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# Mechanochemistry assisted aqueous two-phase extraction: an efficient technique to extract high-purity juglone from the bark of *Juglans mandshurica*

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The bark of *Juglans mandshurica* (BJM) is a Chinese herbal medicine containing a variety of nutrients and pharmacologically active compounds. Juglone, one of the important active ingredients in BJM, has been shown by many pharmacological studies to have antioxidant, antibacterial, antiviral, anti-tumor, immunoregulatory and other pharmacological properties. Mechanochemistry assisted aqueous two-phase extraction (MAATPE) was successfully established to extract juglone from BJM, combining the purification effect of an alcohol/salt aqueous two-phase system (ATPS) with the high efficiency of mechanochemistry. The key parameters of MAATPE were systematically optimized through single factor analysis and response surface methodology. Under the optimal conditions of an ethanol/NaH<sub>2</sub>PO<sub>4</sub> aqueous two-phase system as the liquid reagent, 21.5% (w/w) ethanol concentration, 28% (w/w) NaH<sub>2</sub>PO<sub>4</sub> concentration, 458 rpm milling speed, 14 min milling time and 59 mL g<sup>-1</sup> reagent–material ratio, the maximum yield of juglone was 15.36 ± 0.16 mg g<sup>-1</sup> with a purity of 9.82 ± 0.12% in dry extracts. Compared with other extraction methods, it was found that MAATPE can not only improve the extraction efficiency of juglone, but also achieve higher juglone purity in the products. Finally, the mechanism of action, recyclability and industrial scalability of MAATPE are discussed. In conclusion, MAATPE is an appropriate alternative for the efficient preparation of high-purity products from natural resources.

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## 1. Introduction

*Juglans mandshurica* (*Juglans mandshurica* Maxim.), a tree species belonging to the family Juglandaceae and genus *Juglans*, is cultivated worldwide. In China, it is mainly distributed in Heilongjiang, Jilin, Liaoning, Inner Mongolia, Shanxi, Henan, Hebei and other provinces.<sup>1–4</sup> Its bark, green fruit, peel and kernel are used as Chinese herbal medicine.<sup>3,5</sup> The bark of *Juglans mandshurica* (BJM) contains a variety of nutrients and pharmacologically active compounds such as juglone, polyphenols, flavonoids, and so on.<sup>3,4</sup> Juglone (Fig. 1), one of the important active ingredients in BJM, has been shown by many pharmacological studies to have antioxidant, antibacterial, antiviral, anti-tumor, immunoregulation and other pharmacological properties.<sup>2–6</sup> Due to its beneficial biological properties, juglone has attracted much attention. Therefore, the extraction of juglone from BJM is of great significance for the value-added utilization of natural resources.

In previous studies, some traditional approaches such as heat reflux extraction (HRE), Soxhlet extraction, maceration, *etc.*

have been widely used to extract juglone.<sup>7,8</sup> These approaches have the limitations of low efficiency, long extraction time, high solvent consumption, high temperature, difficulty in separating the target components from the solvent, *etc.*, which do not align with the concept of energy saving and environmental protection in today's society.<sup>7,8</sup> As a result, many novel approaches have been proposed to extract juglone, for example, supercritical CO<sub>2</sub> extraction, ultrasound assisted extraction, microwave assisted extraction, and so on.<sup>8–11</sup> These methods have shown higher extraction yields and shorter extraction time compared to conventional methods. However, these methods also have their limitations. Supercritical CO<sub>2</sub> extraction has the disadvantages of high pressure, high equipment cost, high consumption of energy and CO<sub>2</sub>, and so on. Ultrasound assisted extraction (UAE) cannot uniformly process raw materials, and harsh noise

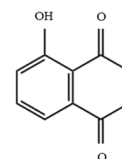


Fig. 1 Chemical structure of juglone.

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is produced during operation. Microwave assisted extraction (MAE) produces high temperature that can lead to degradation of heat-sensitive components. Therefore, it is necessary to seek a method for extracting juglone from BJM which is efficient, economical, non-thermal, and allows easy separation of juglone from the solvent.

Mechanochemistry extraction (ME) is an efficient method with the advantages of being non-thermal, green, environmental friendly, and so on.<sup>12–14</sup> This method utilizes mechanical forces generated during ball milling, including compression and shear forces, to pretreat raw materials and solid reagents.<sup>14</sup> On the one hand, these mechanical forces can improve the crushing ratio and degree of the raw materials, enlarge the solid reagent–material contact area, and reduce the activation energy required for the reaction, thus promoting the release of bioactive components. On the other hand, these mechanical forces can also enhance the chemical reaction of bioactive components with solid reagents, forming water-soluble salts that are easier to extract at a later stage.<sup>12,13</sup> The ME process can be divided into two steps. In the first step, a mixture of raw materials and solid reagents is ground and chemically reacted in a ball mill. In the second step, water is added to the ground mixture for dissolution and extraction. It is necessary to adjust the pH value of the solvent in order to convert the formed water-soluble salts into the target compounds. For acidic target components, solid alkaline reagents such as  $\text{Na}_2\text{CO}_3$ ,  $\text{NaHCO}_3$ ,  $\text{NaOH}$ , *etc.* can be selected, while for alkaline target components, solid acidic reagents such as oxalic acid, citric acid, boric acid, *etc.* can be selected.<sup>13,14</sup> However, due to the two-step operation of ME, it is labor-intensive and time-consuming. In recent years, many studies have been carried out to improve traditional ME.<sup>12,14–18</sup> New solvents such as reverse micelle surfactants, ionic liquids, deep eutectic solvents and organic bases have been combined with ME instead of traditional solid reagents, integrating pretreatment and extraction into one step, thus increasing the extraction efficiency and making the process more convenient.

