A Novel Method to Extract Juglone from *Juglans mandshurica* Waste Branches Using a Water-in-Oil Microemulsion

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Abstract
Pruning of *Juglans mandshurica* produces many branches, which are potentially a rich source of juglone although they are usually discarded as waste. Herein, we describe a water-in-oil microemulsion-based microwave-assisted extraction (MBMAE) method to isolate the juglone found in waste branches from *J. mandshurica*. In our experiment, *J. mandshurica* waste branch powder was added to the MBMAE system. Using the optimized microemulsion system comprising Tween 80:n-propanol:n-hexane:water at 27:13.5:4.5:55 (w/w/w/w), and a pH of 5.6, at an operating temperature of 40 °C, operating time of 63 s, and operating power of 400 W, the juglone yield was 4.58 mg/g from a ratio of microemulsion to branch power of 20:1 (mL/g). The juglone extraction yield using the MBMAE method was 1.86- and 6.65-fold better than that obtained from microwave-assisted extraction by ethanol (Ethanol-MAE) and heat reflux extraction by ethanol (Ethanol-HRE), respectively. The MBMAE method may be useful as an alternative to traditional extraction methods to isolate juglone.

Graphic Abstract

Keywords *Juglans mandshurica* · Juglone · Waste branches · Microemulsion

Statement of Novelty
Many waste branches from *Juglans mandshurica* pruning are often discarded and hence, a rich source of juglone is lost. Traditional extraction methods for juglone are limited by their low efficiency, high cost and complicated operation. On
the basis of green technology, a special water-in-oil microemulsion-based microwave-assisted extraction (MBMAE) system for juglone was established for the first time. This work provides data support for applications of this method to the extraction of juglone from other materials or plants.

**Introduction**

*Juglans mandshurica*, a fast-growing deciduous broadleaf tree and a member of the *Juglandaceae* family, is widely distributed in the northeast of China, South America, North America, Oceania, Southeastern Europe and Eastern Asia [1–4]. As a precious tree species, *J. mandshurica* not only produces an excellent quality of wood, but also has multiple medicinal benefits [5–7]. Researchers have shown that the stem barks, leaves, roots and fruits of *J. mandshurica*, which contain polyphenols, naphthoquinones and tetralones [8], have obvious pharmacological activities that can be used to treat cancer, kidney issues and inflammation [9, 10].

In forestry production, pruning measures are often taken to ensure a reasonable distribution of nutrients, control pests and disease, protect soil and restrain excessive growth so as to maintain tree health [11]. Failure to properly prune trees will make them more susceptible to disease, which may reduce the wood quality or yield of active components [12, 13]. Therefore, pruning is an essential skill for tree growers. However, pruned branches are often discarded as waste even though they are usually rich in biologically active ingredients. Researchers found that the waste branches obtained from *J. mandshurica* pruning contain a certain amount of juglone [14, 15]. The structure of juglone (5-hydroxy-1,4-naphthoquinone) is shown in Fig. 1. Juglone has been suggested as a potential drug candidate for treating cancer, anemia, diabetes, and inflammation [16, 17]. Agricultural waste reutilization has been adopted as an alternative to improve sustainable technologies and resource utilization [18, 19]. Therefore, this research was undertaken to develop an efficient and novel method to obtain juglone from the waste branches of *J. mandshurica* to maximize resource utilization.

Methods such as dipping, decocting, Soxhlet extraction and supercritical CO₂ extraction have been used for juglone extraction [20–22]. However, these traditional extraction methods have limitations, such as low efficiency, complicated operation and consumption of large amount of solvents that may have some toxicity [23–25]. Therefore, it is necessary to develop a novel and efficient extraction method for juglone.

Microemulsion is a transparent or translucent stable liquid mixture system that is composed of oil, water, surfactant, co-surfactant and an electrolyte [26–28]. Compared with conventional extractants, a microemulsion system has several advantages, such as its nanometer scale, large specific surface area, high selectivity and rapid coalescence [29, 30]. On the basis of the benefits of microemulsion extraction (ME), an increasing number of researchers have applied it to the fields of food, medicine, sewage treatment, heavy metal recovery and protein extraction [31–34].

Considering the advantages of ME, a water-in-oil microemulsion was proposed for the first time and thus, a novel and efficient microemulsion-based microwave-assisted extraction (MBMAE) method was developed to obtain juglone from *J. mandshurica* waste branches. This method is in alignment with the full use of waste resources to ensure practices of green development are followed [35–37]. Moreover, response surface analysis (RSM) based on Box–Behnken design (BBD) was applied to optimize the MBMAE of juglone.

