Optimization of Deep Eutectic-like Solvent-based Ultrasound-assisted Extraction of Polysaccharides from *Leonurus* Residues

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Effects of the different types of deep eutectic-like solvents (DESs), molar ratio, water content, extraction temperature, extraction time, and ratio of liquid to solid were studied relative to the yield of *Leonurus* residue crude polysaccharide (LRCP). Extraction amounts of LRCP were determined. The extraction process parameters were optimized by response surface methodology, and an optimal extraction process was achieved. The results showed that DESs comprising choline chloride and ethylene glycol (CCEG) were suitable for the extraction. The optimum extraction process was as follows: water content, 27%; extraction temperature, 62 °C; and extraction time, 48 min. With the abovementioned parameters, the predicted extraction yield of LRCP was 14.1%. It was found that with these optimal extraction conditions, a 52.9% higher extraction yield could be achieved compared with hot water extraction. Therefore, a type of DES was found to be an excellent extraction solvent alternative for the extraction of polysaccharides and has a practical value.

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Keywords: Angelica Residue; Polysaccharides; Deep eutectic-like solvent; Ultrasound-assisted extraction; Response surface methodology

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INTRODUCTION

Leonurus japonicus Houtt (LJ) is widely distributed throughout China. It has the effect of activating blood and regulating menstruation, diuresis, and detumescence, clearing heat, and detoxification. It is used for the management of irregular menstruation, dysmenorrhea, lochia, edema, insufficient urine, and sore and swollen poison (Xiong *et al.* 2013; Liu *et al.* 2014). LJ contains various active ingredients such as alkaloids and polysaccharides. It has been shown that *Leonurus japonicus* polysaccharide (LJP) has anti-oxidative and immunomodulatory effects. LJP is an acidic polysaccharose, which is mainly composed of galacturonic acid, glucose, and galactose. LJP showed mild anticoagulant activity, low toxicity, and less spontaneous bleeding compared with heparin sodium (Hu *et al.* 2020). Therefore, it is of practical significance for optimizing the extraction conditions of LJP to further study LJP activity (Zhang *et al.* 2023).

The extraction of polysaccharides includes several methods, such as traditional water extraction and ethanol precipitation, microwave-assisted extraction, and ultrasonic extraction among others. Water extraction with ethanol precipitation is the most commonly used extraction method, and it is based on the solubility characteristics of polysaccharides (Chen *et al.* 2024). Water extraction has wide applicability in the extraction and separation of plant polysaccharides. However, this method results in a large amount of impurities in the polysaccharide products such as proteins, nucleic acids, and pigments, making the downstream purification process complex and time-consuming (Chen *et al.* 2015; Zhang *et al.* 2016; Chen *et al.* 2024).

Ionic liquids (ILs) and deep eutectic-like solvents (DES), as a new generation of green solvents, are widely used in the extraction of natural components (Antunes *et al.* 2023). ILs have the advantages of negligible vapor pressure, good thermal stability, and good solubility and extractability, but their cost is higher than that of ordinary organic solvents and they have potential biological toxicity and poor biodegradability (Szabó *et al.* 2023). In contrast, DESs are eutectic-like mixtures comprising hydrogen bond acceptors (usually choline chloride) and hydrogen bond donors (usually natural plant-based organic ions such as amino acids, organic acids, and sugars) (Türker and Doğan 2022; Cajnko *et al.* 2023). Though DESs are related to true eutectic solvent mixtures, the widely reported mixtures are usually formulated to achieve a eutectic condition, *i.e.* a minimum temperature of melting. Instead, investigators adjust the compositions as a means of varying the solvent characteristics. The DESs, so formulated, have the advantages of biodegradability, low cost, easy synthesis, high solubility, and low or no toxicity, enabling it to dissolve a variety of substances, including salts, proteins, drugs, amino acids, surfactants, sugars, and polysaccharides (Qu *et al.* 2023). Therefore, DES can be used for efficiently extracting polar and non-polar components from plants.

