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Natural Deep Eutectic Solvents for the Extraction of Mulberroside A from White Mulberry Root Bark

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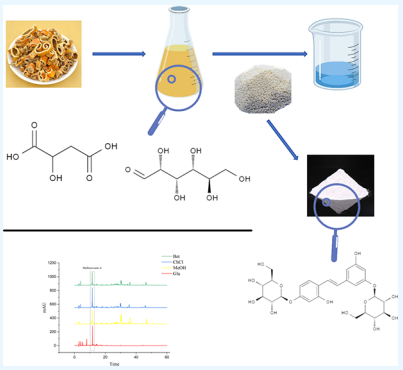
ABSTRACT: In this study, an ultrasonic extraction method of mulberroside A from *Morus alba* L. based on natural deep eutectic solvents (NADESs) was developed. A total of 24 natural deep eutectic solvents (NADESs) with different formulations were designed to extract *M. alba* L. powder, and high-performance liquid chromatography (HPLC) was used as the detection method, with the content of mulberroside A as the only evaluation index. The results showed that malic acid:glucose (1:3) contained the highest mulberroside A content of 0.06% with excellent extraction specificity. In addition, the parameters such as water content, extraction temperature, extraction time, and feed/liquid ratio, which have a great influence on NADESs, were optimized, and the antioxidant activity of NADES extracts was tested. Finally, further fine separation of mulberroside A by semidynamic adsorption on porous polymer beads yielded a refined product of mulberroside A under NADES extraction conditions with a purity of 82.9%.



1. INTRODUCTION

White mulberry root bark is the dried root bark of the *Morus alba* L. family Moraceae, also known as mulberry root white bark, mulberry root bark, mulberry bark, white mulberry bark, etc. It contains a variety of polysaccharides, flavones, alkaloids, and other natural active substances.[1,2] Mulberroside A is one of the important active substances, a stilbene derivative, which has the effects of cough suppressant, anti-inflammatory, analgesic, antioxidant, and hypolipidemic, and regulating metabolic enzymes and transporters.[3−5] Therefore, the extraction of mulberroside A from white mulberry root bark is of great significance for application in food and pharmaceutical fields. Currently, the extraction media for extracting mulberry root bark A from mulberry twigs and white mulberry root bark are mainly organic solvents such as methanol and ethanol, which have the problems of poor extraction specificity, low extraction efficiency, high input cost, and serious pollution. Therefore, it is important to find a new extraction medium for mulberroside A extraction.

Deep eutectic solvents (DES) are considered green solvents with adjustable physicochemical properties. On the one hand, DES is biodegradable, less toxic, and less costly; on the other hand, DES can be widely used as a "nontraditional medium" and "nontraditional solvent" for the extraction of phenolic acids,[6] alkaloids,[7] polysaccharides,[8] and other active ingredients. The extraction of active ingredients such as phenolic acids, alkaloids, and polysaccharides is highly efficient, green, and economical. Natural deep eutectic solvents (NADESs) are composed entirely of small-molecule metabolites such as choline derivatives, alcohols, sugars, and urea, which are safer and more environmentally friendly than traditional DES; they have been studied in the direction of the extraction of active ingredients in traditional Chinese medicines and the synergistic effect of enzymatic reactions.[9,10]

Ultrasonic extraction is a relatively new extraction method, which has a wider application in the extraction of NADESs as a solvent environment.[10,11] Since NADESs tend to have high viscosity, the traditional reflux extraction and leaching methods are less applicable. Ultrasonic extraction can improve the extraction efficiency by virtue of its cavitation effect, mechanical effect, and thermal effect, which makes NADESs have full contact with plant materials.