**Fig. 1** HPLC chromatograms of juglone in *J. mandshurica* waste branches (a) and standard (b)
**Materials and Methods**

**Materials and Reagents**

Discarded branches of *J. mandshurica* were picked from the Forest Experiment Station, Maoer Mountain, Harbin City, Heilongjiang Province, China. The branches of *J. mandshurica* were placed into a 40 °C dryer and then they were smashed into a powder (60 mesh).

Juglone standard (98%) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Other reagents were from Yuanye Chemical Reagent Co. (Shanghai, China).

**Determination of Juglone**

The juglone content was determined by high performance liquid chromatography (HPLC) (1206 Infinity, Agilent Technology Co., Ltd.). A Diamonsil C18 column (250 nm × 4.6 mm, 5 μm, Dikma, Beijing, China) was used for HPLC analysis. The juglone was isocratic eluted over 0–30 min using 50% of a 0.2% phosphoric acid aqueous solution (A) and 50% methanol (B). The flow rate and injection volume were 1.0 mL/min and 20 μL, respectively. The detection wavelength and column temperature were 250 nm and 30 °C, respectively. The HPLC chromatogram of juglone is shown in Fig. 1. The peak of juglone in the HPLC chromatograms was determined by comparing the chromatograms of the waste branch extract of *J. mandshurica* with a standard of juglone. By plotting the concentration of the juglone standard on the abscissa and the average value of the chromatographic peak area on the ordinate, a linear regression equation and determination coefficient were generated (y = 100599x – 133.58, R² = 0.9990).

**Establishment of the Microemulsion System**

To determine the solubility of juglone in different mixtures, suitable components for the juglone microemulsion extraction system were evaluated. A total of 0.5 g powder, 10 mL of the oil phase (walnut oil, olive oil, isoctane, tea oil or n-hexane) and co-surfactant (n-butanol, n-pentanol, PEG-400, propanol or Transcutol HP) were placed into a centrifuge tube and subjected to ultrasonication until the mixture was fully dissolved. Then the mixture was shaken for 48 h to reach dissolution equilibrium, centrifuged, and the supernatant was passed through a 0.45 μm organic filter membrane prior to HPLC analysis. Then, 1.0 g of a surfactant (CTMAB, P204, SDSBS, AOT, tea saponin or Tween 80) was placed into the sieved oil phase and co-surfactant mixture and the volume of liquid consumed for the complete dissolution was recorded. A guideline for the assessment of the solubility of the surfactants is shown in Table 1. Thus, surfactants with greater solubility in the co-solvent and oil phase were obtained by sieving.

Mixed surfactants comprised surfactants and co-surfactants in a certain ratio (w/w). After preparing the mixed surfactant, it was added to the oil solvent and then water was added until the mixture was clear and transparent. To judge whether the mixed solution was a microemulsion system, it was evaluated for its ability to scatter light (determination of the Tyndall phenomenon). The particle size of the microemulsion system was determined using a Zeta analyzer (Brook Haven Instruments Corporation, Holtsville, New York State, USA).

To verify the type of optimal microemulsion system obtained, 400 μL of methylene blue and Sudan red were added separately into the microemulsion system and the diffusion rate of each was measured.

**Extraction Procedure**

To determine if the MBMAE method was better for the extraction of juglone from the branches of *J. mandshurica* compared with traditional methods, the extraction of juglone using the MBMAE method, microwave-assisted ethanol extraction (Ethanol-MAE) and traditional ethanol heat reflux extraction (Ethanol-HRE) were compared (Table 2).

**MBMAE**

A total of 2.0 g of *J. mandshurica* waste branch powder was added to a 100 mL conical flask. Next 40 mL of microemulsion was added to the conical flask at a certain pH. Then the microemulsion and sample mixture were extracted using a microwave under the conditions shown in Table 2. The solutions were filtered and collected, centrifuged at 12,298×g for 10 min, and the supernatant was passed through a 0.45 μm organic filter membrane for HPLC analysis.

| Table 1 | Judgment of solubility type |
|---------|----------------------------|
| Type of solubility | Solute weight (g) | Solvent volume (mL) |
| Extremely soluble | 1.0 | <1 |
| Easily soluble | 1.0 | 1–10 |
| Dissolution | 1.0 | 10–30 |
| Slightly soluble | 1.0 | 30–1000 |
| Very slightly soluble | 1.0 | 1000–10,000 |
| Almost insoluble or insoluble | 1.0 | ≥10,000 |
MAE was completed according to the above method described for MBMAE.