Although the extraction yield of DES for phenolic compounds (García *et al.* 2016; Chanioti and Tzia 2018), flavonoids (Qi *et al.* 2015), and carrageenan (Das *et al.* 2016) is higher than that of traditional extraction solvents, DESs have rarely been used for the extraction of polysaccharides from pharmaceutical residues to date, except for polysaccharide extraction from Chinese yam (Zhang and Wang 2017). At present, the comprehensive utilization rate of angelica residue is still very low. However, this species not only occupies a large amount of land and pollenates the environment, but it also represents a serious waste of resources (Sharma *et al.* 2018). To date, there has been no report about DES used for the extraction of polysaccharides in LJ.

This study investigated the effects of a type of DES, molar ratio, liquid to solid ratio, water content, extraction temperature, and extraction time on the extraction yield of *Leonurus* residues crude polysaccharide (LRCP). The response surface model was applied to design the experiment to optimisze the LRCP extraction process, providing a reference for efficient and green re-extraction of active ingredients from Chinese medicinal residues.

EXPERIMENTAL PROCEDURE

Materials

Leonurus residue (LR), which was the residue carrageenan after alcohol extraction, was provided by Guangxi Pharmaceutical Group Co., LTD. It was dried in the oven at 50 ℃, and then passed through an 80-mesh sieve. Choline chloride, urea, 1, 4-butanediol, lactic acid, and oxalic acid, were purchased from Shanghai Maclin Biochemical Technology Co., LTD and were analytically pure. All other chemicals were of analytical grade.

Preparation of DES

Five types of DESs were prepared by heating method (Hessel *et al.* 2022). That is, two components of molar ratio of each DES was 3:1, which were heated and stirred in a water bath at 50 °C for 1 h until the solution was homogeneous. The five types of DES were as follows: choline chloride-urea (CCU), choline chloride-1,4-butanediol (CCB), choline chloride-ethylene glycol (CCEG), choline chloride-lactic acid (CCLA), and choline chloride-oxalic acid (CCOA).

DES-based Ultrasound-assisted Extraction of LRCP

DES-based ultrasound-assisted extraction process

First, 5 g of LR powder was mixed with an appropriate amount of DES according to the corresponding ratio of material to liquid (20, 30, 40, 50, and 60 mL/g), and double ultrasound-assisted extraction was performed using the Ultrasonic Cell Destruction System (1000 W) for 30 min. The mixture was then distilled to a fifth of its initial volume via vacuum distillation. The concentrate was precipitated by adding anhydrous ethanol to a final concentration of 80%. After centrifugation and freeze-drying, *Leonurus* residues crude polysaccharide (LRCP) was obtained. With D-glucose as the standard, the contents of LRCP were determined via the phenol-sulfuric acid method (Wang *et al.* 2013), and LRCP was calculated as follows:

Extraction yield of LRCP = weight of LRCP (g)/ absolute dry weight of the sample (g)

Hot water extraction process

To evaluate the effect of DES-based LRCP extraction, LRCP was also extracted with hot water. For this, 5 g of LR was extracted with boiling water for 2 h according to the solid-to-liquid ratio of 1:40. After carrying out extraction three times, the extracted solution was mixed and alcohol precipitation was performed to obtain LRCP.

Single-factor experimental design

The effects of types of DES, molar ratio of DES, water content, extraction temperature, extraction time, and ratio of liquid to solid on the extraction yield of LRCP were investigated by single-factor experimental design. Each experiment was done five times.

Experimental design

A Box-Behnken experimental design (BBD) with three independent parameters and three levels was adopted using the Design-Expert 8.0.6 software. A total of 17 experiments at a central point were employed to determine the variables that influence the extraction yield of LRCP. This method allows the establishment of statistical relationships between the experimental variables and response variables to describe the nature of the response surface and elucidate optimal manufacturing conditions. These features should, in turn, allow for predicting an optimal extraction yield of LRCP. Table 1 lists the experimental design of coded factors and results of BBD for extraction yield of LRCP.

The three critical parameters that affected the extraction yield of LRCP were water content (A), extraction temperature (B), and extraction time (C). These parameters were selected as the independent variables based on preliminary experiments, and the dependent variable (*i.e.*, the extraction yield of LRCP) was then evaluated. An analysis of variance (ANOVA) was performed for each response with a confidence interval of 95%. All data are expressed using the average of three replicates along with their coefficient of variation (CV).