In the traditional extraction of plant active ingredients by NADESs, although better extraction results are often achieved, the high boiling point and high viscosity of NADESs lead to the problem of difficulty in adequately separating NADESs from the target substances in the subsequent separation and purification process. In previous studies on the extraction of plant active ingredients from NADESs, the NADES extracts were usually directly diluted with methanol and then directly analyzed using HPLC and other analytical methods for further analysis, which made it difficult to realize the reuse of NADESs.[10] Therefore, at present, there is not only a lack of research on the use of

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NADESs for mulberroside A extraction but also a lack of a technical method to separate NADESs from the extracted plant active ingredients.[11,12]

Therefore, in this study, NADESs were used as the extraction medium for the ultrasonic extraction of white mulberry root bark; then, the main substances were enriched and coarsely separated using dynamic adsorption with porous polymer resins, and in vitro antioxidant activity experiments were carried out on the mulberrosroside A-refined product. A complete method for the isolation of mulberroside A from white mulberry root bark using NADESs as the extraction medium and multiple separation methods in parallel was established.

2. MATERIALS AND METHODS

2.1. Plant Material. White mulberry root bark was stored in the laboratory of B213, Sichuan Industrial Institute of Antibiotics, Chengdu University, and was identified as the dried root bark of *Morus alba* L. by Associate Researcher He Zhengyou of Chengdu University. The raw material of *M. alba* L. was dried at 40 °C for 2 h to constant weight, crushed, and prepared for use.

2.2. Chemical Reagents and Instruments. Chemical reagents and instruments used for this study were methanol and acetonitrile (Chron Chemicals), D101 porous polymer beads (Tianjin Balance Biotechnology Co., Ltd., Product No. HG 2-885-76), Agilent 1260 Infinity II High Performance Liquid Chromatograph (1260 refractive index detector, 1260 diode array detector, Agilent OpenLab CDS software, GPC data analysis software); chromatographic column (Nanochrom UniSil 5-120 C18 Ultra 5 μm), rotary evaporator (BUCHI), and ultrasonic cleaner (KH5200DE, Hechuang Ultrasonic).

Mulberroside A standard (Chengdu Purui Technology Co., Ltd., purity: 98%) was also used. 2.29 mg of mulberroside A control was precisely weighed, dried to constant mass, put in a 10 mL volumetric flask, with methanol added to dilute to the scale, and prepared into a solution of mulberroside A control with a mass concentration of 0.229 mg/mL for spare.

All solutions were filtered through 0.45 μm nylon membranes before being used for HPLC.

2.3. HPLC Conditions. Chromatographic column: Nanochrom UniSil 5-120 C_{18} Ultra 5 μm;

Mobile phase: referring to the liquid phase conditions in ref 11, 0.1% aqueous phosphoric acid solution (A) − acetonitrile (B);

Gradient elution (0−5 min, 10% B; 5−10 min, 10−20% B; 10−25 min, 20−55% B; 25−60 min, 55−70% B);

Detection wavelength: 270 nm; column temperature: 30 °C; injection volume: 10 μL.

2.4. Extraction Procedures. *2.4.1. Natural Deep Eutectic Solvent Preparation.* NADESs are mainly composed of a series of natural metabolites from plants and animals. By heating in a water bath at 85 °C, the hydrogen bond acceptor (HBA) and the hydrogen bond donor (HBD) are bonded to each other by hydrogen bonding to form a low eutectic mixture.[7−9]

A certain proportion of HBA and HBD were mixed well and placed in a water bath at 85 °C with constant stirring, and distilled water was added several times to bring the water content to a preset proportion. After the whole NADES system was clarified and transparent, it was cooled to room temperature and set aside.[10]

2.4.2. Extraction with Different Solvents. A total of 24 combinations of NADESs were designed in this study, as shown in Table 1 (Table 1 shows the abbreviations of the DESs.).[11]