HRE

A total of 2.0 g of *J. mandshurica* branch powder was added to 40 mL of ethanol and refluxed at 60 °C for 2.5 h to carry out HRE. The solutions were filtered and collected, centrifuged at 12,298 × g for 10 min and the supernatant was passed through a 0.45 μm organic filter membrane for HPLC analysis.

**Table 3** Factors and levels of response surface

| Symbol | Independent variables       | Levels |  |
|--------|----------------------------|--------|---|
| X₁     | Microemulsion pH           | 5      | 5.5 | 6 |
| X₂     | Extraction temperature (°C)| 30     | 40  | 50|
| X₃     | Microwave power (W)        | 300    | 400 | 500|
| X₄     | Extraction time (s)        | 50     | 60  | 70|

**MBMAE optimization**

**Single-Factor Experimental Design**

The liquid–solid ratio, microwave power, microemulsion pH, extraction temperature and extraction time were chosen as single-factor parameters from which an initial range of extraction parameters were acquired.

**Experimental Design of Response Surface Analysis**

The relationship between the independent variable and dependent variable was analyzed by RSM. Based on the single factor experiment, four factors were selected to perform a three-level BBD (Table 3) from which the interaction of each factor on juglone yield could be determined (Table 4).

**UV–Vis Analysis of Juglone After Extraction**

According to the literature [38], a pretreated D101 macroporous resin was used to separate the juglone in the extract. The concentration of the diluted juglone extract was 0.2 mg/mL. The diluted juglone extract and D101 macroporous resin were added into an Erlenmeyer flask at 20:4 (mL/g), and the pH was adjusted to 2. Then the Erlenmeyer flask was shaken at 150 r/min at room temperature for 6 h. After the adsorption was completed, an aliquot of 95% acetone was added to the Erlenmeyer flask at a ratio of acetone to resin of 20:4 (mL/g). The desorption was carried out on a shaker at 150 r/min for 6 h at room temperature and a pH of 4. The desorbed solution was rotary evaporated to dryness and redissolved with methanol for UV analysis.

The juglone standard was dissolved in methanol. The standard and extracted samples were analyzed by full-wavelength scanning in the range of 200–500 nm using a UV–vis spectrophotometer (UV-2600; Shimadzu Corporation, Kyoto, Japan).

**FTIR Analysis of Juglone After Extraction**

The pretreatment method for the infrared samples was the same as that of the ultraviolet samples. After the sample was pretreated, it was rotary evaporated to dryness. Subsequently, the powder was subjected to KBr tableting and the FTIR measurement was performed using an infrared spectrometer (IRAffinity-1, Shimadzu Corporation).

**Statistical Analysis**

All experiments were repeated three times. The results are expressed as the mean ± SD (n = 3). The juglone yield was calculated as follows:

$$Y = \frac{k \times C \times V}{M}$$

where Y is the juglone yield (mg/g), k is the dilution factor, C is the juglone concentration (mg/mL), V is the solvent volume (mL) and M is the mass of the *J. mandshurica* branch powder (g).

**Results and Discussion**

**Preparation and Determination of the Microemulsion Prescription**

**Selection of the Oil Phase, Surfactant and Co-surfactant**

The formation of a microemulsion requires that the oil phase molecules and the interface membrane molecules maintain
proper permeability and contact, and the molecular weight of the oil phase should not be too large [39]. The ability of the oil phase to form a microemulsion is related to the molecular size of the oil phase. Generally, microemulsions are easy to form in small molecular oil phases, while it is not easy for microemulsions to form in macromolecular oil phases [40]. Therefore, all the oils selected for this study, walnut oil, olive oil, isooctane, tea oil, n-hexane and castor oil, have short molecular chains and are commonly used in medicinal microemulsions. Additionally, these oils are non-toxic and non-irritating to the body. The oil phase of the microemulsion was selected on the basis of the solubility of juglone in the oil phase. As shown in Fig. 2a, the solubility of the juglone extract in n-hexane, castor oil and isooctane was relatively high. Therefore, n-hexane, castor oil and isooctane were selected as the oil phase for the subsequent screening of the microemulsion system components.

Co-surfactants may increase the solubility of surfactants, reduce the surface tension of the interfacial film of the microemulsion system and increase its fluidity, therefore promoting its formation [41]. Commonly used co-surfactants include lower alcohols, organic amines and mono- and di-alkyl glycerides [42]. In this experiment, n-butanol, n-pentanol, PEG-400, n-propanol and Transcutol HP was selected as co-surfactants. As shown in Fig. 2b, juglone was more soluble in n-propanol and n-butanol than the other tested co-surfactants. Therefore, n-pentanol, n-propanol and n-butanol were selected as the co-surfactants to test in the microemulsion system.

