening of the antioxidant Q-markers, a partial least squares analysis (PLS) model, a supervised classification method, was constructed using common peak areas and antioxidant ability by SIMCA 14.1 software according to the literature precedent [50]. The model verification results (Figure S7A) showed that the variance ratio of response (R^2Y, 0.671) and the model's predictive ability (Q^2, 0.502) were both more than 0.5, indicating our constructed model had a good fitting degree and prediction ability. The result of the replacement test (Figure S7B) showed the regression line slopes of R^2 and Q^2 were positively inclined, and the intercept of the regression lines of R^2 (0.222) and Q^2 (−0.144) was negative, suggesting that our proposed model was not over fitted and had robust predictive capabilities. The correlation results (Figure S6B) of peak areas and antioxidant ability showed that six common peaks had a positive correlation, and three common peaks had a negative correlation. The detailed order for positive correlation peaks was as follows: peak1 > peak9 > peak6 > peak3 > peak2 > peak8, which was consistent with the results of the GRA. To screen the components with a higher antioxidant contribution, the average variable importance plot (VIP) value was investigated, and the results (Figure S6C) showed that the VIP values of peak1, peak6, and peak9 were greater than 1, indicating these compounds made a significant contribution to the antioxidant ability of LJF extracts [6], which were the screened antioxidant Q-markers of LJF. According to the identification results in Section 3.6, the three antioxidant Q-markers were identified as chlorogenic acid (peak1), rutin (peak6), and 3,5-O-Dicaffeoylquinic acid (peak9) by UPLC-MS.

In addition, to verify the antioxidant capacity of the screened Q-markers, the IC_{50} values (concentration of antioxidants required to eliminate 50% of free radicals) of chlorogenic acid, rutin, and 3,5-O-Dicaffeoylquinic acid for DPPH· were investigated and compared with cynaroside (a Q-marker of LJF prescribed by the Chinese Pharmacopoeia). The results showed (Figure S8A–D) that the IC_{50} values of chlorogenic acid, rutin, and 3,5-O-Dicaffeoylquinic acid were obviously less than that of cynaroside, indicating our proposed Q-markers had excellent antioxidant performance.

In order to explore the application potential of the screened antioxidant Q-markers, various LJF samples and counterfeit LF from different origins were collected, and the contents of the three screened Q-markers were measured by HPLC-DAD combined with our proposed tailored DES extraction method (the constructed standard curves of the three Q-markers are shown in Table S11). As shown in Table S12, the content of the three Q-markers in LJF and LF varies with the different origins, indicating the screened Q-markers had the potential to distinguish the origin and variety of LJF. In addition, the concentration of chlorogenic acid in LJF from different regions ranged between 24.18 mg/g and 36.93 mg/g, which was significantly higher than the standards (not less than 1.5%) of the Chinese Pharmacopoeia (2020 Edition), indicating that our collected LJF met the standard. To distinguish the authenticity and origin of LJF, the cluster analysis method was adopted, using the content of Q-markers as variables in SPSS 26.0 statistical analysis software according to the literature precedent [51]. The results (Figure 6C) showed that four classes of samples appeared with a Euclidean distance of 5. The detailed four classes of samples included Henan Province LJF (five samples), Hebei Province LJF (five samples), Shandong Province LJF (five samples), and LF (eight samples), which confirmed that our screened antioxidant Q-markers could be used to distinguish between LJF and LF and LJF from origins.

4. Conclusions

In this study, a novel optimization strategy was proposed with antioxidant ability as the evaluation index and a tailored DES as the extraction solvent. Compared with conventional solvents, the DES showed excellent extraction performance for antioxidants. After

