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A new green alternative solvent for extracting echinacoside and acteoside from *Cistanche deserticola* based on ternary natural deep eutectic solvent

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ABSTRACT

Echinacoside and acteoside, the representative constituents of *Cistanche deserticola* Y. C. Ma (C. *deserticola*), are known for its many important biological activities. In this study, a novel ternary natural deep eutectic solvent (NADES) (citric acid, fructose and glucose in a 1:2:1 molar ratio, 30 % water content) was obtained through the adjustment and optimization of single-factor experiments. And through the Box-Behnken design optimization, the optimal extraction conditions were obtained, at the moment, the yields of echinacoside and acteoside were 6.16 and 2.13 mg/g, respectively. In addition, through computer molecular simulation, it was found that there are strong hydrogen bonds and interactions between the molecules of NADES, and the hydrogen bonds, van der Waals forces and π-π interactions played an important role in the extraction of echinacoside and acteoside from C. *deserticola* by NADES. Finally, compared with the traditional method, NADES ultrasound-assisted extraction of echinacoside and acteoside has a shorter time (15 min), the maximum extraction yield is 1.54–2.86 times and 1.26–2.52 times than that of traditional extraction methods for echinacoside and acteoside, respectively. This study demonstrates that NADES can provides a new direction for developing new sustainable alternatives, improving green extraction process and efficiency for extracting natural active substances.

Introduction

Cistanche deserticola Y. C. Ma (C. *deserticola*) is a traditional Chinese medicinal herbs, which is mainly parasitic on the roots of desert plants such as Haloxylon Bunge and Tamarix Linn [1,2]. It is mainly distributed in China's Inner Mongolia, Xinjiang, Qinghai, Ningxia and other northwestern desert regions and warm and dry regions in Eurasia [3,4]. C. *deserticola* has anti-aging, anti-fatigue, anti-inflammatory, liver-protecting, anti-oxidant, anti-osteoporosis, anti-depression, neuroprotection, immune regulation, treatment of impotence and other effects [5–8]. Echinacoside and acteoside, which is a representative compound of phenylethanoid glycosides and have high content in C. *deserticola* and various pharmacological activities, such as anti-neurodegeneration, anti-osteoporosis, anti-oxidation, anti-cancer, anti-inflammatory, hepatoprotective and cardiovascular protective effects [9–11]. Therefore, echinacoside and acteoside have development prospects that cannot be ignored in the fields of medicine, health care and food. [12–15]. The traditional extraction methods include immersion, reflux, Soxhlet, ultrasonic and microwave extraction [16–18]. Although these methods have their own advantages, there are problems such as low efficiency, long time, high processing cost, and large consumption of organic solvents [19,20]. Also, considering the environmental impact, these methods cannot meet the requirements of green chemistry. As a result, exploring a solvent with green, sustainable and environmentally friendly properties for the extraction of echinacoside and acteoside is indispensable.

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The hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) are complexed to form a new type of solvent called deep eutectic solvent (DES) [21,22]. DES has many advantages, such as simple experimental steps, low cost, adjustable physical and chemical properties, and so on [23–25], but its components usually have poor biocompatibility, which limits their applications in food and pharmaceutical industries [26]. Natural deep eutectic solvents (NADES) are new green and sustainable solvents based on natural ingredients such as choline derivatives, amino acids, sugars, polyols, organic acids and non-toxic quaternary ammonium salts, etc [27,28]. Due to its natural ingredients, NADES is more advantageous in terms of low toxicity, biodegradability, high solubility, sustainability and lower preparation cost [29–31], in line with the requirements of green chemistry, and compared with traditional organic solvents, NADES has higher extraction efficiency, high solubilizing ability and stabilizing ability, have been widely used as an ideal medium for the extraction of different biologically active compounds such as phenols and terpenoids and the sustainable value-added of plant materials [32–35], as a new technology that can replace traditional media, it has attracted great attention. V. Huber, W. Kunz et al. have used a series of NADES to extract the polyphenol compound curcumin, showing excellent extraction performance [36–39].

In this study, ternary NADES was attempted to extract echinacoside and acteoside from phenolic glycosides and phenylpropanoid compounds. A facile, efficient, and environmentally friendly method for NADES combined with ultrasound-assisted extraction (NADES-UAE) of echinacoside and acteoside from C. *deserticola*. Eighteen different binary and ternary NADES composed of HBA and HBD were prepared. The types, proportions, water content, liquid–solid ratio and extraction time of NADES preparation were investigated, and the optimal conditions for extraction of echinacoside and acteoside from C. *deserticola* were obtained via optimizing the moisture content, liquid–solid ratio and sonication time using a Box-Behnken design (BBD). Then, extracting echinacoside and acteoside via various methods, and their yields were compared. Finally, the extraction mechanism was investigated by computer molecular simulation technology.

Experimental

Materials

C. deserticola was bought in the local medicinal bazaar. Choline chloride (98 %), fructose (98 %), glucose (98 %), citric acid (98 %), malic acid (98 %), and glycerol (98 %) were purchased from Kemeiou Chemical Reagent Co., ltd (Tianjin, China). Echinacoside (98 %) and acteoside (98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The information of the chemicals, including name, CAS No., purity in mass, source and purity confirmation method was seen in Table S1. The structure of echinacoside and acteoside is shown in Fig. 1.

HPLC analysis

Refer to the HPLC method previously explored by our research group [40]. The HPLC conditions were as follows: an HIQ sil C_{18}W column (4.6 mm × 250 mm, 5 μm) was used for separating echinacoside and acteoside. The mobile phases were 0.4 % formic acid solution (A) and acetonitrile (B), and the elution process was 0–6 min, 90 % (A); 6–16 min, 90–80 % (A); 16–20 min, 80–75 % (A); 20–25 min, 75–80 % (A); 25–30 min, 80–90 % (A) at a wavelength of 333 nm and a 1.0 mL/min flow rate.

Preparations of NADES

Mix glucose, fructose, glycerol, citric acid, malic acid and choline chloride in a conical flask according to a certain molar ratio. Stir until the mixture of HBD and HBA is uniform and clarified, and there is no precipitation after standing, that is, NADES is prepared, which is transferred to a drying oven and dried with P_2O_5 before use. The preparation composition and ratio is shown in Table 1 and Table 2.

Establishment on extraction conditions of NADES

Screening of NADES types

The physical and chemical properties of NADES can be adjusted by changing the types and molar ratios of HBD and HBA, and the closer the NADES to the natural matrix properties, the higher the effect of solubilization and extraction of target compounds. In this experiment, the effects of binary and ternary NADES on the yields of echinacoside and acteoside were investigated. The extraction of echinacoside and acteoside in *C. deserticola* was carried out under the following conditions: 0.5 g of *C. deserticola* powder was weighed precisely in a 50 mL centrifuge tube, 5 mL of different binary and ternary NADES water systems were added, and the extract was sonicated for 15 min under the same power, and the supernatant was aspirated through a 0.45 μm membrane by centrifugation at 10000 rpm after the extraction. The appropriate amount of filtrate was aspirated, diluted 10 times with deionized water, mixed by vortex shaking, and used as the test solution. Three parallel determinations were carried out by HPLC to calculate the yields of echinacoside and acteoside, and select NADES with the best extraction efficiency.

Optimization of NADES preparation ratios

According to the experimental results in Section 2.4.1, the molar ratio of NADES with the optimal extraction effect was optimized, and the yields of echinacoside and acteoside were studied under five molar ratios (1:1:1, 2:1:1, 1:2:1, 3:2:1, 5:2:1) and ultrasound-assisted extraction with citric acid as HBA and fructose and glucose as HBD, the NADES with the best extraction efficiency was used for the subsequent experiments.

