Ultrasound-assisted deep eutectic solvents extraction of glabridin and isoliquiritigenin from *Glycyrrhiza glabra*: Optimization, extraction mechanism and *in vitro* bioactivities

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**ABSTRACT**

Licorice (*Glycyrrhiza glabra*) is extensively used owing to the superior pharmacological effects. However, its maximum application potential has not been fully exploited due to the limitation of currently available extraction solvent and methods. In this study, an eco-friendly deep eutectic solvent (NADESs) based ultrasound-assisted extraction (DES-UAE) method was applied to prepare licorice extracts. The DES-UAE using choline chloride and lactic acid as solvent was optimized and modeled by using response surface methodology to maximize the extraction yields of glabridin (GLA) and isoliquiritigenin (ISL). The optimized extracts possessed higher contents of GLA and ISL than available extraction methods, and the enriched products showed superior pharmacological activities in vitro. Furthermore, scanning electron microscopy (SEM) and molecular dynamic simulation analyses were performed to deeply investigate the interaction between solvent and targeted compounds. This study not only provides an eco-friendly method for high-efficient extraction of GLA and ISL from licorice but also illustrates the mechanism of the increased extraction efficacy, which may contribute to the application of licorice and deep insight into extraction mechanism using DES.

1. Introduction

Licorice (*Glycyrrhiza glabra*), which belongs to the Leguminosae family, is a kind of traditional Chinese medicine with homologous medicine and food. Its dried roots have been frequently used in the Traditional Chinese Medicine (TCM) prescriptions for the treatment of a variety of diseases, such as cough, stomach pain, and inflammation [1]. These medical applications are mainly attributed to the pharmacological effect of bioactive contents, such as saponins, flavonoids, poly-saccharides, and amino acids. Glabridin (GLA) and isoliquiritigenin (ISL) are two active flavonoid constituents (Fig. 1) that can be extracted from the roots of licorice. Modern pharmacological studies have demonstrated that GLA and ISL possessed superior antioxidant capacity, anti-inflammatory and antimicrobial activities [2,3]. Owing to their promising properties, GLA and ISL show great potential to apply in the pharmaceutical, food, and cosmetic industries. Currently, traditional organic solvents, such as methanol and ethanol, have been applied in the extraction of GLA or ISL from licorice [4]. However, the extraction efficacy is limited due to the poor solubility and low abundance of GLA and ISL in licorice [5]. Additionally, most of the organic solvents show intrinsic disadvantages including strong volatility, high toxicity, and non-degradability, which threatens human health and the environment [6]. To address the challenge, environment-friendly and sustainable solvents have attracted considerable attention.

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Deep eutectic solvents (DESs) are made up of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) [7]. The HBD and HBA can form intermolecular hydrogen bonds, resulting in stable fluid systems with lower melting points and distinct solubilizing behavior [8]. Compared with organic solvents, DESs show distinct advantages such as low cost, biodegradability, ease of preparation, and non-toxicity [9]. Owing to the outstanding properties, DESs are preferable alternatives to organic solvents for extracting bioactive components from medicinal herbs.

Except for the solvents, extraction methods also have significant effect on the extraction of active components from medical plants [4]. Ultrasound assisted extraction, which could enhance extraction efficiency by the destruction of the cell wall and triggering turbulence, has been paid increasing attention [10,11]. To enhance the extraction efficiency, the combination of DESs and ultrasound-assisted extraction (DES-UAE) for extraction active components from medical been widely studied and the improved extraction yields of bioactive compounds, such as anthocyanins, anthraquinones, and polysaccharides, have been demonstrated [12–14]. Despite these advantages and recent advancement, the application of DES to the extraction of bioactive compounds in licorice remains limited to a single analyte (glycyrrhizinic acid). As far as we know, the DES-UAE method has not been used for the simultaneous extraction of GLA and ISL from licorice. In addition, during the development of DES-based extraction methods, most researchers mainly focused on demonstrating the advantages of DES to other solvents, such as extraction efficiency, but spend less time and effort on understanding why better results were obtained. So far, the mechanism of difference in extraction efficiency among different DES or other solvents was still limited on the physicochemical properties of DES, such as pH, and solubility, deep insight into the mechanism of efficacy difference among different solvents was still unclear, which demands further investigation.

Based on the aforementioned, eco-friendly ultrasound-assisted DES extraction was first applied to simultaneously maximize the extraction yields of GLA and ISL from licorice. The optimal DES components were determined and the process of DES-UAE was optimized by response surface methodology (RSM). The obtained optimal extracts were enriched by microporous resin, and finally, the enriched products were subjected to antioxidant, anti-inflammatory, and antibacterial assays to verify the biological activity. Furthermore, SEM and molecular dynamic simulation were applied to deeply investigate the interaction between solvent and targeted component.

## 2. Materials and methods

### 2.1. Plant materials

Licorice (Glycyrrhiza glabra) root, which was prepared by natural drying of fresh licorice roots under controlled conditions of temperature and humidity, was purchased from the local market in Harbin, Heilongjiang Province, China, and identified by Professor Yanyan Liu (College of Veterinary Medicine, Northeast Agricultural University, Harbin, China). The dried roots were ground into powder with a mill (TaiSite, FW100, Tianjin, China) and passed through a 200-mesh sieve. Before extraction, the powdered samples were sealed and stored in a desiccator at room temperature.