Table 1. NADES Formulation Design

serial number	HBA	HBD	molar ratio
NADESs-1	betaine	fructose	1:1
NADESs-2	betaine	fructose	1:2
NADESs-3	betaine	glucose	1:1
NADESs-4	betaine	glucose	1:2
NADESs-5	betaine	ethylene glycol	1:1
NADESs-6	betaine	ethylene glycol	1:2
NADESs-7	betaine	glycerin	1:1
NADESs-8	betaine	glycerin	1:2
NADESs-9	malic acid	fructose	1:1
NADESs-10	malic acid	fructose	1:2
NADESs-11	malic acid	glucose	1:1
NADESs-12	malic acid	glucose	1:2
NADESs-13	malic acid	ethylene glycol	1:1
NADESs-14	malic acid	ethylene glycol	1:2
NADESs-15	malic acid	glycerin	1:1
NADESs-16	malic acid	glycerin	1:2
NADESs-17	choline chloride	ethylene glycol	1:1
NADESs-18	choline chloride	ethylene glycol	1:2
NADESs-19	choline chloride	glycerin	1:1
NADESs-20	choline chloride	glycerin	1:2
NADESs-21	choline chloride	glucose	1:1
NADESs-22	choline chloride	glucose	1:2
NADESs-23	choline chloride	fructose	1:1
NADESs-24	choline chloride	fructose	1:2

Then, 10 g of crushed *M. alba* L. was weighed precisely and placed in a conical flask, and 50 g of NADESs was added and stirred thoroughly and then sealed using a parafilm; the conical flask was placed in an ultrasonic cleaner, and ultrasonic extraction was carried out at 40 °C and 200 W for 60 min. The crude extraction solution of *M. alba* L. was obtained.

2.4.3. Monofactor Analysis of NADESs. NADES water content (20, 25, 30, 35, 40, 45, 50, 55, 60, 70%, m/m); reaction temperature (15, 25, 35 °C); reaction time (20, 40, 60 min); and solid−liquid ratios (1:10, 1:20, 1:30).

2.5. Methodology Validation. *2.5.1. Linear Relationship.* The standard solution of mulberroside A from solution in Section 2.2 was used for gradient dilution and injected into the liquid chromatograph with the HPLC conditions in Section 2.3. The standard curve with concentration (milligrams per milliliter) as the horizontal coordinate (X) and peak area as the vertical coordinate (Y) was plotted.

2.5.2. Precision Test. 10 μL of the control solution was precisely absorbed, the sample was injected five times continuously under chromatographic conditions in Section 2.3, and the peak area was recorded.

2.5.3. Repeatability Test. Five white mulberry root bark herbs were taken, and five test solutions were prepared according to the method under Section 2.4.2. 10 μL was pipetted and analyzed under the chromatographic conditions of Section 2.3, and the peak area was recorded.

2.5.4. Sample Recovery Test. Three portions of white mulberry root bark with a known amount, 1.25 g each, were weighed; the control product was added according to 50, 100, and 150% of half of the sample amount; six test solutions were prepared according to the method under Section 2.4.2; and the peak area was recorded and analyzed under the conditions of HPLC in Section 2.3. The peak areas were recorded and analyzed under the HPLC conditions.

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2.6. Recovery of NADESs. The newly purchased porous polymer bead D101 was placed in a beaker and soaked in anhydrous ethanol for 12 h. The suspended inactivated porous polymer resin particles in the upper layer were discarded. The remaining porous polymer was loaded into a chromatographic column without a sand core, washed with 80% ethanol until no white turbid liquid was present, drenched with distilled water until the eluate was free of alcoholic odor, and set aside.

The NADES extract was evaporated using a rotary evaporator until its volume was constant. The extract was slowly and uniformly added into the porous polymer bead chromatography column, and the valve was opened until about 0.5 cm of the liquid level remained before it was closed. After standing for 30 min, the column was fully eluted using 10 times the column volume of distilled water and then about six times the column volume of 50% ethanol until the eluate became colorless.

Since the components of NADESs have good solubility in distilled water, most of the NADES components remained in the distilled water eluate. The distilled water eluate was spin-evaporated using a rotary evaporator to a constant volume to obtain the NADES regeneration solution, which was used for subsequent repetitive recovery and extraction experiments.

2.7. In Vitro Antioxidant Activities. Previously, mulberroside A has been reported in the literature to possess whitening and antioxidant activities,[13] but the antioxidant activity of mulberroside A with NADESs as extraction solvent has not been reported.

We evaluated the antioxidant activity of mulberroside A under NADES extraction solvent by using the scavenging rate of mulberroside A extracts against DPPH radicals, ABTS radicals, and hydroxyl radicals.