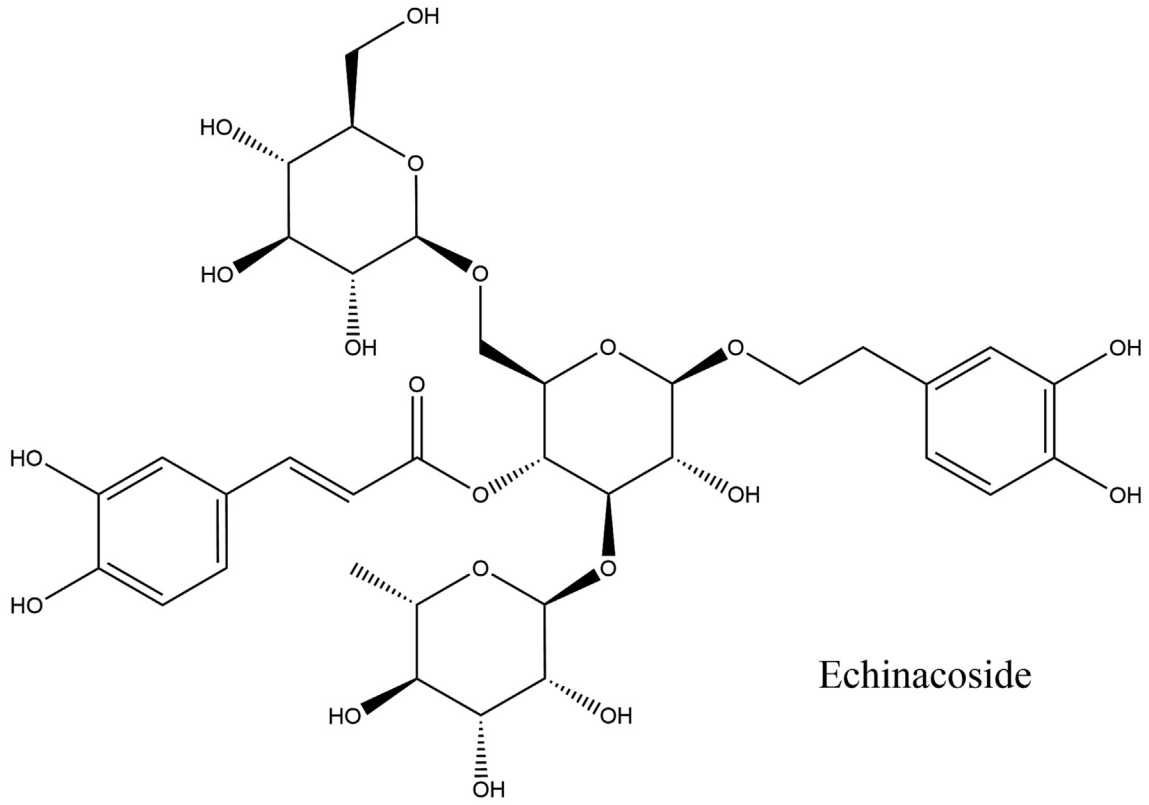
Accurately weigh 5 parts of 0.5 g *C. deserticola* powder into a 10 mL centrifuge tube, add 5 mL of NADES water system with a water content of 50 %, and perform ultrasonic-assisted extraction for 15 min. After the extraction, centrifuge at 10000 rpm, take the supernatant for dilution, accurately measure 2 mL of the supernatant, add 18 mL of 0.4 % formic acid aqueous solution, vortex the mixed solution and let it stand at room temperature, and filter it through a 0.45 μm filter. Analysis was detected using HPLC, and the yields of echinacoside and acteoside were calculated.

Optimization of NADES water content

According to the NADES with the best extraction effect in Section 2.4.2, deionized water was added in the preparation process according to the mass fraction to prepare the optimal NADES solutions with water contents of 20 %, 30 %, 40 %, 50 %, 60 % and 70 %, NADES with different water contents were fully dried with P_2O_5 in the drying oven, then it is used to extract echinacoside and acteoside. Weigh 0.5 g of *C. deserticola* powder, the liquid–solid ratio was 10 mL/g, and the ultrasonic time was 15 min. The content of echinacoside and acteoside was determined using HPLC. The viscosity of NADES was measured by using a NDJ-5S rotational viscometer (Shanghai pingxuan scientific instrument Co., ltd, Shanghai, China).

Optimization of liquid–solid ratio and extraction time

On the basis of selecting the optimal NADES solution, the liquid–solid ratio and extraction time in the extraction process were



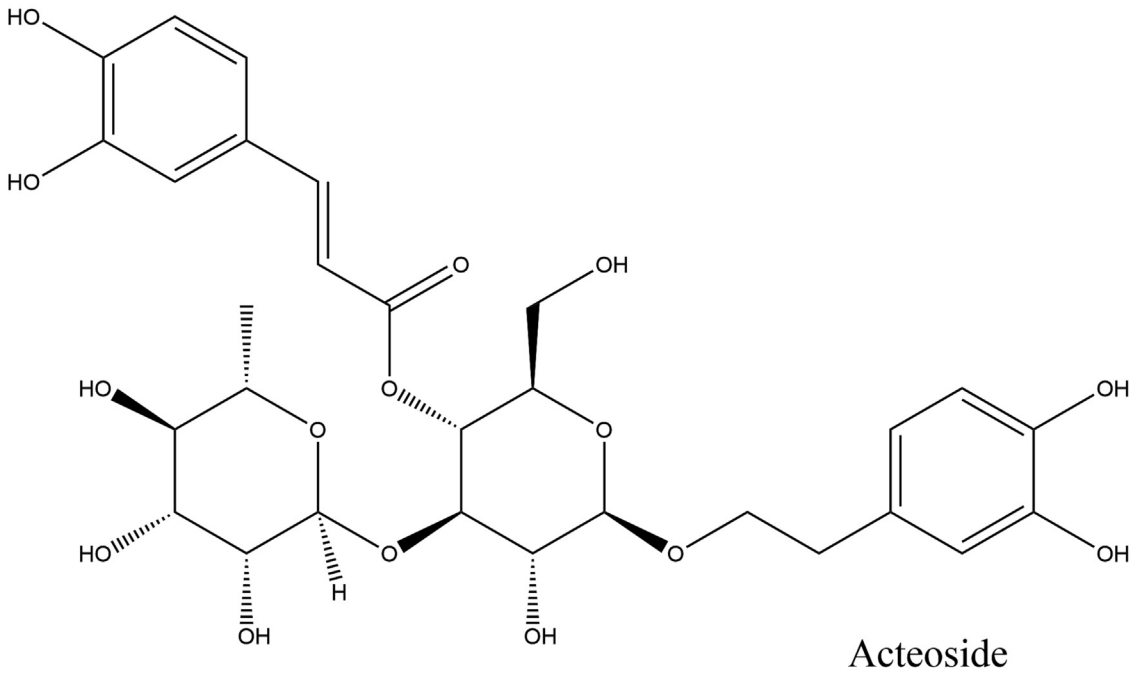


Fig. 1. The struture of echinacoside and acteoside.

Table 1
Composition and ratio of binary NADES.