### 2.2. Chemicals and reagents

Choline chloride, proline, glycyrrhizic acid, citric acid, malic acid, malonate, tartaric acid, oxalic acid, lactic acid, acetic acid, sulfuric acid, diclofenac sodium, ascorbic acid, and 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). AB-8 microporous resin was purchased from Tianjin BSF resin technology Co., Ltd. (Tianjin, China). The bacterial strains of Staphylococcus aureus (ATCC 700,404), Staphylococcus xylosus (ATCC 43,300), Staphylococcus xylosus ATCC 700,404 (S. xylosus), and Escherichia coli ATCC 25,922 (E. coli) were purchased from the American Type Culture Collection (ATCC).

### Table 1

| Abbreviation | Composite of DES | Molar ratio | pH | Viscosity (mPa.s) | GLA extraction yield (μg/g) | ISL extraction yield (μg/g) |
|--------------|-----------------|-------------|----|-----------------|-----------------------------|-----------------------------|
| ChLa         | Choline chloride: Lactic acid | 1:1 | 1.26 | 28.2 | 6875.26 ± 230.17 | 818.03 ± 31.28 |
| ChCit        | Choline chloride: Citric acid | 1:1 | 0.56 | 203.6 | 5134.69 ± 282.71 | 679.15 ± 7.76 |
| ChMa         | Choline chloride: Malic acid | 1:1 | 0.19 | 548 | 5006.92 ± 234.48 | 618.44 ± 4.72 |
| ChMal        | Choline chloride: Malonate | 1:1 | 0.17 | 147.3 | 4757.8 ± 114.21 | 695.89 ± 6.27 |
| ChTa         | Choline chloride: Tartaric acid | 1:1 | 0.03 | 458 | 5582.17 ± 275.35 | 688.02 ± 5.41 |
| ChOxa        | Choline chloride: Oxalic acid | 1:1 | 0.02 | 55.2 | 5348.3 ± 99.01 | 667.15 ± 10.54 |
| ChLe         | Choline chloride: Lactic acid | 1:1 | 2.41 | 35.1 | 5649.22 ± 117.71 | 724.12 ± 8.38 |
| ProLa        | Proline: Lactic acid | 1:1 | 3.61 | 137 | 5903.52 ± 120.83 | 743.67 ± 11.35 |
| GlyLa        | Glycyrrhizic acid | 1:3 | 2.58 | 66.5 | 3853.54 ± 100.05 | 667.46 ± 9.3 |
| Bla          | Betaine: Lactic acid | 1:1 | 4.11 | 88.5 | 5339.36 ± 120.36 | 694.96 ± 5.01 |
| LaGlu        | Lactic acid: Glucose | 3:1 | 0.92 | 71.8 | 6768.41 ± 186.3 | 483.28 ± 12.37 |
| LaFr         | Lactic acid: Fructose | 3:1 | 1.02 | 63.4 | 4029.87 ± 322.53 | 508.93 ± 6.11 |
| ChUre        | Choline chloride: Urea | 1:2 | 8.82 | 18.3 | 4588.11 ± 495.7 | 563.37 ± 36.89 |
| ChAce        | Choline chloride: Acetamide | 1:1 | 7.73 | 51.6 | 4369.17 ± 193.51 | 535.61 ± 12.02 |

**Fig. 1.** The structures of GLA and ISL.
2.3. Preparation and characterization of DESs

The DESs were prepared using a stirring heating method as previously reported [15,16]. Briefly, different kinds of HBA and HBD were mixed and stirred at 80 °C with a magnetic stirrer for 30–60 min until a clear liquid was formed. Then 20% (v/v) of water was added. Homogeneous and clear DESs were obtained after stirring for 30 min. The prepared DESs and corresponding mole ratios of HBA and HBD are shown in Table 1.

The pH values of prepared DESs were measured using a pH-electrode (Sartorius, PB-30, China). The viscosities of each DESs were determined at 25 °C using a rheometer (Brookfield, YR-1, USA). The spectra of ChCl and LA system were evaluated using a Fourier-transform infrared (FTIR) spectrometer (Thermo Scientific Nicolet 6700 model, USA) in the wavenumber range of 4000–400 cm⁻¹.

2.4. HPLC analysis

The extracted samples were diluted with methanol and filtered through a 0.22 μm membrane filter. Then the contents of GLA and ISL were measured using a Waters 2695 High-Performance Liquid Chromatography instrument (Milford, MA, USA). The chromatographic separation was achieved on an Agilent Extend-C18 column (250 mm × 4.6 mm, 5 μm) at 25 °C. Two isocratic elution programs were established and used for HPLC analysis [17]. For GLA analysis, the mobile phase was composed of acetonitrile-0.04% formic acid (45:55, v/v), and the detection wavelength was set to 280 nm. For ISL analysis, the mobile phase was composed of acetonitrile-0.04% formic acid (45:55, v/v), and the detection wavelength was set to 367 nm. The flow rate of the mobile phase and the injection volume was 1.0 mL/min and 10 μL, respectively. Each sample was conducted in triplicate. According to the results, the HPLC retention time for GLA and ISL were 12.1 min and 4.3 min, respectively (Fig. S1).

The applicability of HPLC analysis for the determination of GLA and ISL was evaluated in terms of calibration-curve linearity, inter-day and intra-day precision, stability, and repeatability (shown in Supplementary data). The regression equations of GLA and ISL were: Y = 21137 X + 4105.8 (R² = 0.9993, X: glabridin concentration, Y: peak area) and Y = 6 × 10⁻⁶ X – 22,709 (R² = 0.9996, X: isoliquiritigenin concentration, Y: peak area), respectively. The small corresponding RSD value of each item (<2.04%) indicated the develop method was well fitted for the chemical analysis (Table S1). In addition, a single gradient elution program was also established for determination the contents of GLA and ISL, which could well be fitted for the chemical analysis as well (Fig.S2 and Table S2).