Table 1. Experimental Design of Coded Factors and Results of BBD for the Extraction Yield of LRCP

Coded	Factors	Range and Levels		
		Low (-1)	Medium (0)	High (1)
A	Water Content (%)	20	30	40
B	Extraction Temperature (℃)	50	60	70
C	Extraction Time (min)	30	40	50
Run	Factors			Extraction Yield of LRCP(%)
	A	B	C	
1	0	0	0	13.56 (6.5)*
2	0	0	0	13.48 (5.2)
3	0	1	-1	11.72 (6.3)
4	0	0	0	13.52 (7.0)
5	-1	-1	0	10.68 (6.7)
6	-1	0	-1	10.71 (5.8)
7	0	0	0	13.72 (7.2)
8	1	1	0	11.96 (9.1)
9	0	-1	1	12.84 (4.9)
10	1	0	1	12.82 (6.4)
11	-1	0	1	13.44 (5.3)
12	0	-1	-1	10.50 (6.9)
13	1	0	-1	12.04 (5.8)
14	0	1	1	13.02 (8.2)
15	1	-1	0	10.92 (7.3)
16	-1	1	0	11.32 (6.4)
17	0	0	0	13.64 (5.8)

*Coefficient of variation

RESULTS AND DISCUSSION

Single Factor Experimental Analysis

Effect of the type of DES on the extraction yield of LRCP

During the process of crude polysaccharide extraction from *Leonurus* residues, it is necessary to focus on the physical and chemical properties of the eutectic-like solvent, such as diffusion, solubility, viscosity, surface tension, polarity, and physical and chemical interactions. These factors directly determine the penetration of the DES into the sample matrix and the mass transfer of the target compound from the sample pool to the solution.

It was found that DES had a great influence on the extraction yield of LRCP when the water content was 30%, molar ratio of DES was 3:1, extraction time was 40 min, extraction temperature was 90 °C, and the liquid–solid ratio was 30 mL/g (Fig. 1). CCEG had a higher extraction efficiency for LRCP, with an extraction yield of 13.4%. This was significantly higher than the yield with other DES types, which may be because of the higher hydrogen bonding capacity of CCEG with LRCP and more electrostatic interaction with LRCP compared with other DESs (Khezeli *et al.* 2016; Mehrabi *et al.* 2023). Therefore, CCEG was determined to be suitable for extracting LRCP.