The hydroxyl radical scavenging activity of mulberroside A was determined using the method previously reported by Lin et al.[14] DPPH radical scavenging activity was performed by the method reported in Liu et al.[15] ABTS radical scavenging activity was performed by the method reported in Mfengwana PMAH.[16] Ascorbic acid (Vc) was used as the positive control at the same concentrations.

3. RESULTS AND DISCUSSION

3.1. Liquid Chromatogram of Mulberroside A Standard. The mulberroside A standard was configured as a 0.125 mg/mL solution according to the method in Section 2.2 and analyzed using HPLC via the liquid phase conditions in Section 2.3. The HPLC results are shown in Figure 1.

3.2. Screening of Natural Deep Eutectic Solvents. The component of DES has a significant influence on its physicochemical properties such as polarity, viscosity, and solubilization ability. It directly affects the extraction efficiency of the target compounds. Aiming to select a suitable DES, 24 kinds of DESs were selected in the study.

The extracts of each formulation were diluted 10 times with methanol and analyzed using HPLC. Among the results, the sets with a higher extraction efficiency are shown in Figure 2.

Compared with the other three groups, the malic acid-glucose group (NADESs-12) has the following advantages:

1. Higher biophilicity of the formulation: the constituents are natural organic acids of plant origin and glucose.
2. Easier removal of impurities: although the extraction rate was higher in the NADESs-22 group, the impurities were mainly concentrated in the 25−40 min peak, whereas in the NADESs-12 group, the impurities were concentrated

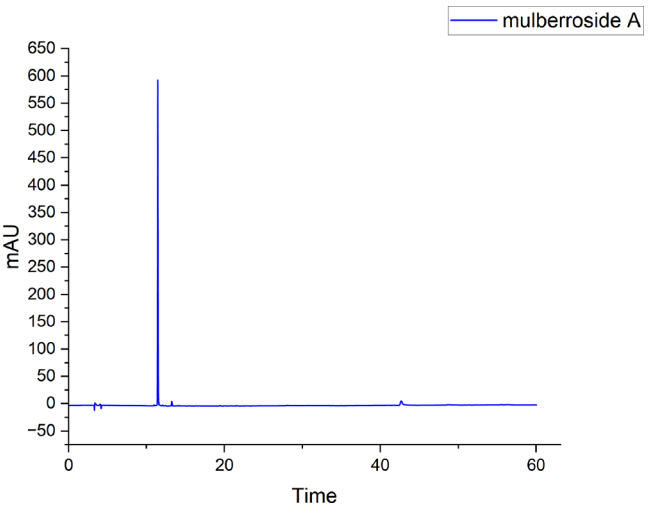

Figure 1. Original HPLC chromatograms of the standards (0.125 mg/mL).

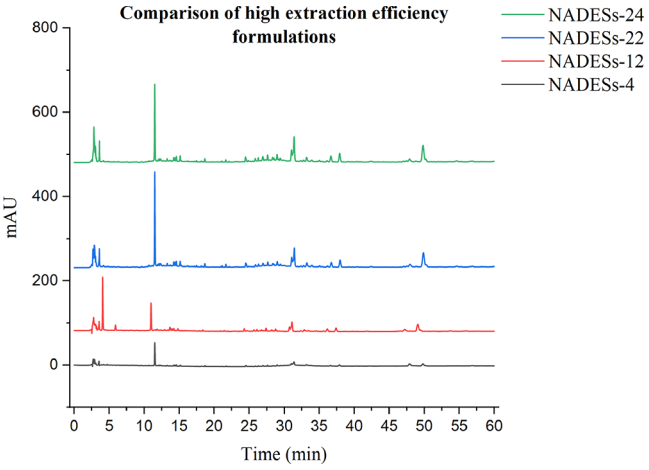

Figure 2. Extraction with different solvents.