2.5. Extraction procedure

For DES-based UAE of GLA and ISL, powdered licorice root (0.5 g) was added to 10 mL of DESs in the tube. Then DES-based UAE was performed in an ultrasonic bath (Scientz, SB-5200DTD, Ningbo, China) at the ultrasonic power of 480 W. After extraction for 20 min at 55 °C, the samples were centrifuged (12000 rpm, 10 min), and the collected supernatant was used for HPLC analysis. In comparison, UAE using water and methanol as solvent were also carried out under the same parameter as that of DES-based UAE. The DESs prepared were shown in Table 1.

Based on the results of DES-based UAE, DES with optimal extraction efficacy (ChLa) was selected for further solvent screening. The effect of the molar ratio of HBA and HBD (1:1, 1:2, 1:3, 1:4, 1:5, and 1:6) and water content (0, 20, 40, 60, 80, and 100 %) on the extraction efficacy of selected DES were evaluated. The sonication input power and centrifugation procedures were the same as described above. Each extraction procedure was performed in triplicate.

2.6. Optimization of GLA and ISL extraction

The single-factor tests were used to optimize the following parameters. Four independent variables, including ultrasound power (320, 400, 480, 560 and 640 W), extraction temperature (35, 45, 55, 65, 75 °C), extraction time (10, 20, 30, 40, 50 min) and liquid-to-solid ratio (10, 20, 30, 40, 50 mL/g), were determined for the preliminary range. Furthermore, the Box-Behnken design (BBD) was employed to optimize the DES-UAE with higher extraction yields of GLA and ISL. Based on the results of univariate tests, the ultrasound power is fixed at 480 W, three independent variables including extraction temperature (°C, X₁), extraction time (min, X₂), and liquid-to-solid ratio (mL/g, X₃) were further optimized at three levels (−1, 0 and +1). Low and high factors were coded as −1 and +1; the center point was coded as 0. The BBD consisted of 17 randomized trials and contained five repetitions of the central point. The BBD generation and the assessment of the experimental results were implemented using the Design-Expert Version. 8.0 (Stat-Ease Inc., Minneapolis, MN, USA). The selection of optimal conditions was based on the extraction yield of GLA (μg/g, Y₁) and ISL (μg/g, Y₂).

The responses were determined under the optimum extraction conditions. Finally, the experimental data were compared with the predicted values based on the standard errors to validate the model.

2.7. Comparison of different extraction methods

Different extraction methods of GLA and ISL were studied for comparison with DES-UAE. Firstly, ultrasound-assisted extraction was performed using methanol or water as solvent at the optimal DES-UAE conditions. Then bath extraction was operated using the screened DES system (ChLa) as solvent. Briefly, powdered licorice roots (0.5 g) and ChLa (12 mL) were added to a round-bottom flask. The mixture was stirred for 180 min at 180 rpm at 62.5 °C. Then Soxhlet extraction was applied for extraction of GLA and ISL from licorice using methanol as solvent. Methanol (100 mL) was placed in a distillation flask, licorice powder (1 g) packed in a filter paper was placed in a thimble chamber where extraction was carried out using freshly condensed solvent from the distillation flask. The extraction process was performed at 85 °C for 3 h. Each extraction procedure was performed in triplicate. After extraction, the mixture was filtered and the supernatant was collected for HPLC analysis.

2.8. Scanning electron microscopy

Scanning electron microscopy (Hitachi, SU8010, Japan) was used to observe the morphology of licorice powder after different treatments, including licorice powder before extraction, after bath extraction, Soxhlet extraction, Meth-UAE, Water-UAE, and DES-UAE. The powder was dried and then analyzed by scanning electron microscopy at 2500 × magnification.

2.9. Molecular dynamic simulation

There different systems, including ChLa and water with GLA, methanol with GLA, and water with GLA, were generated using PACKMOL [18] in a cube of box of 10 nm × 10 nm × 10 nm. The number of molecules in the box was determined according to the molar ratio. Generally, the ChLa box consists of 800 Ch, 800Cl, 800 LA, 40 molecules of GLA and 2200 water molecules; the methanol box consists of 4900 methanol molecules and 40 molecules of GLA; the water box consists of 11,100 water molecules and 40 GLA molecules.

The original structure of molecules or ions, such as methanol, choline cation (Ch), chloride anion (Cl), lactic acid (LA), GLA, was downloaded in PubChem web server and optimized with 83LNP theory and def2-SVP basis [19] by the ORCA package [20]. The topology information of the molecules or ions was generated using the CGenFF server (https://cg...
4

2.10. Enrichment of GLA and ISL from extraction solution

The enrichment of GLA and ISL was conducted using AB-8 microporous resin. Microporous adsorbent resin (10 g) and extraction solution (20 mL) obtained under optimal variables of DES-UAE were added into a conical flask. The flask was shaken at 100 rpm for 24 h at room temperature to reach adsorption equilibrium. After that, the microporous adsorbent resin was rinsed with deionized water to remove polar ingredients and DES, and then a 70% aqueous ethanol solution was used to desorb the target compounds. All the ethanol solution was collected and concentrated under a vacuum using a rotary evaporator. The contents and recoveries of GLA and ISL in the concentrated product were measured by HPLC analysis. For comparison, the extraction solution obtained in the Soxhlet extraction was enriched under the same condition.