Name	HBA	HBD	Molar ratio
NADES2-1	Choline chloride	Glycerol	1:1
NADES2-2	Choline chloride	Fructose	1:1
NADES2-3	Choline chloride	Glucose	1:1
NADES2-4	Citric acid	Fructose	1:1
NADES2-5	Citric acid	Glucose	1:1
NADES2-6	Malic acid	Fructose	1:1
NADES2-7	Malic acid	Glucose	1:1
NADES2-8	Malic acid	Glycerol	1:1
NADES2-9	Citric acid	Glycerol	1:1

optimized. The liquid–solid ratios were 8, 10, 12, 14 and 16 mL/g, and the extraction time were 10, 15, 20, 25 and 30 min, respectively, to investigate the effects of the two elements on the yields of echinacoside and acteoside.

BBD design for NADES ultrasound-assisted extraction

The three factors of water content, liquid–solid ratio and sonication time of NADES were selected to design the BBD test, with echinacoside and acteoside as the response values, and the test factor levels are shown in Table 3.

Extraction strategies

Echinacoside and acteoside from *C. deserticola* were extracted with NADES-UAE, ethanol ultrasonic-assisted extraction (Ethanol-UAE), water ultrasonic-assisted extraction (Water-UAE) and water thermal reflux extraction (Water-HRE), extraction method as shown in Table 4. After extraction, centrifugation was performed and the supernatant was filtered through a 0.45 μm filter for HPLC analysis.

Molecular simulation

Further explore the interaction mode of citric acid, fructose and glucose (molar ratio, 1:2:1) in NADES (system 1), and the extraction mechanism of NADES for echinacoside and acteoside (system 2). First, experimental conditions were simulated using the Foxcite plate in Material Studio, and kinetic simulations at 5000 ps were performed under the isothermal-isobaric (NPT) ensemble. Then, the 1:2:1 molecular fragment after kinetic optimization in system 1 and the 1:1 molecular fragment after kinetic optimization in system 2 were taken out, and optimized using B3LYP/6-31G* in Gaussian 16 respectively, DFT-D3 is used for dispersion correction [41–43]. After the structure is optimized, the energy calculation is carried out under the same method, and the energy calculation of the single molecule in the system is carried out, and the binding energy of the system 1 and system 2 are calculated according to the following equation (1) and (2). Finally, reduced density gradient (RDG) and the gradient isosurfaces analysis was performed using Multiwfn v.3.8 and VMD v.1.9.3 [44,45].

$$\Delta E_1 = E_{system1} - \left(E_{cit} + E_{fru} + E_{glu}\right) \quad (1)$$

$$\Delta E_2 = E_{system2} - \left(E_{system1} + E_{ech} + E_{act}\right) \quad (2)$$

Where ΔE_1 is the binding energy of system 1, E_{cit}, E_{fru}, E_{glu} and $E_{system1}$ is the energy of citric acid, fructose, glucose and system 1, respectively; ΔE_2 is the binding energy of system 2; E_{ech}, E_{act} and $E_{system2}$ is the energy of echinacoside, acteoside and system 2, respectively.

Results and discussion

Effect of different types of NADES

The physicochemical properties of NADES are related to the type and ratio of HBA and HBD, and the ratio of HBA and HBD influences the stability of NADES. NADES suitable for the physicochemical properties of the target compounds can be extracted from the natural matrices of plants with high content of active ingredients. Firstly, the type of NADES was optimized. It can be seen from Fig. 2, among the nine types of NADES based on acids, bases, sugar groups and polyols, those based on choline chloride and glycerol and fructose glucose, NADES2-3 had a higher content of echinacoside, while the content of acteoside was higher in NADES2-2 than that in NADES2-3, probably due to the structural difference between fructose and glucose. And it was observed in the experiment that the viscosity of NADES containing glucose was greater than that of NADES containing fructose, and the viscosity affected the mass transfer to the solvent [46]. In addition, the alkaline environment was not conducive to the structural stability of echinacoside and acteoside, which in turn inhibited the release of active ingredients into the solvent. NADES based on citric acid and glucose showed good results for the extraction of echinacoside; NADES based on malic acid and fructose for the extraction of acteoside showed good results, probably malic acid and fructose are structurally easier to interact with acteoside and increase the possibility of contact between acteoside and solvent. The more acidic NADES with citric acid facilitated the solubilization and mass transfer of echinacoside and acteoside in *C. deserticola*. The acid-based NADES had a lower pH, which enhanced the ability of the solvent to provide protons and accept electrons, improving the solubilization performance of echinacoside and acteoside and increasing the extraction efficiency instead. The extraction effect of NADES2-8 on echinacoside and acteoside was satisfactory, but the yields were lower than those of NADES2-5. In the alcohol-based NADES, no ionization occurs in the solvent, so the yields of echinacoside and acteoside are relatively stable.

According to the binary NADES, the specificity of each ligand for the extraction of echinacoside and acteoside was investigated, and ternary NADES were prepared and examined for their extraction efficiency of echinacoside and acteoside. The polarity of individual NADES in Fig. 3 is different, but they have the potential to act as alternative media for the matrix in plants, thus enhancing the sol-

Table 2
Composition and ratio of ternary NADES.

Name	Component 1	Component 2	Component 3	Molar ratio
NADES3-1	Choline chloride	Glucose	Fructose	1:1:1
NADES3-2	Glycerol	Malic acid	Glucose	1:1:1
NADES3-3	Glycerol	Citric acid	Glucose	1:1:1
NADES3-4	Citric acid	Fructose	Glucose	1:1:1
NADES3-5	Citric acid	Malic acid	Fructose	1:1:1
NADES3-6	Malic acid	Fructose	Glucose	1:1:1
NADES3-7	Malic acid	Citric acid	Glucose	1:1:1
NADES3-8	Glycerol	Citric acid	Fructose	1:1:1
NADES3-9	Glycerol	Malic acid	Fructose	1:1:1

Table 3
Test factor levels of BBD.

Order	Water content, % (X_1)	Liquid-solid ratio, mL/g (X_2)	Extraction time, min (X_3)	Yield of echinacoside (mg/g)	Yield of acteoside (mg/g)
1	30	10	20	6.18	2.17
2	35	12	20	4.51	1.05
3	30	10	20	6.15	2.15
4	30	10	20	6.09	2.13
5	30	8	25	4.02	1.34
6	35	8	20	5.01	1.40
7	25	10	25	4.56	1.19
8	30	12	15	4.55	1.46
9	35	10	15	4.90	1.22
10	25	10	15	4.20	1.18
11	30	12	25	4.80	1.38
12	30	8	15	4.84	1.23
13	30	10	20	6.28	2.27
14	25	12	20	4.08	1.43
15	35	10	25	5.42	1.47
16	25	8	20	4.30	1.10
17	30	10	20	6.18	2.12

Table 4
Strategies of different extraction methods for the extraction of echinacoside and acteoside.