Surfactants, known as amphiphiles, are the main component of the microemulsion that functions to solubilize the target compound. In our experiment, the surfactants most commonly used in pharmaceuticals, CTMAB, P204, SDBS, AOT, tea saponin and Tween 80, were selected for preliminary screening. Since the selected surfactant
contains both solid and liquid, the solubility of the surfactant in the selected oil phase and co-surfactant was used as an index to determine the surfactant solution. As shown in Tables 1 and 5, Tween 80, OTAC and P204 were extremely soluble or easily soluble in the selected oil phase and co-surfactant. Therefore, it was determined that Tween 80, OTAC and P204 were the most suitable surfactants for this system.

Screening of the Microemulsion System

The oil phase, co-surfactant and surfactant were tested for emulsion formation. Since castor oil, \( n \)-pentanol, OTAC and P204 cannot form an emulsion, four emulsification systems were finally obtained, namely Tween 80-\( n \)-hexane-\( n \)-propanol, Tween 80-\( n \)-hexane-\( n \)-butanol, Tween 80-iso-octane-\( n \)-propanol and Tween 80-iso-octane-\( n \)-butanol. The particle size analysis and Tyndall effect analysis were performed on the above four systems. As shown in Fig. 3, except for the Tween 80-iso-octane-\( n \)-propanol system, the other three systems all satisfied the microemulsion particle size requirements of 10–100 nm and all four systems produced the Tyndall effect (Fig. 3). Therefore, three suitable types of microemulsion systems were obtained. Additionally, by drawing a pseudo-ternary phase diagram and calculating the microemulsion area, it was further determined that the microemulsion area formed by the Tween 80-\( n \)-hexane-\( n \)-propanol system was the largest (Fig. 4a). Therefore, the Tween 80-\( n \)-hexane-\( n \)-propanol microemulsion system was selected for further characterization.

| Table 5 Solubility of surfactant in co-surfactant and oil phase |
|----------------|---------------|----------------|---------------|----------------|---------------|---------------|
|                | CTMAB (mL)    | SDBS (mL)     | Tea saponin (mL) | OTAC (mL) | Tween 80 (mL) | P204 (mL) |
| \( n \)-Propanol | 432           | 754           | 884           | 1           | 0.8           | 1            |
| \( n \)-Butanol  | 317           | 835           | 676           | 3           | 0.5           | 1            |
| \( n \)-Pentanol | 206           | 668           | 832           | 3           | 0.5           | 0.6          |
| \( n \)-Hexane   | 472           | 741           | 851           | 5           | 1             | 0.6          |
| Isooctane       | 561           | 732           | 915           | 5           | 1             | 0.6          |
| Castor oil      | 360           | 575           | 743           | 6           | 1             | 1            |
Screening of the Distribution Ratio of Different Components in the Microemulsion

The proportions of the individual components of the Tween 80-n-hexane-n-propanol microemulsion system were adjusted to obtain a higher juglone yield. As shown in Table 5, when the proportion of Tween 80:n-propanol:n-hexane:water was 27:13.5:4.5:55 (w/w/w/w), the juglone yield was the highest at 3.28 mg/g (Table 6). Therefore, the juglone-specific microemulsion system comprised Tween 80:n-propanol:n-hexane:water at a ratio of 27:13.5:4.5:55 (w/w/w/w). The structure of each component in the microemulsion is presented in Fig. 5.

Determination of Microemulsion Type

The diffusion rate of Sudan red and methylene blue dye in the juglone-specific microemulsion system was compared to determine the type of the newly developed juglone microemulsion system. As shown in Fig. 6, the time for the Sudan red to diffuse through the full bottle was shorter than that for methylene blue. This indicated that the juglone-specific microemulsion system was a water-in-oil (W/O) type, which was consistent with reports of fat-soluble components being suitable for extraction by a W/O type of microemulsion [43].