2.11. Determination of antioxidant activities

The antioxidant activities of the enriched products were determined by evaluating the ABTS radical scavenging activity assay. ABTS radical scavenging activity was measured according to the methods described by Luo et al [25]. The ABTS reaction working solution was prepared by mixing equal quantities of 7.4 mM ABTS solution and 2.6 mM K3[Fe(CN)6] solution; and left to react for 12~16 h at room temperature (23 ± 2 °C) in the dark. The working solution was then diluted with water until the absorbance reached 0.700 ± 0.01 at 734 nm. The 10 μL enrichment products solution sample was added to the test tube with 290 μL diluted ABTS reagent solution, and then shaken and placed in a dark place to react for 30 min, to determine the absorbance A1 at 734 nm. At the same time, the blank control group A0 (replacing the sample with ultra-pure water) and the sample background group A2 (using ethanol instead of ABTS reaction working solution) were set up, and the ABTS radical scavenging activity (%) was calculated according to equation (1).

\[
\text{Inhibition} (%) = \frac{1 - (A_1 - A_2)/A_0}{1} \times 100\% (1)
\]

Finally, the concentration of the enrichment products that half-maximal inhibitory concentration of free radicals present in the solution (IC50) was calculated, VC was used as the standard radical scavenger.

2.12. Determination of Anti-inflammatory activities

The anti-inflammatory activities of the enriched products from DES-UAE and Soxhlet extraction were measured using an anti-hyaluronidase assay [26]. Briefly, the enzyme (25 μL, 30 U/mL), the incubation buffer (25 μL), the enrichment product solutions (10 μL) and the acetate buffer (15 μL, pH 4.5) were mixed and pre-incubated at 37 °C for 10 min. After that, the hyaluronic acid (25 μL, 0.3 mg/mL) was added and incubated at 37 °C for 45 min. The undigested hyaluronic acid was precipitated with NaOH cetyltrimethylammonium bromide solution (200 μL, 2.5%). The mixture was kept at room temperature for 10 min, and then its absorbance A1 was measured at 600 nm. The experiment set up three groups of control groups, of which the A0 group contains a mixture of enzyme and substrate, the A1 group does not contain an enzyme mixture, and the A2 group is a blank control group. In addition, 25 μL of buffer solution was used instead of 25 μL of HA solution as the sample background group A4. The inhibition of hyaluronidase activity (%) was calculated according to equation (2).

\[
\text{Inhibition} (%) = \frac{1 - (A_1 - A_2)/A_0}{1} \times 100\% (2)
\]

The half-maximal inhibitory concentration value IC50, defined as the substance concentration necessary to obtain 50% inhibition of hyaluronidase activity, was determined. Diclofenac sodium was used as the standard radical scavenger.

2.13. Determination of antibacterial activities

Minimum inhibitory concentration [7], which is defined as the lowest concentration of an antimicrobial agent that prevents the growth of bacteria, was measured to evaluate the antibacterial activities of the extracts using a microtiter broth dilution method [3]. In brief, strains of S. aureus, S. xylosus, and E. coli were harvested, and adjusted to a density of 1 × 10^8 CFU/mL. Thereafter, 180 μL of the bacterial suspension was inoculated into each well of a 96-well plate. Subsequently, 20 μL of the enriched extracts from DES-UAE and Soxhlet extraction were added. The treated bacterial specimens were then incubated under aerobic conditions at 37 °C with shaking and were used for turbidity observations after 24 h.

2.14. Statistical analysis

All the experiments were carried out in triplicates. SPSS 11.0.0 statistical software (IBM, USA) was used for standard deviation calculations, pictures were done using GraphPad Prism 8.0.2 software.

3. Results and discussion

3.1. Screening of DES

The structure of DES was important for the extraction efficacy owing to the different physicochemical properties, such as viscosity, polarity, and pH [27]. In this study, fourteen DES systems were prepared for screening their performance in extracting GLA and ISL from licorice. It was reported that the high viscosity of DES could lead to a slow mass transfer and restrict their application [28]. Thus, all the DESs were blended with a moisture content of 20% (v/v). The physicochemical properties and extraction yields of different DESs were shown in Table 1. For DES with choline chloride (ChCl) as HBA, acid-based DES was more efficient for extraction of GLA and ISL than that of alkaline urea or acetamide. For DES with LA as HBD, sugar-based DES showed a lower extraction yield of ISL, but higher extraction yields of GLA, than that of amino acid. Among all the DESs, ChLa with low pH and viscosity showed higher extraction yields of GLA (6875.26 ± 230.17 μg/g) and ISL (818.03 ± 31.28 μg/g) than other DESs under the same extraction conditions (liquid-to-solid ratio of 20 mL/g; extraction time of 20 min; extraction temperature of 50 °C). Moreover, ChLa offered better performances in extracting GLA and ISL compared to conventional organic solvents methanol. Hence, ChLa was selected for further optimization.

3.2. Effects of physicochemical properties of DESs

Previous studies have shown that the intermolecular interaction between solute and solvent will be affected by the pH value of DES so that the extraction efficiency of target compound from the sample matrix is different [29]. Based on the above results (Table 1), most acidic DESs were more effective for ISL and GLA extraction than alkaline DESs. This may be due to the existence of phenolic hydroxyl groups in the GLA and ISL. In the study of Zhao and coworkers [30], it was also found that acid-based DESs exhibited higher extraction efficiency than amino-
Based DESs for the extraction of flavonoids. The acidic environment could increase the electrostatic and hydrogen-bonding interactions between the solvent and target components, which promoted the dissolution of GLA and ISL.