Table 2. Peak Area Ratio of Mulberroside A at Different Water Ratios

serial number	water ratios	peak area
1	20%	3.6702
2	25%	3.4314
3	30%	5.2474
4	35%	8.6192
5	40%	10.1708
6	45%	10.1379
7	50%	10.6036
8	55%	11.5607
9	60%	17.1149
10	70%	16.1413

Table 3. Peak Areas of Mulberroside A in Different Reaction Temperatures

serial number	reaction temperatures (°C)	peak areas	proportion of peak area
1	15	156.69054	11.7197
2	25	69.70760	6.0984
3	35	126.12462	7.2220

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Table 4. Peak Areas of Mulberroside A in Different Solid−Liquid Ratios

serial number	solid−liquid ratios	peak areas
1	1:10	69.70760
2	1:20	64.72943
3	1:30	104.89952

Table 5. Peak Areas of Mulberroside A in Different Reaction Times

serial number	reaction times (min)	peak areas
1	20	89.10558
2	40	69.70760
3	60	147.29759

Table 6. Peak Areas of Mulberroside A Detected in the Eluents

eluents	peak areas
D101 water elution	93,325
D101 50% ethanol elution	109,539
DA201 water elution	142,480
DA201 50% ethanol elution	116,479
HPD-100 water elution	121,927
HPD-100 50% ethanol elution	93,002
AB-8 water elution	239,475
AB-8 50% ethanol elution	105,224

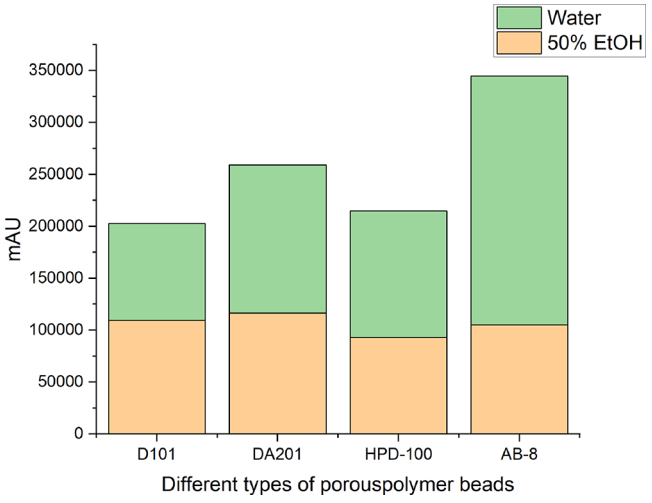

Figure 3. Schematic comparison of peak areas of eluents from various classes of porous polymer beads.

in the peak before 10 min. According to related papers,[13,17] the impurities before 10 min were plant organic acids such as chlorogenic acid, while the impurities peaked at 25−40 min were flavonoid derivatives such as mulberrin. Mulberrin is much more difficult to remove than organic acids during subsequent purification of the polymer porous microspheres.

In summary, we believe that NADESs-12 is more technically superior in our study.

3.3. Results of Monofactor Analysis of NADESs. *3.3.1. Effect of Different Water Contents on the Extraction Rate of Mulberroside A.* Water content is one of the important parameters affecting the extraction capacity and extraction efficiency of NADESs, and it plays an important role in

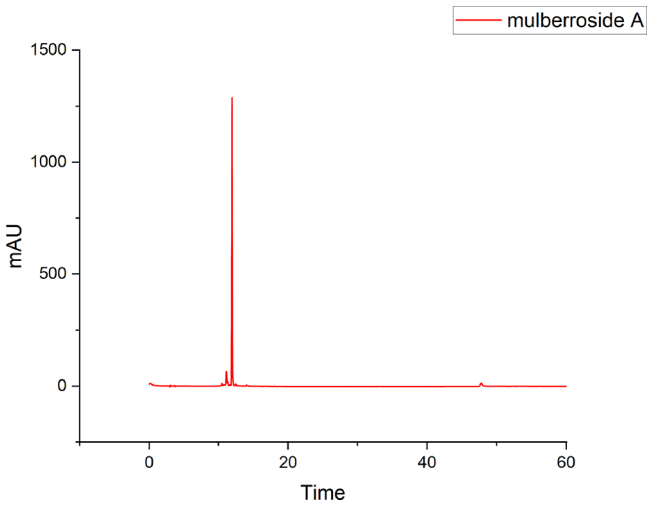

Figure 4. HPLC liquid chromatogram of the mulberroside A purified product.

regulating the viscosity and polarity of NADESs. Since the identified NADES formulations are highly susceptible to producing insoluble white solids at low water content, and it is difficult to form a stable and homogeneous solution system, the monofactor analysis of water content was based on 20% as the minimum ratio.