Extraction Mechanism

Tween 80 is nonionic surfactant with a high surface activity, strong solubilization, low toxicity and hemolysis, and a broad pH range [44]. As shown in Fig. 7, it contains one ester group, three hydroxyl groups and five ether groups. During extraction, the oxygen atoms on the ester group, ether group and hydroxyl groups in the Tween 80 form hydrogen bonds with the phenolic hydroxyl groups in the juglone to achieve a strong hydrogen bond extraction effect. Additionally, the oxygen atoms contained in the Tween 80 and juglone have different electronegativities because of differences in their connecting groups. Therefore, the empty orbitals of the oxygen atoms in the ester,
hydroxyl and ether groups in Tween 80 and the lone pair of electrons in the oxygen atom in the hydroxyl group of juglone can form a coordination bond, which plays a critical role in coordination extraction. As a special covalent bond, the coordination bond extends the conjugated chain of the juglone molecule by forming a coordination bond.

**Table 6** The effect of microemulsion distribution ratio on juglone yield

| Run | Tween 80 (%) | n-Propanol (%) | n-Hexane (%) | Water (%) | Juglone yield (mg/g) |
|-----|--------------|----------------|--------------|-----------|---------------------|
| 1   | 27.0         | 13.5           | 4.5          | 55.0      | 3.28                |
| 2   | 22.1         | 11.0           | 8.3          | 58.6      | 3.07                |
| 3   | 18.3         | 9.2            | 11.8         | 60.7      | 3.01                |
| 4   | 14.7         | 7.4            | 14.7         | 63.2      | 2.65                |
| 5   | 10.8         | 5.4            | 16.3         | 67.5      | 2.23                |
| 6   | 8.0          | 4.0            | 17.9         | 70.1      | 2.11                |
| 7   | 2.7          | 5.5            | 19.0         | 72.8      | 2.02                |
| 8   | 3.3          | 1.6            | 19.4         | 75.7      | 1.93                |
| 9   | 1.5          | 0.7            | 19.9         | 77.9      | 1.78                |
bond between the O atom in the carbonyl group of juglone and the O atom of the –O– in the Tween 80 ester group, or between the –O of the ionized juglone anion and the O atom in the C=O of the Tween 80 ester group. This results in the rearrangement of the electron cloud between juglone and Tween 80, which forms an electron-deficient π,π-conjugated system. The formation of the conjugate system improves the stability of the juglone and Tween 80 combination. Additionally, the H+ from the dissociation of juglone could also coordinate with the oxygen atom in Tween 80 to make Tween 80 positively charged, which could then form an electrostatic interaction with the dissociated juglone anion to complete the extraction process. The chemical effect of Tween 80 extracting juglone is theoretically the result of coordination, hydrogen bonding, and electrostatic and conjugate interactions.

The co-surfactant can adjust the polarity of the microemulsion system by adjusting the surface activity of the surfactant. n-Propanol was used as the co-surfactant as it contains a polar hydroxyl group that is soluble in water, and a hydrocarbon chain that is soluble in oil, and thus, n-propanol can fill in the cavities between the aligned Tween 80 molecules. On the one hand, the insertion of n-propanol can adjust the distribution and arrangement of Tween 80 in the oil–water interface film, thus increasing its strength and flexibility, reducing interfacial tension, increasing the stability of the system, and improving the solubilizing ability of the microemulsion to water. On the other hand, the insertion of n-propanol can also weaken the forces among the Tween 80 molecules, increase the solubility of Tween 80 in solvents, and make the reverse micelle system more stable, in turn improving the extraction effect of the microemulsion system on the target component [45].

The oil phase is one of the most important components of the self-microemulsion system. n-Hexane was selected as the oil phase as not only can it dissolve a large amount of juglone, but also it promotes self-microemulsification. Its synergistic effect with the water phase can maximize the solubilization of juglone into the microemulsion system and maximize its extraction efficiency [46].

**Single Factor Experiments**

To define the experimental domain for each factor and establish a control method, some factors that may have a greater impact on the results were explored [47]. Each factor was investigated while other parameters were set at constant values [48, 49]. Five main influencing factors, the microemulsion-powder ratio (10–30 mL/g), experimental temperature (30–50 °C), experimental time (50–70 s), microwave power (300–500 W) and microemulsion pH (5–6) were studied, and the results are summarized below.

**Effect of the Liquid–Solid Ratio**

The number of “pools” contained in the microemulsion determines its extraction capacity for juglone. The extraction saturated easily because of an insufficient amount of...
extractant. As shown in Fig. 8a, the juglone yield was improved as the liquid–solid ratio increased from 10 to 20 mL/g. When the liquid–solid ratio reached 20:1, the juglone was completely extracted, and any further increase in the liquid–solid ratio did not result in any further improvement in the juglone yield. Therefore, in terms of the yield and cost, the liquid–solid ratio of 20:1 was the most suitable for further optimization experiments.