optimization, the antioxidant ability and antioxidant content of the LJF extracts obtained by our proposed method were obviously superior to the Chinese Pharmacopoeia method. The extraction mechanism investigation showed that the strong damaging effect of the DES on plant cells and its proper polarity for forming the hydrogen bond interaction promoted the extraction performance. Polyphenols and flavonoids are the main antioxidants in LJF extracts. After combining the HPLC fingerprint with the PLS model, chlorogenic acid, rutin, and 3,5-O-Dicaffeoylquinic acid were screened as the antioxidant Q-markers. By precisely analyzing their content and combining cluster analysis, the screened Q-markers could successfully identify the origins of LJF and distinguish it from LF. This study proposed a novel optimization strategy for the preparation of antioxidant additives for natural food. Therefore, the constructed extraction could be adopted for the quality evaluation of natural plants.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/separations11060189/s1. 1. Supplementary Experimental Section: 1.1 Determination of Content of Antioxidants; 1.2 Surface Morphology Characterization of LJF Powder; 2. Supplementary Results and Discussion: 2.1 Classification Analysis of Antioxidant Components; 2.2 Construction of HPLC Fingerprints; 3. Supplementary Figures: Figure S1. The external morphological characteristics of LJF (A). The microscopic characteristics of LJF. (B): non-glandular hair; (C): calcium oxalate clusters; (D): pollen grains; (E): glandular hair; Figure S2. The FT-IR spectrum of TMAC-EG-1 DES; Figure S3. The antioxidant ability standard curve of Trolox on the DPPH· scavenging; Figure S4. The correlation between TFC content of LJF and the α (A), β (B) and π* (C) subtraction value of DESs; The correlation between TPC content of LJF and the α (D), β (E) and π* (F) subtraction value of DESs; Figure S5. The HPLC-fingerprint of LJF extracts at different UV detection wavelengths. The dots in the graph indicate the presence of chromatographic peaks; Figure S6. Characteristic chromatogram of LJF (A). The partial regression coefficient of PLS model (B) and variable importance plot of PLS model (C); Figure S7. The permutation test results (A) and replacement test results (B) of PLS model based on tailored DES extract from different LJF; Figure S8. The elimination of DPPH· with different concentrations of chlorogenic acid (A), rutin (B), 3, 5-O-dicaffeoylquinic acid (C), and cymaroside (D); 4. Supplementary Tables: Table S1. The information of collected LJF and LF samples and their water content; Table S2. Experimental design and results of Box-Behnken Design; Table S3. ANOVA of the established BBD model; Table S4. K-T parameters of prepared DESs and organic solvents; Table S5. The results of total polyphenols (TPC), total flavonoids (TFC), total reducing sugars (TRS) content and antioxidant ability of LJF extracts from different origins; Table S6. The results of grey relation analysis between total polyphenols (TPC), total flavonoids (TFC), total reducing sugars (TRS) content and antioxidant capacity of LJF extracts; Table S7. The precision, repeatability and stability evaluation of HPLC fingerprint method of LJF extracts; Table S8. The results of similarity analysis of HPLC fingerprint of LJF different origins.; Table S9. MS data for characteristic peaks of compounds of LJF by HPLC-MS; Table S10. The result of grey relation analysis between the area of common peaks and antioxidant capacity of LJF extracts; Table S11. The standard curves of chlorogenic acid, rutin and 3,5-O-Dicaffeoylquinic acid; Table S12. The content of chlorogenic acid, rutin and 3,5-O-Dicaffeoylquinic acid in LJF and LF from different origins [38,47,48,52–57].

Author Contributions: Conceptualization, W.-W.D., H.L. and J.W.; data curation, B.S., H.Y., X.-J.H., Y.-J.Z., T.-X.G., X.-Y.C., A.Y., X.-Y.D. and C.-Y.Z.; formal analysis, H.Y.; investigation, W.-W.D. and B.S.; validation, W.-W.D.; writing—original draft, W.-W.D. and B.S.; writing—review and editing, W.-W.D., B.S., H.Y., X.-J.H., Y.D., Y.-Q.X. and R.G.; project administration, W.-W.D., H.L. and J.W.; methodology, H.Y.; visualization, B.S.; formal analysis, H.Y.; supervision, H.L. and J.W.; funding acquisition, H.L. and J.W. All authors have read and agreed to the published version of the manuscript.

Funding: We thank the Natural Science Foundation of Jiangxi Province (20232BAB216138), the China Academy of Chinese Medical Sciences (XJ2023000203), Hubei University of Technology 2023 Graduate Research and Innovation Project, the National Natural Science Foundation of China TUBITAK (Turkey)–NSFC (China) Bilateral Cooperation Grant (82061138005) and "Research and application of redox medicine in severe diseases" International Collaboration Project-Ministry of Science & Technology of Hubei Province, "The role and mechanism of cellular protein folding regulated by

chaperone proteins in autism" High Foreign Talent Program-Ministry of Science & Technology of China (G2022027010L) for financially supporting this project.

Data Availability Statement: The original contributions presented in this study are included in this article/the Supplementary Materials. Further inquiries can be directed to the corresponding author.

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

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