Methods	Extraction solvent	Liquid-solid (mL/g)	Extraction time (min)	Extraction temperature (℃)	Extraction instrument
NADES-UAE	NADES	10	5–30	50	Ultrasonic extractor, XM-500UVF model, Xiaomei Ultrasonic Instrument Co., ltd., Kunshan, China
Ethanol-UAE	50 % Ethanol	10	10–90	50	The same as above
Water-UAE	Water	10	10–90	50	The same as above
Water-HRE	Water	20	10–180	100	Electric heating sleeve, SKM model, Shandong Juancheng Hualu Electric Equipment Co., ltd., Heze, China

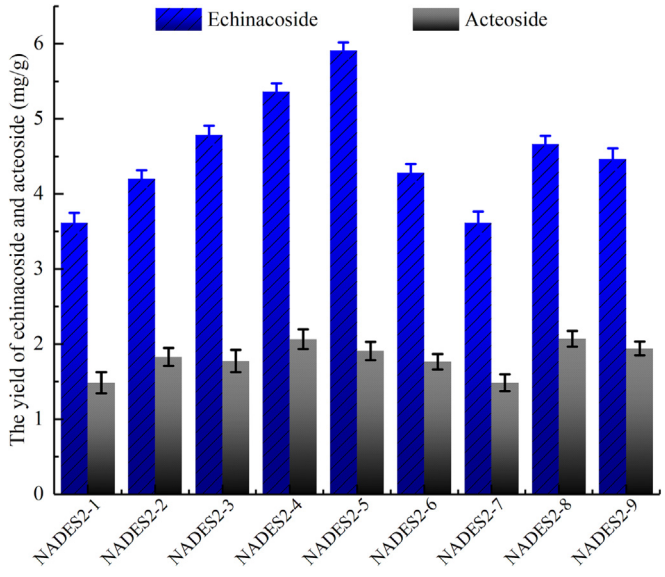


Fig. 2. The yields of echinacoside and acteoside from *C. deserticola* with different types of binary NADES.

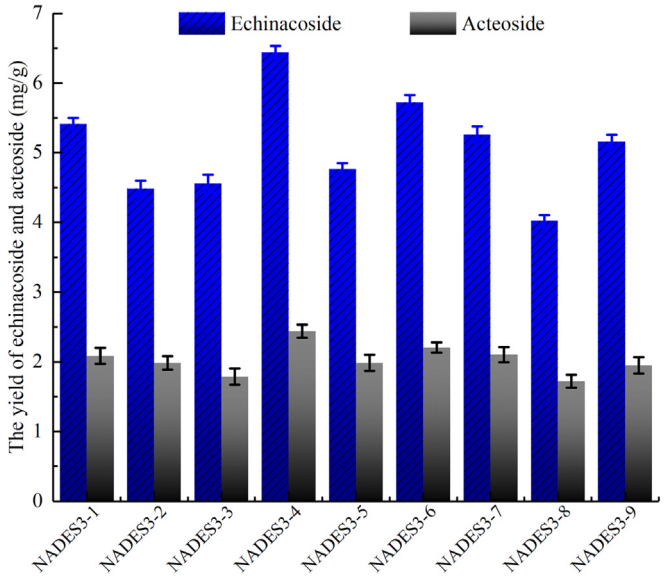


Fig. 3. The yields of echinacoside and acteoside from *C. deserticola* with different types of ternary NADES.

ubilization of the medicinal active ingredients, which may be one of the reasons to explain the increased yield of echinacoside and acteoside. According to the principle of similar solubility increase in polarity, the position and number of hydroxyl groups of individual ligands influence the polarity of NADES, echinacoside and acteoside molecules have more hydroxyl groups, while sugars and organic acids are both HBD and HBA, so the multi-hydroxyl group NADES system is more likely to bind to echinacoside and acteoside, which have stronger hydrogen bonding accepting ability.

In addition, there is a strong interaction between the HBA, HBD and the proton on the water hydroxyl group. In the extraction system, the hydrogen bonding between solute and solvent will significantly improve the extraction efficiency. Echinacoside and acteoside are polyhydroxy compounds, and these hydroxyl groups can easily form hydrogen bonds with NADES or between water.

Citric acid is highly acidic, and NADES solutions containing citric acid also become acidic, which may be similar to the nature of the natural substrates that make the structures of echinacoside and acteoside stable. The presence of excess hydroxyl or carboxyl groups in the ligand leads to more hydrogen bonding, which increases the stability of the liquid. Citric acid can form stable liquids with a variety of sugars, while malic acid has fewer carboxylic acid groups, which could explain the stronger extraction of NADES containing citric acid than NADES containing malic acid. The total extraction yield of NADES synthesized from citric acid with fructose and glucose was highest, 8.87 mg/g for echinacoside and acteoside. Therefore, NADES3-4 was selected for further experimental extraction studies.

Optimization of NADES-UAE

Extraction time and liquid-to-solid ratio

It can be seen from Fig. 4(A) that the yield of echinacoside and acteoside increased gradually with the increase of extraction time, and the yield started to decrease and leveled off after 15 min, probably because it took some time to reach the mass transfer equilibrium between NADES solution and *C. deserticola* powder, and the concentration of active ingredients in the extraction solution leveled off after reaching the equilibrium. In addition, ultrasound will lead to the increase of the solution temperature, when the temperature increases, the molecular movement speed is accelerated, and the molecules gain enough kinetic energy to overcome the intermolecular force, and it will significantly reduce the viscosity of NADES and increase the mass transfer rate, so the yield of echinacoside and acteoside increases, and the yield tends to be stable when the time exceeds 15 min, so 15 min was chosen as the best extraction time.

According to Fig. 4(B), the viscosity of NADES was larger than that of conventional solvents, and the low liquid–solid ratio led to the low efficiency of solvent transfer, and with the increase of solvent amount, the content of dissolved echinacoside and acteoside could be obviously observed to increase, but when the liquid–solid ratio exceeded 10 mL/g, the yield of both compounds began to decrease and then stabilized, probably because more solvent would increase the energy consumption of ultrasonication, leading to the poor cavitation of ultrasonication. Therefore, it was determined that the liquid–solid ratio of 10 mL/g gave the highest extraction efficiency for both compounds.

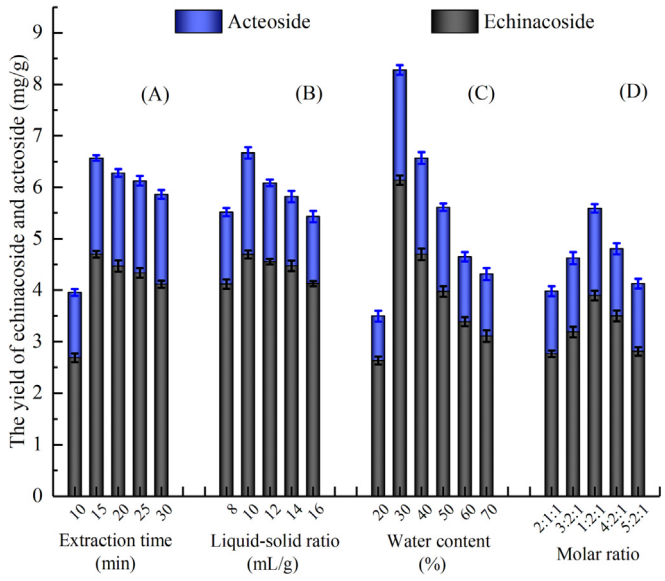


Fig. 4. The effect of the extraction time, liquid–solid ratio, water content and molar ratio of NADES on the extraction yields of echinacoside and acteoside.

Water content of NADES

Water in NADES exists in the form of bonded water, and appropriate water content can change the physical properties of NADES, such as the viscosity of the solvent, and also affect the hydrogen bonding in the NADES system. The effect of different water contents of NADES on the yield of echinacoside and acteoside was investigated by changing the water content of NADES, and the water content of NADES was changed after the optimal molar ratio of ternary NADES had been determined, and NADES with water contents of 20 %, 30 %, 40 %, 50 %, 60 % and 70 %were configured, it can be seen from Fig. 4(C), during the preparation process. Table S2 shows the NADES viscosity data with different water content, as the water content increases, the decrease of NADES viscosity could be obviously observed, and the lowest yield of echinacoside and acteoside was obtained at 20 % of water content, because the high viscosity of solvent affected the mass transfer effect and could not extract the target compounds effectively. Adding water would significantly reduce the viscosity of solvent and improve the mass transfer effect. Water as a strong polar solvent can change the polarity of NADES with different water contents, which can regulate the generation of hydrogen bonds in the system to a certain extent. The decrease of the extraction rate after the water content was more than 40 % was due to the disappearance of a large number of hydrogen bonds in the NADES solvent caused by the excessive addition of water, which destroyed the special structure of the NADES solution, and also made the polarity of the solvent is too high, so the optimal water content of NADES is chosen to be 30 %.