**Effects of Microwave Power**

The extraction power was varied from 300 to 700 W to determine how it impacted the juglone yield. The juglone yield was the highest at 400 W, and when the power was further increased, the yield of juglone decreased (Fig. 8b). This phenomenon may be related to the fact that the electromagnetic field generated by microwave extraction increased with the increase of power. With the increase of power, the gradually increasing magnetic field promoted the acceleration of electron migration in the extract. When the power was too strong, the dissociation of juglone molecules in the microemulsion system was accelerated under the induced magnetic field. The charged ions in the microemulsion were more concentrated at the end of the gel core, forming an electrostatic shielding field, which reduced the binding ability of the dissociated juglone ions with the Tween 80. Thus,
Fig. 8 Effects of different extraction parameters on the yield of juglone. (a) liquid–solid ratio; (b) extraction power; (c) microemulsion pH; (d) extraction temperature; (e) extraction time)
the juglone yield was reduced under the above conditions. Therefore, taking environmental protection and yield into account, 400 W was selected as the power condition for subsequent experiments.

The Effect of Different Microemulsion pHs

The pH of the microemulsion system is another important factor affecting the extraction results. Because a phenolic hydroxyl group is present in juglone, juglone was stable in the relatively acidic microemulsion solution. The effect of the microemulsion solution at different pHs on the juglone extraction yield was investigated from pH 4.0 to 6.0. The microemulsion pH was adjusted with 1.0 mol/L hydrochloric acid. As shown in Fig. 8c, pH 5.5 was the most suitable pH for extracting juglone. The pH mainly affects the extraction rate by influencing the electrostatic interactions. When the pH was too low, although it was beneficial for Tween 80 to form positively charged ions, excessive H⁺ may interact with the negatively charged polar head on the surface of the reverse micelle gel core to produce a shielding effect. This environment resulted in negatively charged juglone ions that could not be extracted into the “pool” and the extraction rate was decreased. Furthermore, excessive H⁺ inhibited the dissociation of juglone and reduced the number of negative ions produced by juglone ionization, therefore weakening the electrostatic extraction effect with Tween 80. Under high pH conditions, the amount of H⁺ coordinated with Tween 80 will also be decreased, which would reduce the positive charge of Tween 80 and decrease the electrostatic effect. Therefore, pH 5.5 was regarded as the most suitable pH for subsequent research.

Effect of Temperature

The microwave temperature is a crucial parameter that influences the juglone yield. As shown in Fig. 8d, the juglone yield gradually increased as the temperature increased from 20 to 40 °C, and then the yield decreased as the temperature further increased. This phenomenon likely resulted from the fact that at low temperature, the movement of juglone was slowed such that it was not well dispersed in the microemulsion, which limited its interaction with Tween 80. When the temperature was higher than 40 °C, the juglone molecules began to decompose, which reduced the extraction rate. Taking energy consumption and production efficiency into consideration, 40 °C was chosen as the optimum temperature for juglone extraction.

Effect of Time

To evaluate the effect of microwave time on the juglone yield, microwave times from 40 to 80 s were investigated. As shown in Fig. 8e, the juglone yield increased as the microwave time increased from 40 to 60 s. At 60 s, the juglone yield reached a maximum and then decreased with a further increase of microwave time. This result may be attributed to the fact that as the extraction time increased, the contact time between juglone and the microemulsion increased, and the yield also increased. However, when the time exceeded 60 s, the temperature of the microemulsion increased, and the stability of juglone was decreased, which led to a decrease in yield. Therefore, an extraction time of 60 s was chosen for the subsequent experiments.

Response Surface Analysis of Juglone Yield

On the basis of the initial experiments, microemulsion pH, microwave power, experimental temperature and experimental time were closely related to juglone yield. Considering the results of the single factor experiments, these parameters were further investigated and then optimized by using RSM. The interaction between the four parameters that had the greatest impact on juglone yield was tested using BBD. A quadratic regression model was established to relate the response to the variables as described by Eq. 2. The arrangement and results are shown in Table 4.

\[
Y = 4.61 + 0.11X_1 - 0.0333X_2 - 0.02X_3 + 0.13X_4 + 0.125X_1X_2 + 0.035X_1X_3 + 0.16X_2X_4 - 0.1X_3X_4 - 0.145X_4^2 - 0.145X_3X_4 - 0.628X_1^2 - 0.463X_2^2 - 0.418X_3^2 - 0.35X_4^2
\]

(2)

Among them, Y was the juglone yield (mg/g), X₁, X₂, X₃, and X₄ were the microemulsion pH, extraction temperature (°C), microwave power (W) and extraction time (s), respectively.