Meanwhile, it was found that the extraction ability of acid-based DES changed with acid molecular structures, which is consistent with the research of Florindo and Vieira [31]. This phenomenon might be because the extensive hydrogen-bonding interaction increased the viscosity. As we know, high viscosity is one of the significant disadvantages of DES, which could hinder the mass transfer and diffusion of the compounds [31]. Therefore, despite of the similar pH, ChLa system with low viscosity (28.2) showed higher extraction yields than that of ChCit (203.6). The results were consistent with previous studies that DES with low viscosity benefited the extraction of flavonoids [32].

Based on the above interpretation, it was demonstrated that pH and viscosity played an essential role in the extraction efficacy of flavonoids from licorice. Generally, acidic DES with low viscosity promoted the extraction. Nevertheless, despite similar pH values, ChTa with relatively higher viscosity (458) was slightly more effective than ChOxa for extraction of ISL. In addition, LaGlu was efficient for extraction of GLA (6768.41 ± 186.3 μg/g), but the extraction yield of ISL (483.28 ± 12.37 μg/g) was the lowest. The results indicated that the physicochemical properties of DES partly affected the extraction efficacy [31]. And in this study, acidic ChLa with low viscosity was the best choice for the extraction of GLA and ISL.

### 3.3. Characterization of DES

DESs are formed via hydrogen-bond interactions between HBA and HBD. Thus, FTIR of LA, ChCl and ChLa DES were measured to analyze hydrogen bonds between ChCl and LA [33]. Fig. 2 depicts FTIR analysis of DES precursors (ChCl and LA), as well as their mixtures with and without addition of water (w/w). A wide peak between 3650 and 3200 cm⁻¹ corresponds to the hydroxyl groups. The peak at about 1716 cm⁻¹ corresponds to the C = O bonds of lactic acid. The stretch of the carboxylate group might attribute to the carboxylate group. Compared with single ChCl or LA, DES showed several frequency shifts and bands widths changes. The most distinct changes were the stretching vibration absorption peak of the hydroxyl group between 3650 and 3300 cm⁻¹ in DES was broader than those in the ChCl or LA, and further increased with the adding of water. These results indicated that hydrogen bonds were formed between ChCl and lactic acid in DES.

### 3.4. Effect of molar ratio and water content in DES on extraction efficiency

For ChCl and LA-based DES selection, the extraction of GLA and ISL was performed by increasing the ratio of choline chloride and lactic acid. As shown in Fig. 3A, the extraction yields of GLA and ISL changed irregularly with the change of molar ratio. Different molar ratios of HBA and HBD can change the physicochemical properties of the DES such as pH, polarity, viscosity, and density, which affect the interactions between the target products and DES [34]. A maximize extraction yield was obtained when the molar ratio of ChLa was 1:1, indicating that this molar ratio was most suitable for the simultaneous extraction of GLA and lactic acid in DES.

As shown in Fig. 3B, ChLa with 20% water contents showed higher GLA and ISL extraction yields than that of other water contents. This is because that the addition of water can reduce the viscosity of the solution and regulate the polarity of the solution, which is beneficial to increase the dissolution rate of the compounds. However, a further increase in water contents reduced the extraction yield because high water content will destroy the interactions between the DES and target components [35,36]. Considering the extraction yield of GLA and ISL, ChLa with 20% water content and a molar ratio of 1:1 was selected for the subsequent extraction experiments.

### 3.5. Single-factor experiments

Multiple factors could affect the yield of extracts from licorice [37]. To determine the correlations between the extraction conditions and target components, the effect of ultrasonic power, extraction temperature, extraction time, and liquid-to-solid ratio on the yield of GLA and ISL
ISL were evaluated using single-factor experiments. As shown in Fig. 3C, with the increase of ultrasound power, the yield of GLA and ISL increased and achieved a maximum level at the ultrasound power of 480 W. However, the yield of ISL and GLA started to decrease when the ultrasound power exceeded 480 W. As we know, ultrasonic could result in the damage of plant cells and promote the dissolution of active compounds. However, once the ultrasound power is high enough for cell destruction, then increasing the power will produce a lot of useless...
Table 2: Independent variables, their levels for the Box-Behnken design, and the responses obtained.

| Run | X1: Temperature (°C) | X2: Time (min) | X3: liquid-to-solid ratio (mL/g) | Y1: GLA extraction yield (µg/g) | Y2: ISL extraction yield (µg/g) |
|-----|----------------------|----------------|-----------------|-----------------|-----------------|
| 1   | 55(-1)              | 20(0)          | 10(-1)          | 6414.96         | 746.88          |
| 2   | 65(0)               | 20(0)          | 10(-1)          | 6927.56         | 857.08          |
| 3   | 55(-1)              | 20(0)          | 20(0)           | 6817.08         | 805.36          |
| 4   | 75(1)               | 20(0)          | 30(1)           | 6573.8          | 793.04          |
| 5   | 55(-1)              | 20(0)          | 30(1)           | 6807.68         | 826.92          |
| 6   | 65(0)               | 30(1)          | 30(1)           | 6317.92         | 843.88          |
| 7   | 65(0)               | 10(-1)         | 30(1)           | 6477.12         | 738.96          |
| 8   | 65(0)               | 30(1)          | 10(-1)          | 5950.08         | 749.12          |
| 9   | 65(0)               | 20(0)          | 20(0)           | 6922.08         | 853.12          |
| 10  | 65(0)               | 20(0)          | 20(0)           | 6918.56         | 845.44          |
| 11  | 65(0)               | 20(0)          | 20(0)           | 6942.08         | 856.48          |
| 12  | 65(0)               | 10(-1)         | 30(1)           | 6905.88         | 809             |
| 13  | 55(-1)              | 20(0)          | 30(1)           | 6942.08         | 856.48          |
| 14  | 75(1)               | 20(0)          | 10(-1)          | 6202.88         | 833.08          |
| 15  | 65(0)               | 20(0)          | 20(0)           | 6929.92         | 859.92          |
| 16  | 75(1)               | 30(1)          | 20(0)           | 5969.04         | 805.72          |
| 17  | 75(1)               | 10(-1)         | 20(0)           | 6557.64         | 771.76          |