Molar ratios of NADES

The number of HBA or HBD groups, the spatial structure and the position of bonding affect the stability of NADES. After screening the kinds of HBA and HBD of ternary NADES, the combination of citric acid and fructose and glucose with the highest yield of echinacoside and acteoside was selected. During the experiment, it was found that the viscosity of NADES containing glucose was much larger than that of NADES without glucose, and the molar ratio of glucose was fixed when optimizing the molar ratio of ternary NADES considering the mass transfer properties of the solvent. The molar ratios of citric acid and fructose were changed to prepare NADES with molar ratios of 1:2:1, 2:1:1, 3:2:1, 4:2:1 and 5:2:1, respectively. The optimal molar ratio of HBA and HBD was determined based on the extraction rate of echinacoside and acteoside.

It is not difficult to see from Fig. 4(D) that when fructose and glucose are used as hydrogen bond donors, the yields of echinacoside and acteoside gradually increase as the molar ratio of citric acid and fructose increases. And the yields of echinacoside and acteoside tended to decrease with the increase of the ratio of citric acid after the ratio of 1:2:1, the possible reason is that the viscosity and surface tension of NADES decrease with the increase of citric acid, which can improve the diffusion and mass transfer effect of deep eutectic solvent. Hydrogen bonding, van der Waals forces and electrostatic interactions can influence the viscosity of NADES, so the number of hydroxyl groups in the NADES system largely affects the mass transfer efficiency of the solvent [47], and a suitable ratio is more favorable for the extraction of echinacoside and acteoside. And when the citric acid continued to increase will influence the interaction of echinacoside and acteoside with hydrogen bonding, probably because the extraction rate of low eutectic solvent with fructose glucose as HBD is mainly affected by intermolecular hydrogen bonding, citric acid competes with sugars for electrons, in addition, the increase of the proportion of

citric acid leads to the decrease of the proportion of hydroxyl groups, the interaction force between echinacoside and acteoside with hydrogen bonding is weakened so the extraction rate is reduced.

BBD experimental results

After determining the factors affecting the extraction of echinacoside and acteoside, BBD was used to study the interaction between three factors, namely, water content of NADES, liquid–solid ratio and extraction time, and the ANOVAs of the experimental results are shown in Table 5. As can be seen that the regression model had a P value < 0.0001, indicating that the experimental model was highly significant, the misfit term was not significant, and the experimental error was small, which indicated that the model could predict the experimental values better. In addition, the correlation coefficient R^2 for the model of echinacoside was 0.9539 and the correlation coefficient R^2 for the model of acteoside was 0.9826, indicating that the model can accurately describe the relationship between the independent variables and the response values. By performing a function fit to the experimental data, the quadratic regression equation for the coded variables can be derived as follows.

$$Y_1 = 6.18 + 0.35X_1 - 0.036X_2 + 0.039X_3 - 0.082X_1X_2 + 0.040X_1X_3 + 0.26X_2X_3 - 0.73X_1^2 - 0.95X_2^2 - 0.67X_3^2 \quad (3)$$

$$Y_2 = 2.17 + 0.028X_1 - 0.030X_2 + 0.037X_3 - 0.17X_1X_2 + 0.059X_1X_3 - 0.048X_2X_3 - 0.50X_1^2 - 0.42X_2^2 - 0.40X_3^2 \quad (4)$$

Where Y_1 (mg/g) is the yield of echinacoside; Y_2 (mg/g) is the yield of acteoside; X_1 (%) is the water content; X_2 (mL/g) is the liquid–solid ratio; X_3 (min) is the extraction time.

The interaction of each factor is shown in Fig. 5. On the basis of the quadratic model, the optimal extraction conditions are obtained as follows: NADES water content is 30 %; liquid–solid ratio is 10 mL/g; extraction time is 15 min. Under the optimal conditions, the predicted yield of echinacoside was 6.18 mg/g; the predicted yield of acteoside was 2.17 mg/g. Three parallel verification experiments were carried out on the optimal extraction conditions, and the experimental results were as follows: the average yield of echinacoside was 6.16 mg/g, and the average yield of acteoside was 2.13 mg/g. The two experimental values are respectively close to the predicted values, indicating that the established model is reliable.

2D NMR analysis

2D $^1H/^1H$ COSY was performed on NADES to identify the coupling spins in NADES. 2D $^1H/^1H$ NOESY was performed on NADES and acteoside, NADES and echinacoside to determine the space between hydrogen bonds. In the 2D $^1H/^1H$ COSY section of Fig. 6 (A), δ_H 4.89 is related to δ_H 3.10, δ_H 4.25 is related to δ_H 2.87, δ_H 3.53 is related to δ_H 3.02, δ_H 2.87 is related to δ_H 3.10, it indicates that there is a hydrogen bond between the hydroxyl group of glucose and fructose. These three components can self orient and form a complex matrix of microstructures, resulting in excellent stability. In the NMR spectrum, the OH peak may disappear because the hydrogen in OH may be replaced by deuterium from the NMR solvent (DMSO-d6). In addition, the intensity of the cross peak is relatively high, indicating that the distance between components is short, successful formation of NADES.

In the 2D $^1H/^1H$ NOESY spectra of Fig. 6(B) and (C), there are many cross peaks, indicating the existence of the intermolecular interaction between NADES and acteoside, NADES and echinacoside and the hydrogen bond formed between their hydroxyl groups. Table S3 and Table S4 have shown partial hydrogen signal attribution of acteoside and echinacoside. In Fig. 6(B), for NADES and acteoside, (9.58, 4.35), (9.15, 4.35) and (8.67, 4.35) indicate that there is a hydrogen bonding force between NADES and glucose H-1 ”; (9.58, 5.13), (9.15, 5.13) and (5.13, 8.67) indicate that hydro-

Table 5
ANOVA for the yields of echinacoside and acteoside.

	Quadratic sum	Degree of freedom (df)	Mean square	F-value	P-value	
Echinacoside						
Model	10.21	9	1.13	16.09	<0.0001	significant
X_1	0.96	1	0.96	13.64	0.0077	-
X_2	0.010	1	0.010	0.14	0.72	-
X_3	0.012	1	0.012	0.17	0.69	-
X_1X_2	0.027	1	0.027	0.38	0.55	-
X_1X_3	6.48×10^{-3}	1	6.48×10^{-3}	0.092	0.77	-
X_2X_3	0.28	1	0.28	3.97	0.087	-
X_1^2	2.27	1	2.27	32.23	0.0008	-
X_2^2	3.84	1	3.84	54.41	0.0002	-
X_3^2	1.89	1	1.89	26.86	0.0013	-
Residual	0.49	7	0.070	-	-	-
Lack of Fit	0.47	3	0.16	32.65	0.0028	not significant
Pure Error	0.019	4	4.84×10^{-3}	-	-	-
Cor Total	10.70	16	-	-	-	-
Acteoside						
Model	2.93	9	0.33	43.85	<0.0001	significant
X_1	6.28×10^{-3}	1	6.28×10^{-3}	0.85	0.39	-
X_2	7.37×10^{-3}	1	7.37×10^{-3}	0.99	0.35	-
X_3	0.011	1	0.011	1.47	0.26	-
X_1X_2	0.12	1	0.12	15.67	0.0055	-
X_1X_3	0.014	1	0.014	1.85	0.22	-
X_2X_3	9.16×10^{-3}	1	9.16×10^{-3}	1.23	0.30	-
X_1^2	1.07	1	1.07	143.82	<0.0001	-
X_2^2	0.74	1	0.74	99.46	<0.0001	-
X_3^2	0.67	1	0.67	90.65	<0.0001	-
Residual	0.052	7	7.43×10^{-3}	-	-	-
Lack of Fit	0.037	3	0.012	3.27	0.14	not significant
Pure Error	0.015	4	3.77×10^{-3}	-	-	-
Cor Total	2.99	16		-	-	-