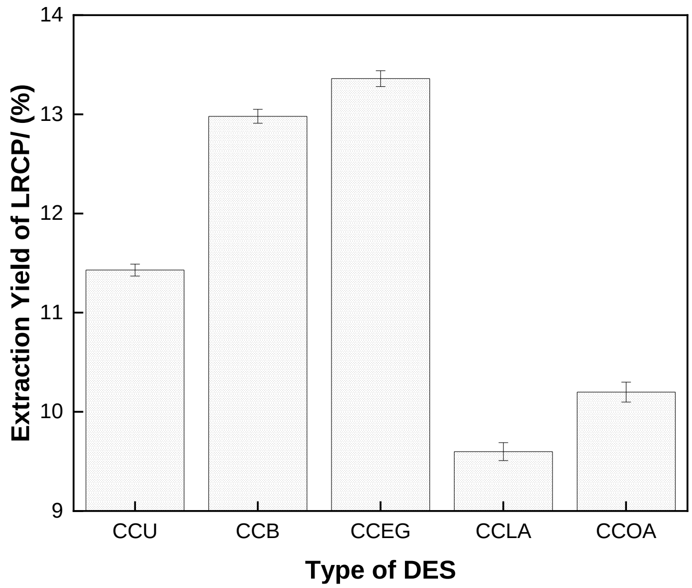

Fig. 1. Effect of the type of DES on the extraction yield of LRCP

Effect of the molar ratio of DES on the extraction yield of LRCP

The composition of CCEG will affect the hydrogen bonding between its hydrogen donor and recipient, thus determining its viscosity and polarity (Zhang and Wang 2017). With the water content of 30%, extraction time of 40 min, extraction temperature of 90 °C, and liquid to solid ratio of 30 mL/g, the effect of CCEG with different molar ratios on the extraction yield of LRCP was studied (Fig. 2). With the initial increase in the molar ratio of glycol to choline chloride, the extraction rate increased, and it finally stabilized. When the molar of ethylene glycol was increased, the extraction yield of LRCP reached a high level of 13.5%. Increasing the proportion of ethylene glycol in DES not only reduced the viscosity and surface tension of DES, but it also increased the diffusion and mass transfer of DES, thereby increasing the extraction rate. However, after increasing the proportion to a certain extent, ethylene glycol in DES weakened the interaction between ethylene glycol and choline chloride, resulting in no further significant change in extraction rate (Qi *et al.* 2015; Zhang *et al.* 2022). Thus, 1:5 was determined to be the suitable molar ratio of CCEG.

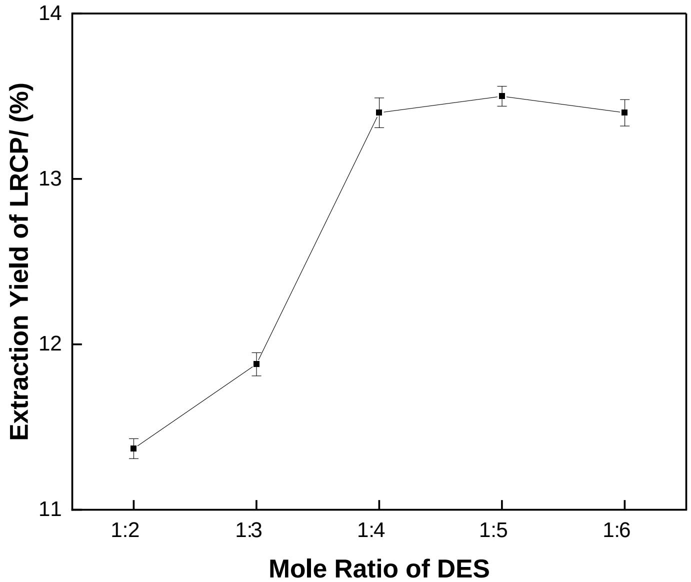

Fig. 2. Effect of the molar ratio of CCEG on the extraction yield of LRCP

Effect of liquid to solid ratio on the extraction yield of LRCP

To investigate the influence of the liquid to solid ratio on the extraction yield of LRCP, under the conditions of water content 40%, molar ratio 1:5, extraction time 40 min, and extraction temperature 80 °C, the liquid to solid ratios of 20, 30, 40, 50, and 60 mL/g were tested. The results are shown in Fig. 3. With an increase in the liquid to solid ratio, the extraction yield of LRCP increased at first and then slightly decreased. The reason for this is that a small amount of extraction solvent can easily achieve extraction equilibrium, that is, incomplete extraction of the target compound. Although a large amount of extraction solvent can improve the leaching rate of the target compound, it will also lead to wastage of the extraction solvent and complexity of the extraction process (Wang *et al.* 2016). Therefore, a liquid to solid ratio of 40 mL/g was determined to be sufficient to ensure the extraction yield of LRCP.