The effect of ultrasonic power was set at 480 W [38]. Then the effect of extraction temperature (35–75 °C) on the yield of GLA and ISL was investigated by fixing the ultrasonic power to 480 W. As shown in Fig. 3D, it was found that the extraction yield of GLA and ISL was promoted when extraction temperature increased from 35 to 65 °C. Many studies showed that high temperature will result in the decomposition of the flavonoid compounds [39]. As the temperature increased to 75 °C, the extraction yield decreased. Therefore, the optimal temperature was around 65 °C.

Next, the effect of sonication time on the extraction efficiency was studied. As shown in Fig. 3E, the extraction yield of GLA and ISL had the maximum value at 20 min. As the extraction time increased and exceeded 20 min, the extraction yield started to decrease. This might owe to the disruption of active compounds that occurred after long exposure to heat or ultrasonic irradiation [11,40].

Finally, the effect of the liquid-to-solid ratio on the yield of GLA and ISL was studied. As shown in Fig. 3F, the extraction yield of GLA and ISL was promoted when the liquid-to-solid ratio was increased to 20 mL/g. Although a higher liquid-to-solid ratio could dissolve more target compounds, the excessive increase of extractant would increase the energy consumption of ultrasound [41,42]. Thus, the extraction yield decreased when the liquid-to-solid ratio exceeded 20 mL/g.

From the results of single-factor experiments, the parameter of ultrasonic power, extraction temperature, extraction time, and liquid-to-solid ratio has a significant effect on the yield of GLA and ISL. While from the perspective of energy saving, the ultrasonic power of 480 W was chosen as the optimal ultrasonic power for extraction. Other factors, including extraction temperature (X1), extraction time (X2), and liquid-to-solid ratio (X3), were considered as key factors to optimize the extraction yield of GLA and ISL from licorice using the response surface method.

3.6. Optimization of extraction conditions by Box-Behnken design

According to the Box-Behnken design, 17 experiments were performed to investigate the impacts of three independent variables (extraction temperature, extraction time, and liquid-to-solid ratio) on the response of variables (the extraction yield of GLA and ISL). The independent variables and response variables of RSM were shown in Table 2. Responses for the extraction yield of ISL and GLA varied from 5950.08 to 6942.08 µg/g and 713.36 to 859.92 µg/g, respectively. The large value ranges demonstrated that the independent variables have a significant effect on the extractions. A second-order polynomial equation was applied to express the proposed model after multiple regression analysis of the experimental data. The second-order polynomial equations for the responses and variables in terms of the coded levels are as follows:

GLA yield (µg/g) = 6928.04–116.58X1–289.72X2 + 194.21X1 + 6.40X1X2–7.10X1X3+15.23X2X3–226.33X3–315.06X12–200.23X22

ISL yield (µg/g) = 854.40–16.05X1–13.34X2 + 40.56X3 + 1.50X1X2–0.09X1X3 + 6.18X2X3–32.80X12–17.61X22–51.55X32

The results of ANOVA can illustrate the reliability of the second-order polynomial models. As shown in Table 3, it was apparent that the model was extremely significant (p < 0.0001) for quantification of extraction yields of GLA (Y1) and ISL (Y2). In addition, no significance was observed in the lack of fit (6.22 and 0.4603, p > 0.05), and the correlation coefficients (R2, 0.9991, and 0.9953) for the two responses were exceeded 0.99, demonstrating that the model was adequate to explain the relationship between the independent variables and the responses [43]. All the results confirmed that the second-order models could well fit the experimental data, and were suitable for the prediction of relevant responses. The linear coefficients (X1 and X2) and quadratic coefficients (X12 and X22) showed the most significant (p < 0.0001) and negative effects on the responses of GLA and ISL. While the linear coefficient X3 showed a highly significant and positive effect on the extraction yield of GLA and ISL. In addition, the interaction of time and ratio and liquid to solid (X1X3) was significant and had a positive effect on the response of ISL. Other variables were insignificant for two
To visualize the interactive effect of the variables on the responses, the 3D response surface plots were constructed based on the regression model equations. As shown in Fig. 4, all the response surface curves exhibited a similar pattern with a maximum point in the experimental domain of each, demonstrating the selected factor ranges were reasonable. The extraction yields of GLA increased with the increase of liquid to solid ratio (Fig. 4A-C). Additionally, higher solid to solid ratio was beneficial to increase the yield of GLA, which could increase the dissolution of GLA in the DES. Meanwhile, a longer extraction time with high liquid to solid ratio or low extraction temperature promoted the extraction of ISL (Fig. 4E and F). However, the extraction yield of ISL decreased with the increased extraction temperature and time. This might be because ISL tended to be destroyed in the high temperature, resulting in a low extraction yield. According to the response surface, the optimal values of the three variables for simultaneously maximizing the two responses were: extraction temperature of 62.5°C, extraction time of 18 min and solid to liquid ratio of 24, and the obtained maximum responses were 7034.56 ± 7.29 µg/g and 859.29 ± 2.79 µg/g, respectively. Furthermore, model validation was conducted under the optimal predicted conditions. The experimental values of the two responses were 7034.56 ± 7.29 µg/g and 859.29 ± 2.79 µg/g, which were consistent with the predicted values. The results demonstrated the established RSM model was accurate and suitable for optimizing the DES-UAE process.