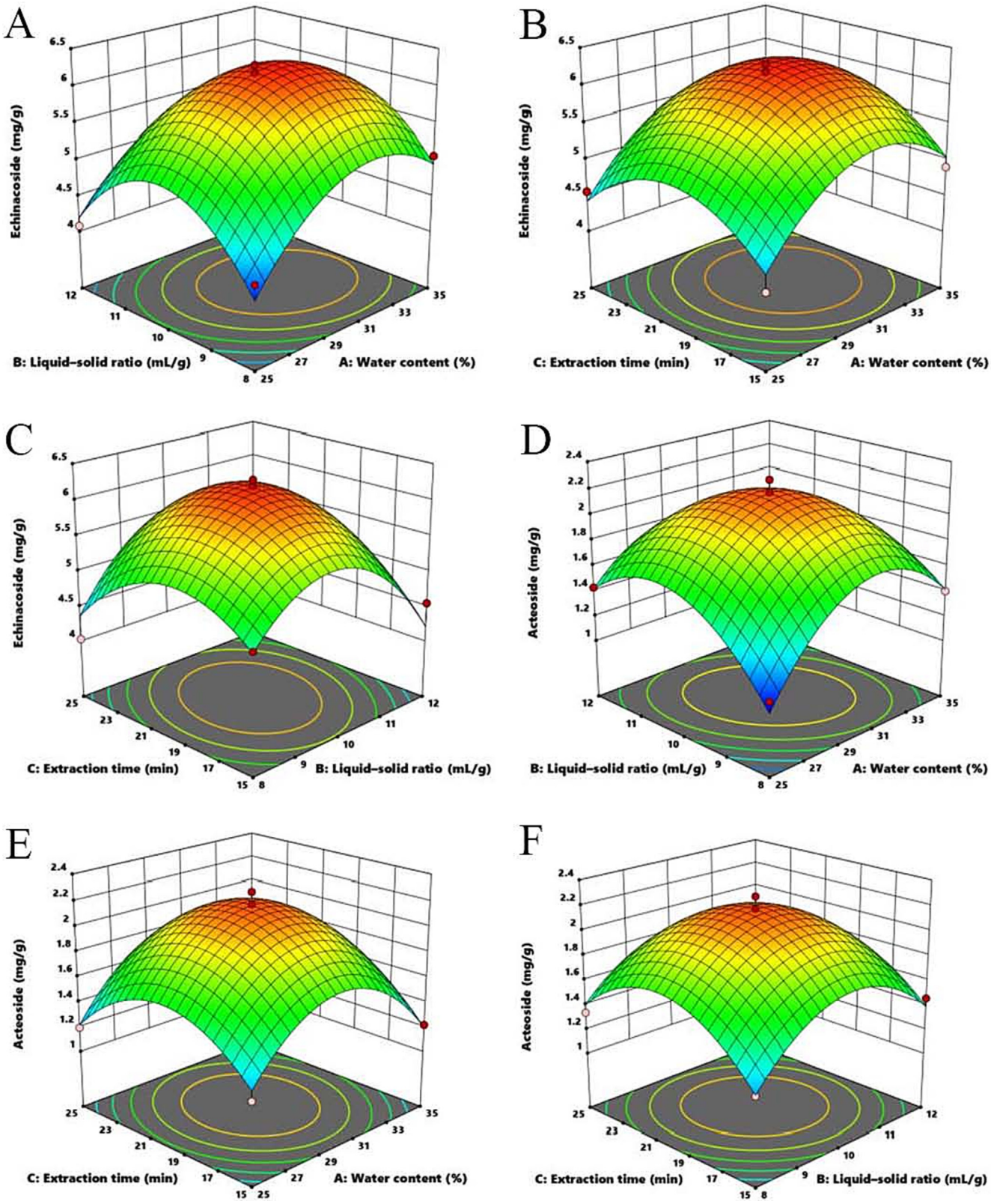


Fig. 5. Response surface plots for the extraction of echinacoside and acteoside from *C. deserticola* using NADES: the interaction between the liquid–solid ratio and water content (A, D); the interaction between extraction time and water content (B, E); the interaction between the liquid–solid ratio and extraction time (C, F).

gen bonding force is formed between NADES and rhamnose H-1 ‴; (9.58, 6.19), (9.15, 6.19) and (6.19, 8.67) indicate that hydrogen bonding force is formed between NADES and caffeoyl H-8. In Fig. 6(C), for NADES and echinacoside, (9.59, 4.37), (9.15, 4.37) and (4.37, 8.65), it is indicated that there is a hydrogen bond force between NADES and glucose H-1 ″; (9.59, 4.72), (9.15, 4.72) and (8.65, 4.72) indicate that there is a hydrogen bond force between NADES and glucose H-4 ″; (9.59, 5.03), (5.03, 9.15) and (5.03,