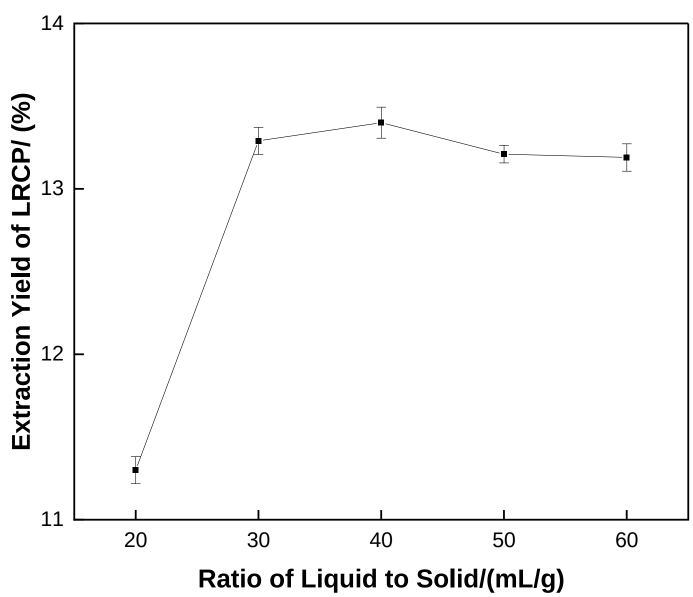

Fig. 3. Effect of liquid to solid ratio on the extraction yield of LRCP

Effect of water content on the extraction yield of LRCP

There is an extensive hydrogen bond network among DES components, resulting in high viscosity and weak mobility of the component ions. Changing the viscosity and mobility is the key to obtaining good pore penetration in the sample matrix and promoting mass transfer from the plant matrix to the solution (Wang *et al.* 2016). The water content of DES affects its viscosity and increases its polarity. The effect of CCEG with different water content was determined relative to the extraction yield of LRCP (Fig. 4), under the conditions of CCEG molar ratio 1:5, extraction time 40 min, extraction temperature 90 ℃, and liquid to solid ratio 40 mL/g. The extraction yield of LRCP increased and reached the highest (13.4%) when the water content ranged from 0% to 30%. However, the extraction yield of LRCP decreased with an increase in the water content from 30% to 50%. It was verified that the extraction performance also depended on the water content of DES solution, and low water content of CCEG was unfavorable for plant cell penetration, making it difficult to achieve a high extraction rate (Türker and Doğan 2022). However, excessive water content of CCEG would increase the polarity of the mixture and reduce the interaction between CCEG and LRCP, thus promoting the formation of simple aqueous solutions of ethylene glycol and choline chloride and reducing the extraction rate of LRCP (Gutiérrez *et al.* 2009). Therefore, the water content of 40% in CCEG was selected for the subsequent extraction process.

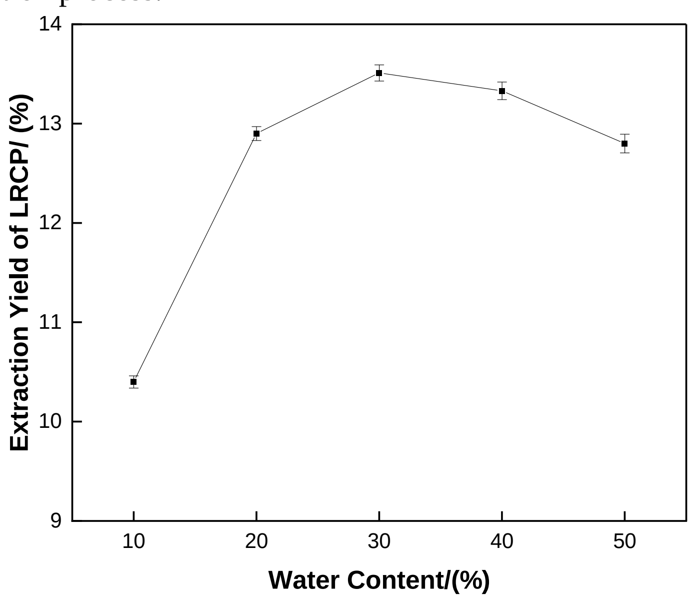

Fig. 4. Effect of water content on the extraction yield of LRCP

Effect of extraction temperature on the extraction yield of LRCP

The extraction temperature is an important factor affecting the extraction yield. The target compounds are adsorbed on the sample matrix by physical adsorption and chemical interaction. High temperatures tend to reduce these physical adsorption and chemical interactions, thereby increasing the leaching of target compounds in the extraction solvent (More *et al.* 2022). In addition, higher extraction temperature can greatly reduce the viscosity of the extraction solvent, increase the diffusion of the extraction solvent, and accelerate the mass transfer of the target compound. As shown in Fig. 5, at the water content of 40%, CCEG molar ratio of 1:5, extraction time of 40 min, and liquid to solid ratio of 40 mL/g, an extraction temperature of 60 °C resulted in a higher extraction yield than other temperatures. Although a higher extraction temperature can improve the extraction performance, it may not be able to withstand the thermal degradation of the polysaccharide (Chen *et al.* 2012; Chen *et al.* 2021). Therefore, the extraction temperature of 60 °C was selected for the study.