From the analysis of the model, the p value was < 0.0001 and the F value was 19.44, which indicated that the model was extremely statistically significant. The value of the lack-of-fit term, 0.0826, was greater than 0.05, which indicated that it was not significant and further confirmed that the model fit the data well and the test error was small. The value for \( R^2 \) was 0.9511, which proved that the equation could fit 95.11% of the results for the juglone yield, that is, 95.11% of the change in juglone yield came from the selected variables. In summary, this model can be used to analyze and predict juglone yield. From the F value in Table 7, the order of the influence of the various factors on juglone yield was experimental time (X₄) > microemulsion pH (X₁) > experimental temperature (X₂) > experimental power (X₃).

The 3D models of the influence of the various factors in the MBMAE on juglone yield are shown in Fig. 9. We found that the output of juglone was positively correlated with the influencing factors, which included microemulsion pH, extraction temperature, extraction time and microwave...
### Table 7 Results of ANOVA

| Variables  | Sum of squares | Degrees of freedom | Mean square | F-value | p-value | Significant<sup>b</sup> |
|------------|----------------|-------------------|-------------|---------|---------|--------------------------|
| Model      | 4.66           | 14                | 0.33        | 19.44   | <0.0001 | **                       |
| $X_1$      | $1.45 \times 10^{-2}$ | 1               | 0.15        | 8.48    | 0.0114  |                        |
| $X_2$      | $1.33 \times 10^{-2}$ | 1               | 1.33        | 0.78    | 0.3925  | ns                       |
| $X_3$      | $0.48 \times 10^{-2}$ | 1               | 0.48        | 0.28    | 0.6048  | ns                       |
| $X_4$      | 2.03           | 1                 | 0.20        | 11.84   | 0.0040  | **                       |
| $X_1X_2$   | $6.25 \times 10^{-2}$ | 1               | 6.25        | 3.65    | 0.0768  | ns                       |
| $X_1X_3$   | $0.49 \times 10^{-2}$ | 1               | 0.49        | 0.29    | 0.6011  | ns                       |
| $X_1X_4$   | 1.02           | 1                 | 0.10        | 5.98    | 0.0283  |                        |
| $X_2X_3$   | 0.04           | 1                 | 0.04        | 2.34    | 0.1487  | ns                       |
| $X_2X_4$   | $8.41 \times 10^{-2}$ | 1               | 8.41        | 4.91    | 0.0438  |                        |
| $X_3X_4$   | $8.41 \times 10^{-2}$ | 1               | 8.41        | 4.91    | 0.0438  |                        |
| $X_1^2$    | 2.56           | 1                 | 2.56        | 149.36  | <0.0001 | **                       |
| $X_2^2$    | 1.39           | 1                 | 1.39        | 81.18   | <0.0001 | **                       |
| $X_3^2$    | 1.13           | 1                 | 1.13        | 66.17   | <0.0001 | **                       |
| $X_4^2$    | 0.81           | 1                 | 0.81        | 47.19   | <0.0001 | **                       |
| Residual   | 0.24           | 1.71              | 2.2          | 4.41    | 0.0826  | ns                       |
| Lack of fit| 0.22           | 10                | 2.2          | 4.41    | 0.0826  | ns                       |
| Pure error | $1.99 \times 10^{-2}$ | 4               | 0.5         | 4.41    | 0.0826  | ns                       |
| Cor total  | 4.90           | 28                | R$^2=0.9511$ | 28 C.V. % = 3.41 |

<sup>a</sup>X<sub>1</sub>: microemulsion PH;  X<sub>2</sub>: extraction temperature (°C);  X<sub>3</sub>: microwave power (W);  X<sub>4</sub>: extraction time (s)  
<sup>b</sup>* means significant, p<0.05;  ** means highly significant, p<0.01;  ns means not significant

### Fig. 9 RSM for juglone yield in branches of *J. mandshurica*.  
**a** Varying microemulsion pH and extraction temperature (°C).  
**b** Varying microemulsion pH and extraction power (W).  
**c** Varying microemulsion pH and extraction time (s).  
**d** Extraction temperature (°C) and extraction power (W).  
**e** Varying extraction temperature (°C) and extraction time.  
**f** Varying extraction power and extraction time.
power. The most suitable conditions optimized by BBD comprised a microemulsion pH of 5.62, an experimental time of 63.24 s, an experimental temperature of 40.81 °C and an experimental power of 378.58 W. The maximum juglone yield from the prediction model was 4.60 mg/g. After taking into account the limitations associated with the actual conditions, the experiment was performed using a microemulsion pH of 5.60, an operating time of 63 s, an operating temperature of 40 °C and an extraction power of 400 W for three cycles. The actual output of juglone was 4.58 mg/g on average, which was in accordance with the predicted value. This correlation showed that the extraction process of juglone optimized by BBD was stable and reliable.