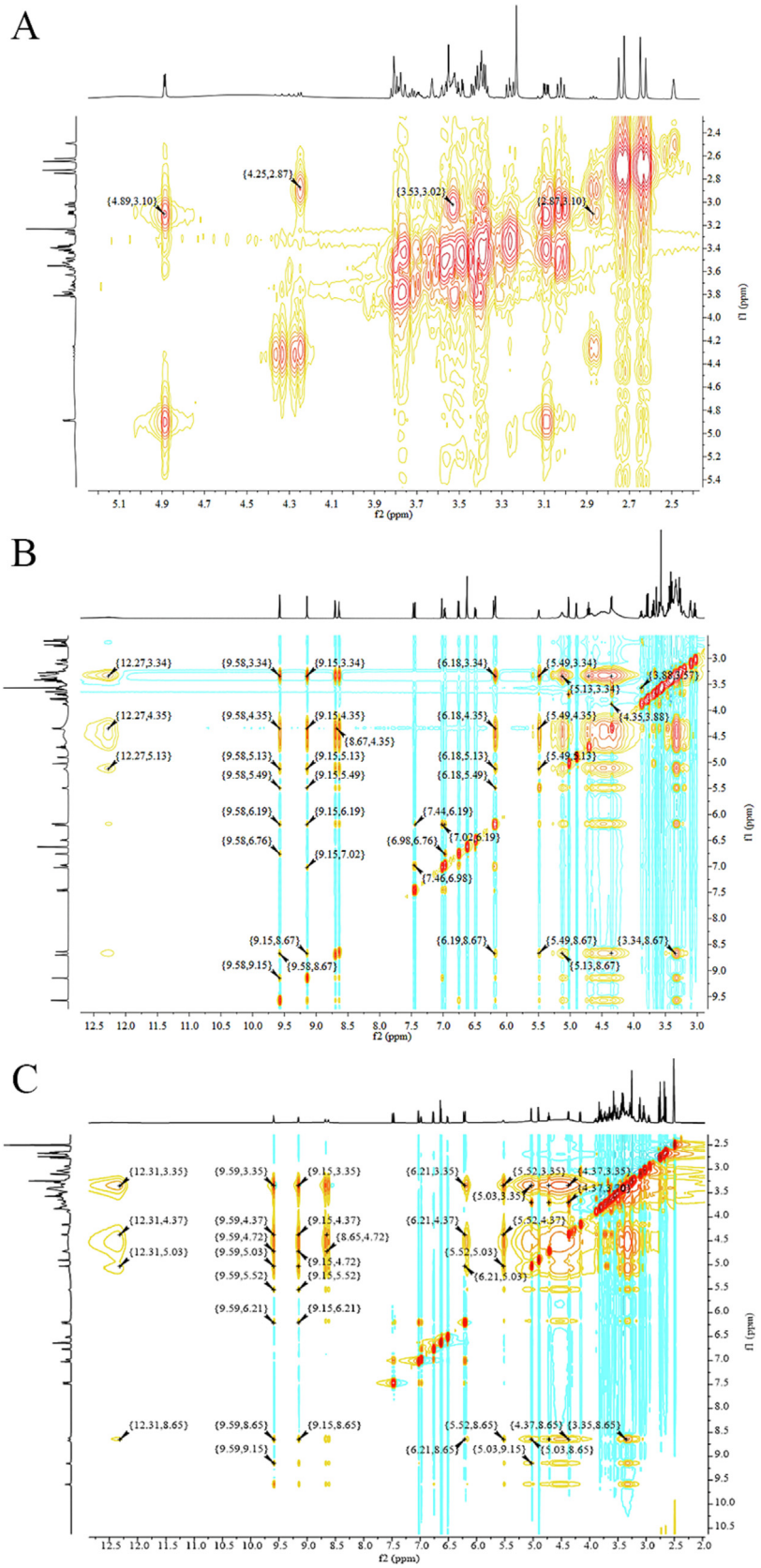


Fig. 6. 2D $^{1}H/^{1}H$ COSY spectrum of NADES (citric acid, fructose and glucose in a 1:2:1 molar ratio) (A); $^{1}H/^{1}H$ NOESY spectrum of NADES and acteoside (NADES: acteoside in a 1:1 molar ratio) (B); $^{1}H/^{1}H$ NOESY spectrum of NADES and echinacoside (NADES: echinacoside in a 1:1 molar ratio) (C).

8.65) indicate that hydrogen bonding force is formed between NADES and rhamnose H-1 ‴; (9.59, 6.21), (9.15, 6.21) and (8.65, 6.21) indicate that hydrogen bonding force is formed between NADES and caffeoyl H-7. In addition, some undefined peaks with low intensity were detected, which may be due to the peak after hydrogen bonding donor bonding of three components in NADES.

Molecular simulation

The possible interaction modes between system 1 and system 2 were further explored using molecular simulations to reveal the extraction mechanism. The optimized structure of echinacoside, acteoside, citric acid, fructose and glucose is shown in Fig. S1. First, as shown in Fig. 7(A) and (D), the simulation boxes of the two systems were established respectively. Then, the types of non-covalent interactions between molecules in system 1 and system 2 are shown using RDG and sign(λ_2)ρ. As shown in Fig. 7(B), in the region where sign(λ_2)ρ is about −0.043, δg^{inter} has a large peak with a height of about 0.076; in Fig. 7(E), in the region where sign (λ_2)ρ is about −0.035, δg^{inter} has a large peak with a height of about 0.066, and their peaks are all located in the negative region, manifesting that exist a strong hydrogen bond interaction between the molecules of system 1 and system 2 [48]. In addition, in the positive region of sign(λ_2)ρ about 0.03, there is a small peak of δg^{inter} in both system 1 and system 2, which reflects the weak interaction region between molecules in system 1 and system 2. The gradient isosurfaces (s = 0.5 au) in Fig. 7(C) and (F) show where the interaction occurs. It is not difficult to find that the blue and green isosurfaces are the main intermolecules in the two systems, and the bluer isosurfaces reflect the more important hydrogen bond interactions, while the green areas in the isosurface are weak van der Waals interactions, two forces play an important role in the formation of NADES and the extraction of echinacoside and acteoside [49]. Typical bond lengths for physical interactions are greater than 2.0 Å, and chemical interactions are usually in the range of 0.5-–2.0 Å [50]. Here, all the intermolecular bond lengths in system 1 are less than 2.0 Å, which clearly indicates that the molecular binding between system 1 is based on chemical interactions and has strong chemical bond forces. In addition, the binding energies between system 1 and system 2 were calculated to quantify their interactions. It can be seen from Table 6 that the binding energy between system 1 is about −650.378 KJ/mol, indicating that there are strong hydrogen bonds and interactions between its molecules, which should be one of the reasons for the good extraction performance of NADES. The binding energy between systems 2 reached −1743.332 KJ/mol, it shows that the interaction force between NADES, echinacoside and acteoside is very strong, and the formed system is very stable. In summary, the extraction of echinacoside and acteoside from *C. deserticola* by NADES mainly relies on hydrogen bonding, van der Waals force and π-π interaction.

Recovery and reusability of NADES

Macroporous resins are commonly used for enrichment and separation of natural products from extracts, and HPD-300 resin has remarkable adsorption and desorption properties for echinacoside and acteoside [51]. After enrichment of echinacoside and acteoside with HPD-300 macroporous resin, echinacoside and acteoside were eluted with deionized water, and the effluent was collected to obtain NADES liquid by rotary evaporation, and then placed in a desiccator to dry for the next cycle of extraction. The results of five cycles are shown in Fig. 8, and it is easy to see that