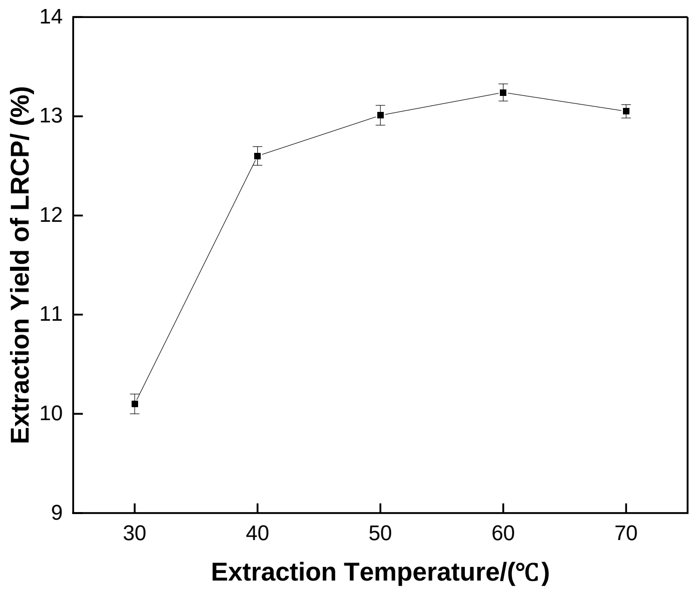

Fig. 5. Effect of extraction temperature on the extraction yield of LRCP

Effect of extraction time on the extraction yield of LRCP

The influence of different extraction times on the extraction yield of LRCP was determined. As shown in Fig. 5, under the conditions of DES water content of 40%, DES molar ratio of 1:5, extraction temperature of 60 ℃, and liquid to solid ratio of 40 mL/g, the extraction rate increased with the increase in extraction time from 20 min to 40 min. However, the extraction rate decreased with an increase in the extraction time from 40 min to 60 min, which may be because of the hydrolysis of the polysaccharide under high temperature and with a long extraction time (Liu *et al.* 2009; Zhang *et al.* 2020). Therefore, the extraction time should be controlled at 40 min.

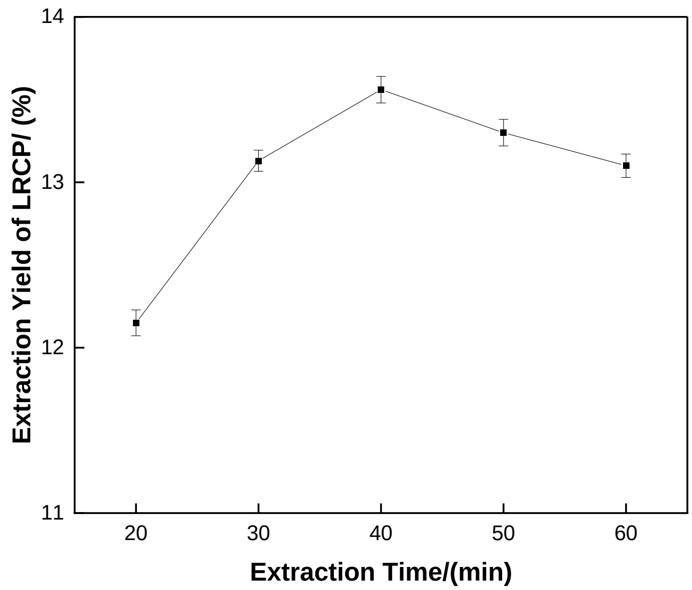

Fig. 6. Effect of extraction time on the extraction yield of LRCP

Data Analysis and Regression Models

The ANOVA p-values for the extraction yield of LRCP are presented in Table 2.