Comparison of Different Extraction Methods

The juglone yields obtained using MBMAE, Ethanol-MAE and Ethanol-HRE were compared (Table 4). The highest juglone yield from the MBMAE method was 4.58 mg/g, which was 1.86-fold and 6.65-fold higher than that obtained from Ethanol-MAE and Ethanol-HRE, respectively. Therefore, MBMAE is an alternative method with stronger comprehensive capabilities to the conventional methods used to extract juglone from the branches of *J. mandshurica*.

UV–Vis Analysis of Juglone After Extraction

The UV–Vis spectrum of the standard and sample of juglone is shown in Fig. 10. The peak shape and peak position of the sample are essentially the same as those of the juglone standard. The UV absorption of the standard occurs at 210 nm, 250 nm and 418 nm. The UV absorption of the sample occurs at 200 nm, 208 nm, 249 nm and 421 nm. The absorption peaks at 210 nm and 208 nm are attributed to the −OH n → σ* transition, the absorption peaks at 250 nm and 249 nm are the π → π* transition and n → π* transition caused by the conjugated structure composed of a benzene ring and a carbonyl group [50] and the absorption peaks at 418 nm and 421 nm may be caused by n−p* transitions in the two carbonyl groups [51, 52]. However, the sample has a strong absorption at 200 nm, which may be related to the presence of extract impurities containing heteroatom functional groups. The lone pair of electrons in the heteroatoms absorb energy and then transition to the σ* reverse orbit, triggering an n → σ* transition, which is lower than the energy required for the σ → σ* transition, which results in stronger end absorption. In summary, the UV characteristic absorptions of juglone in the sample after extraction are basically consistent with those of the juglone standard, which indicates that the MBMAE method is suitable for extracting juglone from *J. mandshurica* branches, and the structure of juglone before and after extraction is the same.

FTIR Analysis of Juglone After Extraction

Fourier transform infrared spectrum (FTIR) measures the ability of molecules to selectively absorb certain wavelengths of infrared light, which causes transitions in the vibrational and rotational energy levels in the molecule, and can be used in the structural analysis of the compound. Figure 11 is the IR spectrum of the extracted sample. The extracted sample has an absorption peak at 3439 cm⁻¹, indicating the presence of phenolic hydroxyl groups in the molecule and this characteristic vibration frequency is consistent with the presence of phenolic hydroxyl groups in juglone as reported in the literature [53]. The two strong
absorption peaks at 1666 cm$^{-1}$ and 1645 cm$^{-1}$ are characteristic absorptions of aromatic ketones, which indicate that there are carbonyl groups connected to the benzene ring in the sample structure. At the same time, the characteristic vibration of the carbonyl group in juglone also appeared near this frequency. The peak at 3057 cm$^{-1}$ is the characteristic stretching vibration of aromatic hydrogen, and 1600 cm$^{-1}$ and 1430 cm$^{-1}$ are the benzene ring skeleton vibrations, which further verifies the presence of benzene rings in the sample [54]. Taken together, the results from the FTIR data indicate that there was juglone in the extract.

Conclusions

In this study, a novel microemulsion system was incorporated into a MBMAE method and used for the first time to obtain juglone from the waste branches of *J. mandshurica*. Under the optimal extraction parameters identified by a single factor experiment and RSM, the juglone yield was 4.58 mg/g, which was 1.86-fold and 6.65-fold higher than that obtained from Ethanol-MAE and Ethanol-HRE, respectively. These results demonstrated that using the MBMAE method, juglone was selectively solubilized into the reverse micelle system through coordination and hydrogen bonding with the microemulsion, and was more selective than MAE and HRE. MBMAE is an efficient, rapid and promising technique for extracting juglone from the branches of *J. mandshurica*. At the same time, this work provides data support for the potential of microemulsions to be an excellent alternative solvent for extracting juglone from other plant materials, products or wastes.

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Data Availability Not applicable.

Code Availability Not applicable.

Declarations

Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service or company that could be construed as influencing the position presented in the manuscript entitled “A novel method to extract juglone from *J. mandshurica* waste branches using a water-in-oil microemulsion”.

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