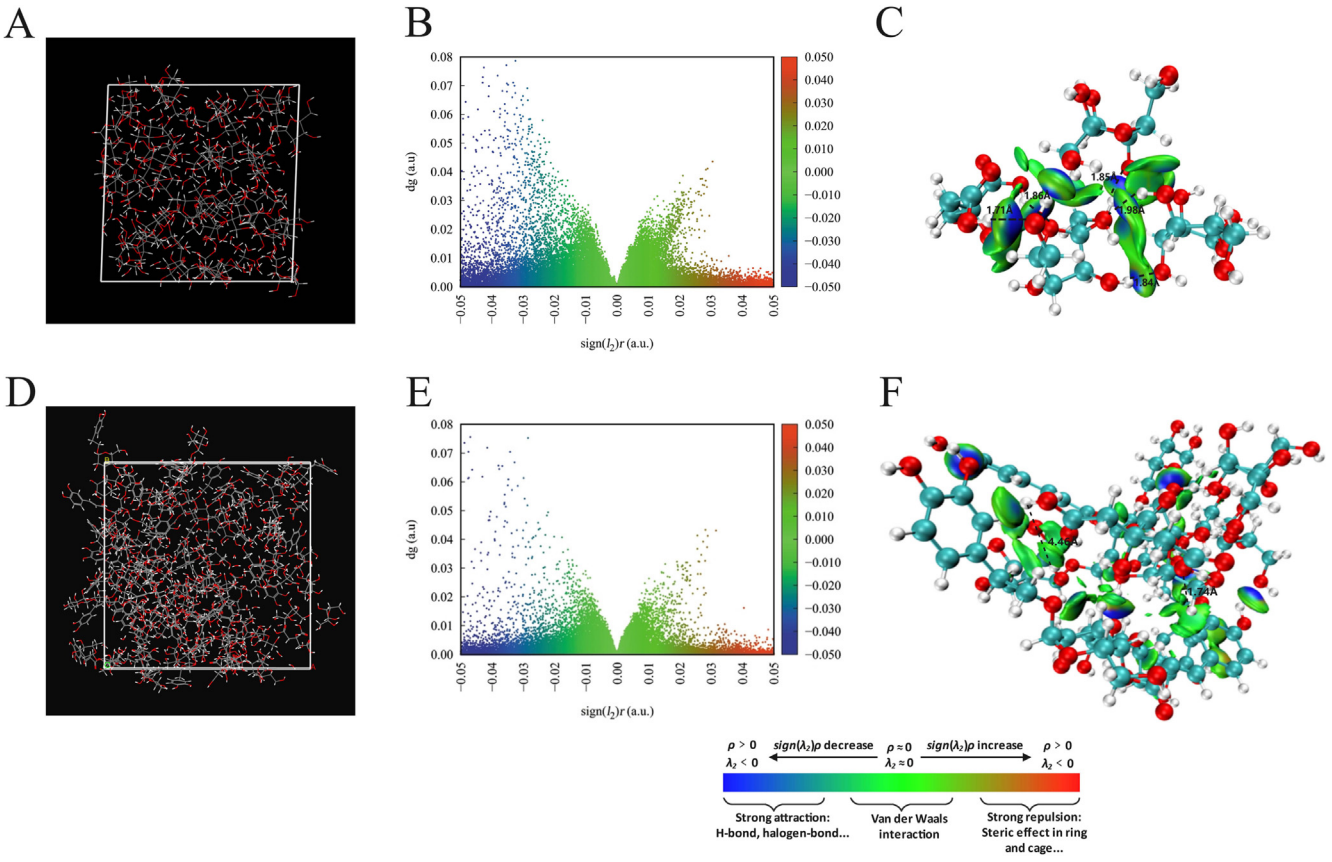


Fig. 7. The simulation box of system 1 (A); scatter diagram of the RDG vs *sign(l_2)ρ* (B) and the gradient isosurfaces (C, s = 0.5 au) for system 1; the simulation box of system 2 (D); scatter diagram of the RDG vs *sign(l_2)ρ* (E) and the gradient isosurfaces (F, s = 0.5 au) for system 2.

Table 6
Calculated energies of citric acid, fructose, glucose, echinacoside, acteoside, system 1 and system 2.

Molecules	*E* (Hartree)	△*E* (Hartree)	△*E* (KJ/mol)
Citric acid	−760.046	-	-
Fructose + fructose	−1374.285	-	-
Glucose	−687.133	-	-
Echinacoside	−2865.724	-	-
Acteoside	−2254.976	-	-
System 1*	−2821.711	−0.248	−650.378
System 2**	−5120.699	−0.664	−1743.332

* NADES: Citric acid, fructose and glucose (molar ratio, 1:2:1).
** NADES, echinacoside and acteoside.

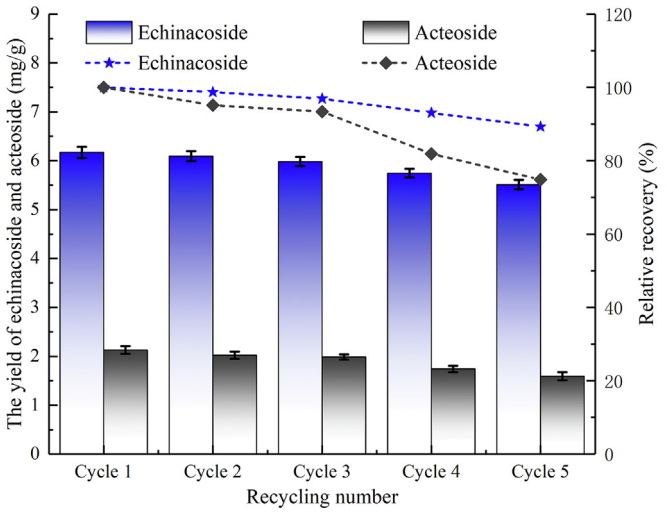


Fig. 8. Reusability of NADES.

the NADES sample recovered after five cycles still has a high extraction capacity. After the fifth cycle, the extracted amount of NADES for echinacoside and acteoside was only reduced by 0.66 and 0.53 mg/g, and the relative recoveries were maintained at 89.30 % and 74.83 % of the original NADES, respectively, indicating that the recovered NADES had satisfactory reusability performance. Therefore, the prepared NADES is highly recoverable and reusable, which can reduce the cost and facilitate industrial production.

Comparison of different extraction processes

The NADES-UAE technique was compared with three traditional techniques for the extraction of echinacoside and acteoside in *C. deserticola*, and the results are displayed in Fig. 9. The largest extraction yield of echinacoside and acteoside using NADES-UAE reached 6.15 and 2.10 mg/g, respectively, which were 1.54–2.86 times and 1.26–2.52 times that of Ethanol-UAE, Water-UAE and Water-HRE, respectively. And contrast with NADES-UAE, Ethanol-UAE needs to consume a large amount of ethanol, which increases the cost and safety hazards. NADES-UAE has the shortest extraction time (15 min), and the ingredients are green and natural, while Ethanol-UAE, Water-UAE and Water-HRE not only take a long time (60–120 min) but also have lower extraction yield for echinacoside and acteoside. Therefore, NADES-UAE is a particularly efficient for the extraction of natural bioactive components from plant material.

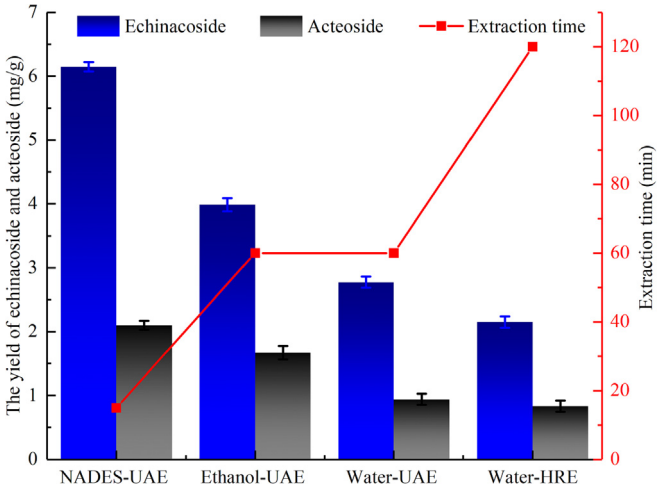


Fig. 9. The effect of different extraction methods on extraction yields of echinacoside and acteoside.

Conclusions

In this study, a total of 18 kinds of green and environmentally friendly binary and ternary NADES were prepared, and a new ternary NADES composed of citric acid, fructose and glucose in a molar ratio of 1:2:1 and 30 % water content was screened out. And the optimal extraction conditions were obtained when the yields of echinacoside and acteoside reached the highest. Three validation experiments were carried out, and the yields of echinacoside and acteoside were 6.16 and 2.13 mg/g, respectively. Then compared with other traditional extraction methods, NADES-UAE has higher extraction capacity and the shortest time. Finally, the interaction between NADES, echinacoside and acteoside was found to be mainly hydrogen bonding, van der Waals force and π-π stacking by molecular simulation. This work not only offers an environmentally friendly, efficient and green way for the extraction of echinacoside and acteoside from C. *deserticola*, but also has broad application prospects in the efficient separation of bioactive compounds from natural plants and the food or pharmaceutical industries.

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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiec.2022.11.033.

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