Table 2. Analysis of Variables for Parameters and their Interactions

Source	Sum of squares	df	Mean square	F--value	P-value
Model	22.35	9	2.48	330.17	< 0.0001**
A	0.32	1	0.32	42.02	0.0003**
B	1.19	1	1.19	157.67	< 0.0001**
C	6.39	1	6.39	849.69	< 0.0001**
AB	0.04	1	0.04	5.32	0.0545
AC	0.95	1	0.95	126.40	0.0001**
BC	0.27	1	0.27	35.95	0.0005**
A^2	4.78	1	4.78	635.90	< 0.0001**
B^2	7.10	1	7.1	943.61	< 0.0001**
C^2	0.30	1	0.3	39.54	0.0004**
Residual	0.053	7	7.521 E-003		
Lack of fit	0.016	3	5.175 E-003	0.56	0.6705
Pure error	0.037	4	9.280 E-003		
Cor total	22.40	16			
R^2 = 0.9976		R^2_{Adj} = 0.9946		C.V.% = 0.70	

All p-values below 0.05 revealed significant model terms, whereas values above 0.05 indicated insignificant model terms. Meanwhile, p-values below 0.0001 implied that all models of mechanical properties were significant and there was only a 0.01% chance that such values could occur because of noise (Guo *et al*. 2010).

The regression equation was as follows: the extraction yield of LRCP = 13.58 + 0.20A + 0.39B + 0.89C + 0.10AB − 0.49AC − 0.26BC − $1.07A^2$ − $1.30B^2$ − $0.27C^2$. As shown in Table 2, the terms A, B, C, AC, BC, A^2, B^2, and C^2 were significant. The F-value of 330.17 presents the model as significant. A p-value < 0.05 is indicative of the significance of the model terms. The high value of R^2 (0.9976) indicates a high level of model fitting. All predicted R^2 values agreed with the adjusted R^2 values. Values of adequate precision greater than four are desirable. The Lack of Fit F-value of 0.56 suggested that the model is non-significant. The low coefficient variance (C.V.% = 0.70) confirmed the accuracy and reliability of the experimental values in the regression model.

Response Surface Interaction Analysis

The three-dimensional (3D) response surface and contour plots for TP are shown in Fig. 7. The 3D response surface plot and the two-dimensional (2D) response contour plot provide a visual interpretation of the interaction between two independent variables. The 3D response surface diagram can show the interaction between independent variables and response variables. The 2D response contour map not only can explain the interaction between independent variables but also reflect the importance of the interaction between variables; that is, the circular response contour plot shows that the interaction between the corresponding variables is negligible, whereas the elliptic response contour plot shows that there is significant interaction between the corresponding variables (Guo *et al.* 2010; Zhang *et al.* 2022). As shown in Fig. 7, the interaction effect between water contents (A) and extraction times (C) was greater than that between different extraction temperatures (B) and extraction times (C); in turn, the interaction effect between different extraction temperatures (B) and extraction times (C) was greater than that between different water contents (A) and extraction temperatures (B), *i.e.*, AC>BC>AB. AC made a better contribution compared with the other two. The most likely reason is that the high water contents or extraction times might affect the force between polysaccharides and DES (Meng *et al.* 2023).

Optimization and Verification Experiment

Based on the optimization analysis, validation experiments were performed following the same method at optimum conditions, *i.e.*, water content, 27%; extraction temperature, 62 °C; and extraction time, 48 min, which led to a predicted LRCP extraction yield of 14.1%. Under these conditions, the observed extraction yield of LRCP was 13.7% ± 0.11%, indicating that the model had high reliability and that the reproducibility of the extraction process was good (Yao *et al.* 2023), compared with the low polysaccharide extraction yield (only 8.94%) from *Leonurus* residue observed via hot water extraction. The DES extraction method reported in this study showed significant improvement, and the extraction rate was increased by 52.9%. Therefore, this model is suitable for the optimization of LRCP extraction.