### Table 4

| Solvent               | Temperature(°C) | Time(min) | liquid-to-solid ratio(g/mL) | GLA extraction yield(µg/g) | ISL extraction yield(µg/g) |
|-----------------------|-----------------|-----------|----------------------------|---------------------------|---------------------------|
| DES-UAE               | DES             | 62.5      | 18                         | 1:24                      | 7034.56 ± 7.29             | 859.29 ± 2.79             |
| Meth-UAE              | Methanol        | 62.5      | 18                         | 1:24                      | 4943.85 ± 85.12            | 673.78 ± 21.77            |
| DES-Bath extraction   | DES             | 62.5      | 180                        | 1:24                      | 5203.99 ± 59.56            | 710.47 ± 28.58            |
| Soxhlet               | Methanol        | 85        | 180                        | 1:24                      | 3922.61 ± 119.76           | 564.69 ± 18.89            |
| Water-UAE             | Water           | 62.5      | 18                         | 1:24                      | 602.37 ± 19.38             | 143.39 ± 8.21             |

#### 3.7. Comparison of DES-UAE and other extraction methods

To demonstrate the effectiveness of DES-UAE in extracting GLA and ISL, a comparative study was performed. As shown in Table 4, higher yields of GLA and ISL were obtained using DES as the extraction solvent instead of methanol and water for UAE, which indicated that DES was more efficient than that of conventional solvents. The results also coincided with those obtained for the extraction of glycyrrhizic acid from Glycyrrhiza glabra. [44] Meanwhile, the DES-based UAE method was more efficient than that of DES-based batch extraction, and this efficiency was demonstrated not only by the increased number of target products extracted but also by the reduced extraction time. This is due to the fact that the ultrasonication approach is more effective in destroying the external structure of the raw materials, allowing for better penetration of the solvent into the materials. Soxhlet extraction is a classical method for extracting compounds from solid substances and is widely used for the extraction of flavonoid natural products [25]. Besides, all the extraction methods used DES as solvent showed a higher yield of GLA and ISL than Soxhlet extraction. And while all used methanol as the extraction solvent, the UAE-based extraction method was less time-consuming and higher yield than the Soxhlet extraction. Therefore, the DES-based UAE could not only overcome the drawbacks of the conventional extraction method, such as large solvent requirement, higher extraction time, and temperature but more efficient
for GLA and ISL extraction.

3.8. Scanning electron microscopy

The disruption of plant cells could increase the release of the target compounds from the plant matrix and result in a higher extraction rate [45]. Therefore, SEM was used to observe the morphology of licorice root after different extraction treatments. As shown in Fig. 5, the surface of the licorice powder was intact before extraction (Fig. 5F). While the surface of the power changed rough and obvious damage was observed on the cell surface after different extraction processes. The destruction degree of the surface was in the following order: A > B > C > D > E > F. For DES-UA extraction, the cell wall could be destroyed by mechanical effect of ultrasound and fibrinolysis effect of DES as well [46]. Thus, a high destruction extent of cell-wall was observed. In addition, the yield of ISL and GLA was related to the destruction degree of the surface. The large destruction extent of cell wall promoted the release of the target compounds from the plant matrix and enhance extraction efficiency.

3.9. Molecular dynamics simulation analysis

The relevant role of intermolecular interaction between extracted compounds and solvents in affecting the dissolution capacity of extractive has been reported [47]. Therefore, the extraction efficacy might be influenced by the interaction between GLA and solvent molecules. To reveal the mechanism why DES was more efficient for extraction than other solvents from an atomic perspective, molecular dynamic simulation was performed. ChCl-La, methanol and water were selected as three typical solvents, and GLA was selected as a typically extracted compound due to the larger variation range of extraction yields among different solvents.

Fig. 6A showed the distribution of GLA in the solvents from 0 ns and 100 ns. It could be observed that GLA molecules immediately aggregated from 0 to 100 ns in water, while the distribution of GLA molecules in DES and methanol slightly changed and remained evenly distributed, demonstrated GLA was more soluble in ChLa and methanol than in water. For quantitative analysis and comparison, solvent accessible surface area (SASA) is employed as a critical index to verify the extent of contact between GLA and solvent molecules. From Fig. 6B, it was observed that the SASA values of GLA in methanol kept stable from 0 to 100 ns. While the SASA values of GLA in DES slightly decreased in the first 40 ns and then kept stable. Upon reaching the stable dissolving state in solvents, GLA molecules possessed 156.92 nm² of SASA in ChLa, which was slightly lower than that in methanol (205.03 nm²) and significantly higher than that in water (65.60 nm²). This trend matched with the formation of GLA clusters in water, and the approximately even distribution of GLA in methanol or ChLa (Fig. 6A).