The effects of different water content ratio gradients (20, 25, 30, 35, 40, 45, 50, 55, 60, and 70%, m:m) on the extraction rate of mulberroside A were investigated by fixing the extraction temperature at 20 °C, the extraction time at 60 min, and the liquid−liquid ratio at 1:20 (m:m), and the results are shown in Table 2. The peak area ratio showed a uniform growth trend when the water content was 20−55%, and the growth trend increased significantly when the water content was 60% and then began to show a decreasing trend when the water content was 70%. Therefore, 60% was chosen as the optimum level of the water content factor.

3.3.2. Effect of Different Reaction Temperatures on the Extraction Rate of Mulberroside A. Water content of 30%, reaction time of 40 min, and material−liquid ratio of 1:10 were fixed to investigate the effect of different ultrasonic temperatures (15 °C, 25 °C, 35 °C) on the extraction of mulberroside A. The experimental results are shown in Table 3. The results showed that the content of mulberroside A was the highest at the ultrasonic temperature of 15 °C; therefore, 15 °C was selected as the optimal level of the ultrasonic temperature factor.

3.3.3. Effect of Different Solid−Liquid Ratios on the Extraction Rate of Mulberroside A. The effects of different solid−liquid ratios (1:10, 1:20, and 1:30) on the extraction of mulberroside A were investigated by fixing the water content of 30%, the reaction temperature of 25 °C, and the reaction time of 40 min, and the experimental results are shown in Table 4. The results showed that the content of mulberroside A was the highest when the solid−liquid ratio was 1:30, and therefore, 1:30 was chosen as the optimum level of solid−liquid ratio factor.

3.3.4. Effect of Different Reaction Times on the Extraction Rate of Mulberroside A. Water content of 30%, reaction temperature of 25 °C, and solid−liquid ratio of 1:10 were fixed, the effect of different sonication time (20, 40, and 60 min) on the extraction of mulberroside A was investigated, and the experimental results are shown in Table 5. The results showed that the content of mulberroside A in the extract continued to