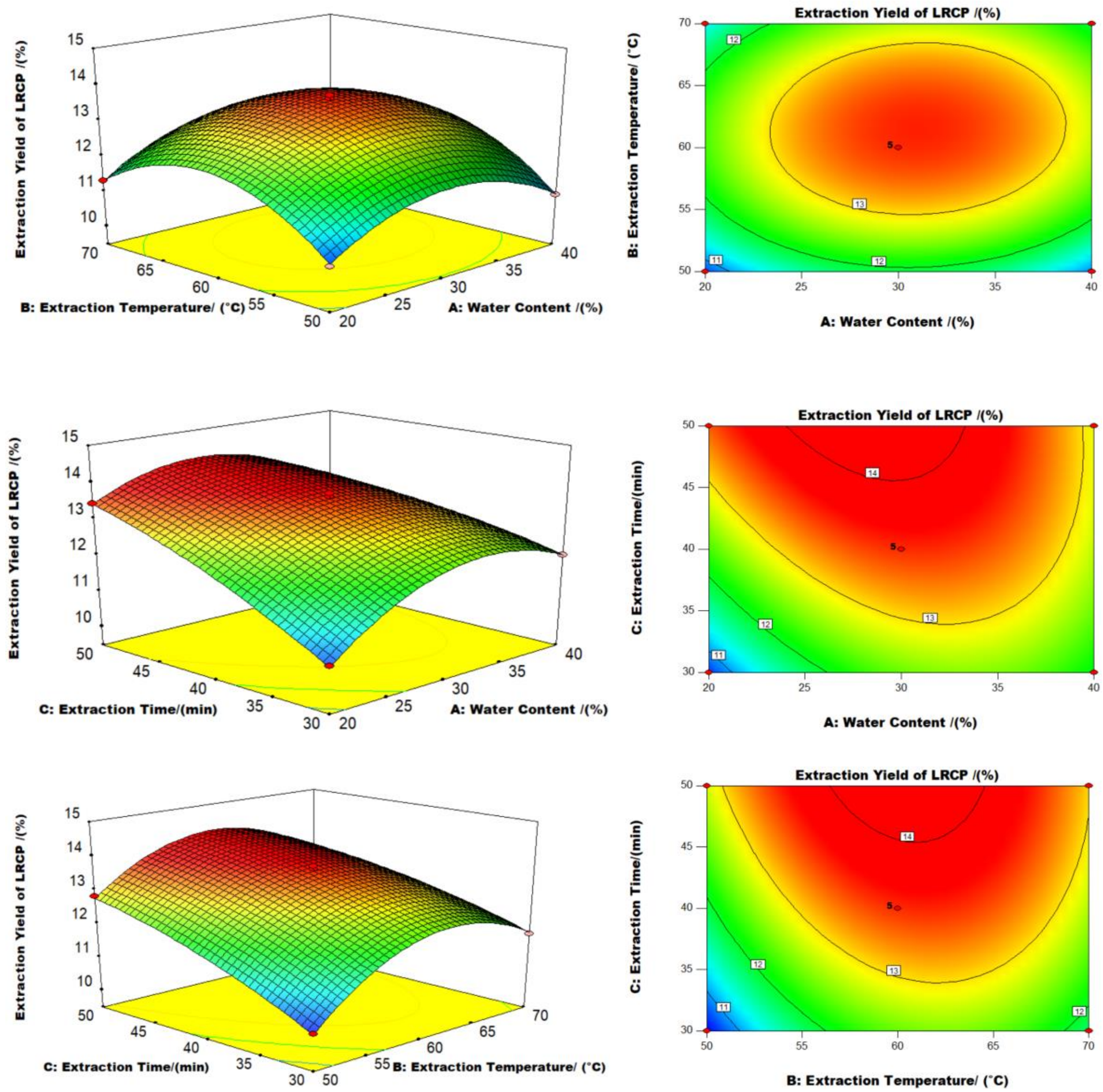

Fig. 7. Response surface plots and response contour plots showing the interaction effect of water content (A), extraction temperature (B), and extraction time (C) on the extraction yield of LRCP

CONCLUSIONS

1. Choline chloride with ethyene glycol (CCEG) was found to be an efficient deep eutectic-like solvent (DES), showing the highest extraction performance for *Leonurus* residue crude polysaccharide (LRCP). Based on the initial screening of single factor experiment, a high degree of model fitting was achieved for LRCP using the green and low-cost CCEG as DES and with ultrasound enhancement.
2. Optimum conditions of extraction were water content of 27%, extraction temperature of 62 °C, and extraction time of 48 min, leading to a predicted LRCP extraction yield of 14.1%.

3. The response surface methodology was revealed to be successful, and extraction times and extraction temperatures had more effect on the extraction yield of LRCP compared with water content of CCEG.
4. The study elaborated that ultrasound-assisted DES could be beneficial for the extraction process of *Leonurus* residue and the development of its polysaccharides for the functional food industry.

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