Furthermore, the average noncovalent interaction (aNCI) between the GLA and solvent molecules were investigated. Several representative noncovalent interactions, such as hydrogen bonds, van der Waals interactions and steric repulsion, were studied [47]. As shown in Fig. 6C, the color calibration from dark blue to red reflected the interaction varied from strong attraction force (hydrogen bonds) to repulsion force (van der Waals force). It was observed that GLA was tightly attracted to the blue surface in the DES, indicating that GLA formed strong hydrogen bonds with the surrounding solvent molecules. Especially the hydrogen bonds bridged with chloride anion were more stable than other solvents. The results attributed to the special characteristic of chloride anion in ChLa, which could serve as a bridge connecting H atoms of hydroxyl groups in GLA and solvent molecules simultaneously. This interaction could also weaken the steric effect of solvent molecules surrounding GLA and increase the formation of hydrogen bonds at the same time. To the contrary, stronger repulsion interaction (red area) was observed between GLA and water, which was resulted by the formation of GLA cluster (Fig. 6A) and further restricted the contact of GLA with water. Besides, it should be noted that the van der Waals interactions (large green area) between GLA and DES was much stronger than water and methanol systems. Previous studies demonstrated that close and strong van der Waals interactions promoted the dispersion of solute molecules in the DES and endowed a more stable state, leading to its solubility in the solvent [48]. Overall, the attracted interaction between GLA and ChLa was more stable (hydrogen bonds) and strong (van der Waals force) than GLA with other solvents, which might eventually contribute to the high extraction yield of GLA.

3.10. Enrichment of GLA and ISL from extraction solution

The AB-8 microporous resin, which possessed the same similar polarity with ISL and GLA, was used to simultaneously enrich ISL and GLA for the first time [49]. After treatment with AB-8 microporous resin, the content of ISL and GLA in DES-UAE enrichment reached 8.63 ± 0.24 %
and 1.01 ± 0.02 %, respectively, with recoveries of 87.52 ± 1.59 % and 83.72 ± 1.58 %. The contents of ISL and GLA in Soxhlet enrichment reached 4.82 ± 0.09 % and 0.73 ± 0.02 %, respectively, with recoveries of 82.80 ± 1.41 % and 86.56 ± 3.42 %. The results proved that the AB-8 microporous resin could effectively enrich the GLA and ISL from the DES solution.

3.11. Pharmacological activities

The application of GLA and ISL as antioxidants, anti-inflammatory, and antimicrobial agents has been extensively studied. In this experiment, the pharmacological activities of the enriched products from two different extraction methods, including antioxidant activities, anti-inflammatory activities, and antibacterial activities, were measured to verify the application potential.

Antioxidant components usually possess a strong ability of free-radical scavenging, which is associated with many diseases. ABTS has been frequently used as a representative reagent for examining the free radical scavenging activities of bioactive compounds. To quantify the antioxidant activities of enriched products, the concentration of the samples required to scavenge 50% of radicals (IC50) was measured. A smaller IC50 value indicates an increase of free-radical scavenging...
ability [50]. As anticipated, the IC50 values of the enriched products of Soxhlet extraction were approximately 2 folds higher than that of DES-UAE (296.18 ± 0.84 μg/mL vs 163.06 ± 1.19 μg/mL). Of note that vitamin C (Vc), the most accepted antioxidant, only showed an IC50 value of 80.06 ± 1.28 μg/mL. [51]. The above results confirmed that the enriched products using DES-UAE exhibited better in vitro antioxidant activity. Then the anti-inflammatory activity of different enriched products was determined by hyaluronidase inhibition assay [26,52]. Similarly, the superior anti-inflammatory ability of enriched products with DES-UAE was detected (Fig. 7). The IC50 value of the enriched products with DES-UAE and Soxhlet extract were 230.56 ± 2.77 μg/mL vs 163.06 ± 0.84 μg/mL, respectively. This is similar to the results obtained from the ABTS radical-scavenging activity assay. In addition, compared with Soxhlet extract, the MIC values of enrichment products with DES-UAE against S. aureus, S. xylosus, and E. coli significantly reduced, showing 125, 250 and 250 μg/mL, respectively (Fig. 7).

The results demonstrated that the enriched products of DES-UAE possessed higher antioxidant, anti-inflammatory, and antibacterial activities than that of Soxhlet extraction. According to the contents of enriched products, it was obvious that the high GLA and ISL contents endowed the superior pharmacological activities of enriched products. The findings of this study will contribute to the implementation of a useful role of GLA and ISL in the management of antioxidant, anti-inflammatory, and antibacterial properties.

4. Conclusions

In summary, an eco-friendly DES-based UAE method was used for the first time to simultaneously extract GLA and ISL from licorice. The optimal DES system was determined, and the variables that affect the DES-UAE process were evaluated and optimized. The verification experiments using optimized conditions confirmed that the method was reliable and more efficient than the conventional extraction method. In addition, the enriched products possessed superior antioxidant, anti-inflammatory antibacterial activities. Furthermore, the mechanism of the high extraction efficacy of ChLa compared with other solvents was revealed by the molecular dynamic simulation. Overall, our study not only provides a green and highly efficient DES-UAE method for extraction GLA and ISL from licorice, but also demonstrates the mechanism of extraction efficacy difference among different solvents.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

Yan-Hua Li and Zhi-Yun Zhang designed the whole experiment; Chen Xing directed the completion of the experiment; Wen-Qiang Cui, Yue Zhang, Xin-Shu Zou, Jing-You Hao, Si-Di Zheng, Ting-Ting Wang, Xiao-Zhen Wang, Tong Wu, Yan-Yan Liu, Xue-Ying Chen and Shu-Guang Yuan were supportive during the experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultraschon.2022.105946.

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