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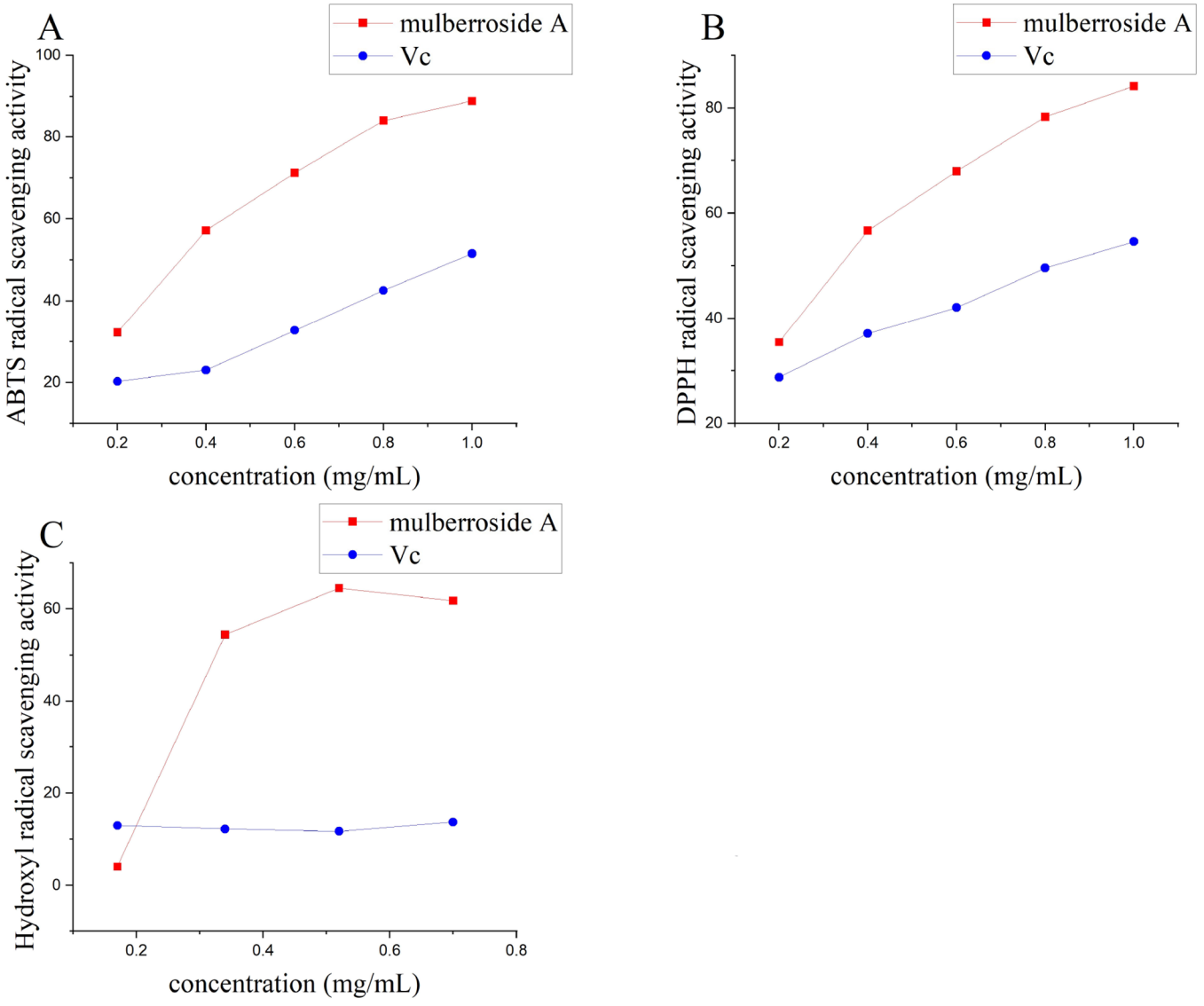

Figure 5. Antioxidant activities of mulberroside A. ABTS radical scavenging assay (A); DPPH radical scavenging assay (B); hydroxyl radical scavenging assay (C). Vc represents ascorbic acid.

increase with the increase of the reaction time and reached the maximum at 60 min. Therefore, 60 min was chosen as the optimum level of reaction time factor.

3.4. Methodology Validation Results. *3.4.1. Linear Relationship.* The standard curve was plotted with the concentration (mg/mL) as the horizontal coordinate (X) and the peak area as the vertical coordinate (Y): $Y = 9719.2X - 40.08$, $R^2 = 0.999$. It showed that the linearity was good.

3.4.2. Precision Test Results. After five injections and analysis, the RSD was 1.86%, indicating the good precision of the instrument.

3.4.3. Repeatability Test Results. After HPLC analysis of five test solutions, the contents were 5.71, 6.12, 6.19, 5.99, and 6.24 mg, with an RSD of 3.50%, which indicated that the method was reproducible.

It was also concluded that the average content of mulberroside A was 6.05 mg per 10 g of white mulberry root bark herb. The abundance of mulberroside A in white mulberry root bark was 0.06%.

3.4.4. Sample Recovery Test Results. After weighing 1.25 g of the sample, the amounts of mulberroside A standard added were 0.38, 0.76, and 1.13 mg. The recoveries of the three gradients were 106.23, 100.73, and 106.61%, respectively, with an RSD of 3.15%.

3.5. Initial Purification of Mulberroside A and Recovery of NADESs. Due to the high viscosity and high boiling point of NADESs, traditional separation methods (extraction, rotary evaporation, etc.) are not effective in separating them from the extracts. We found that column chromatography using a porous polymer bead can effectively separate NADESs from the active substances through our preliminary study.

3.5.1. Screening of Porous Polymer Beads. A total of four types of porous polymer resins, AB-8, D101, HPD-100, and DA201, were selected for the screening of porous polymer resins for the purification process.

Certain amounts of AB-8, D101, HPD-100, and DA201 porous polymer beads were weighed into a beaker and prepared for use after activation using the method in Section 2.5.

The activated AB-8, D101, HPD-100, and DA201 porous polymer resins were weighed and loaded onto the chromatography column, and a quantitative amount of NADESs white

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mulberry root bark extracts was taken on the sample, which was sequentially eluted with 10 times the amount of water and 50% ethanol. The two eluents were rotary-evaporated and dried separately and then reconstituted with 10 mL of methanol. The methanol resolution was diluted 10-fold and analyzed by HPLC according to the HPLC conditions in Section 2.3, and the results are shown in Table 6.

Comparing the aqueous and alcoholic elution groups of the major porous polymer resins, we found that the aqueous eluate of D101 macroporous resin contained the smallest percentage of mulberroside A, and the overall peak area was lower than that of the other three macroporous resins. This proved that D101 possessed a stronger adsorption and retention capacity for mulberroside A than AB-8, HPD-100, and DA201, was able to effectively adsorb and separate mulberroside A in the extracts of white mulberry root bark NADESs, and had a significant separation and purification effect in the purification process of mulberroside A extracts. The experimental results are shown in Figure 3.

3.5.2. HPLC Chromatogram of the Purified Product of Mulberroside A. The extract of white mulberry root bark NADESs was purified using D101 porous polymer beads to purify mulberroside A. The 50% ethanol eluate from Section 2.5 was spin-dried sufficiently to obtain the mulberroside A purified product.

A 20 mg portion of the mulberroside A purified product was taken and fully dissolved in a 10 mL volumetric flask to obtain a 2 mg/mL solution of mulberroside A purified product. It was analyzed under the HPLC conditions in Section 2.3, and the content of mulberroside A was 82.9%, as shown in Figure 4.

3.5.3. Recovery of NADESs. Most of the NADES components remained in the aqueous eluate, because of their good solubility in water.

The distilled water eluate in 2.5 mL was spun using a rotary evaporator until the volume was constant to obtain the NADES regeneration solution, which had almost the same extraction capacity of mulberroside A as the freshly formulated NADESs.

3.6. In Vitro Antioxidant Activity Assay Results. The experimental results are shown in Figure 5. From the results in Figure 5, it could be seen that the scavenging rate of free radicals by mulberroside A was increased with the increase of concentrations (0.2 to 1 mg/mL). Moreover, its scavenging capacity was higher than that of Vc in both DPPH and ABTS radical scavenging experiments, and its scavenging capacity was significantly higher than that of Vc in hydroxyl radical scavenging experiments in the high concentration range (Vc represents ascorbic acid).

4. CONCLUSIONS

In this experiment, a novel ultrasonic extraction method using NADESs as solvents was employed to extract mulberroside A from white mulberry root bark. The results showed that among the 24 designed solvent systems of NADESs, the malic acid−glucose group had the highest extraction efficiency of 0.06%. In addition, we found that the malic acid−glucose group possessed very high specificity for the extraction of mulberroside A under certain experimental conditions. After certain optimization, the optimal extraction conditions were 60% water content, reaction temperature 15 °C, reaction time 60 min, and solid−liquid ratio 1:30. On the other hand, we successfully separated NADESs from mulberroside A by porous polymer bead adsorption, and the preliminary refined mulberry piroside A content was 82.9%, which possessed a higher purity; the NADESs were reclaimed and recycled, which made the ultrasonic extraction of NADESs more economical, environmentally friendly, and green. These results indicate that the NADES ultrasonic extraction method is an efficient, environmentally friendly, and green method for extracting mulberroside A from white mulberry root bark; furthermore, the extracted mulberroside A possesses high free radical scavenging capacity and is an excellent natural antioxidant.

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Notes

The authors declare no competing financial interest.

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