Although both traditional and modified ME can achieve high efficiency, these methods often co-extract unwanted components along with the target compounds. This results in reduced product purity and complicates subsequent purification processes. For example, when solid alkaline reagents are used to extract punicalagin with water as the solvent, hydrophilic impurities such as proteins, polysaccharides, and pectin are inevitably co-extracted.<sup>19</sup> Therefore, it is critical to identify a suitable reagent that enhances ME efficiency while ensuring high product purity.

An aqueous two-phase system (ATPS) is a water-based biphasic solvent formed by mixing two or more kinds of immiscible hydrophilic substances in water.<sup>20,21</sup> It is often used to separate and purify various biomacromolecules, *e.g.*, enzymes, proteins, viruses, DNA, and so on.<sup>21</sup> There are many types of ATPS, such as polymer systems, surfactant systems, ionic liquid systems, deep eutectic solvent systems, and so on.<sup>21</sup> However, most of the polymers are expensive and difficult to recycle, have slow phase separation and exhibit high viscosity.<sup>22,23</sup> Similarly, surfactants also exhibit the disadvantages of slow phase separation and high viscosity.<sup>22,23</sup> Deep

eutectic solvents and ionic liquids may pose potential safety risks, and whether they can be applied in the pharmaceutical and food industry is a problem that cannot be ignored.<sup>24,25</sup> In recent years, short-chain alcohol/salt ATPSs have attracted attention due to their excellent phase demixing effect, low viscosity, high selectivity, easy preparation, low cost, and easy recycling.<sup>21</sup> They have been used to extract low molecular weight biomolecules such as polyphenols, flavone, alkaloids, and so on.<sup>26–28</sup> In short-chain alcohol/salt ATPSs, weakly polar compounds are enriched in the alcohol-rich phase, while strongly polar compounds are collected in the salt-rich phase due to their different partition coefficients between the two phases, resulting in a purification effect.<sup>26,28</sup> Considering these characteristics, in order to improve product purity, short-chain alcohol/salt ATPSs can be used as the liquid reagent in ME, which can not only simplify the subsequent purification operation, but also achieve higher extraction efficiency.

In this study, a short-chain alcohol/salt ATPS solvent was selected as the liquid reagent for ME, namely, mechanochemistry assisted aqueous two-phase extraction (MAATPE), to extract juglone from BJM. The effects of the types of short-chain alcohol/salt ATPS,  $\text{NaH}_2\text{PO}_4$  concentration, ethanol concentration, reagent–material ratio, milling speed and milling time on the yield and recovery of juglone were systematically optimized through single factor analysis and response surface methodology (RSM). Then, MAATPE was compared with other extraction methods in terms of extraction efficiency of juglone, and juglone purity in dry extracts. Finally, the mechanism of action, recycling and industrial scalability of MAATPE are discussed.

## 2. Materials and methods

### 2.1 Raw materials and reagents

The barks of *Juglans mandshurica* (BJM), collected from Harbin (Heilongjiang province, China) in September, 2023, were provided by Sichuan BencaoTang Pharmaceutical Co., Ltd (Chengdu, China). They were dried, crushed and then sieved through a 60-mesh screen. The obtained fine brown powder was carefully collected in sealed brown glass bottles and stored in a glass desiccator for future experimental studies.

Analytical grade absolute ethanol, methanol, formic acid, phosphoric acid,  $\text{NaH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ , and  $(\text{NH}_4)_2\text{SO}_4$  were provided by Chengdu Kelong Chemistry Co., Ltd (Chengdu, China). Juglone (HPLC grade standard reagent), purity > 97%, was provided by Chengdu Shengnuo Biotechnology Co., Ltd (Chengdu, China). Deionized water was used for all experiments, unless otherwise specified.

### 2.2 MAATPE procedure

The process of MAATPE of juglone from BJM is illustrated as follows. The desired ethanol/ $\text{NaH}_2\text{PO}_4$  ATPS was prepared on the basis of the phase diagram reported by Huang *et al.*<sup>29</sup> According to the reagent–material ratio, a certain mass of raw materials and a certain volume of ATPS solvent were placed into each grinding tank with the same number of 4 mm diameter zirconia balls. Then, the mixture of raw materials and ATPS



solvent was ground using a planetary ball mill (DECO-PBM-V-2L-A, Changsha Deco Equipment Co., Ltd, China) equipped with four 500 mL grinding tanks at room temperature at a certain speed for a specific period of time. After that, the solid impurities in the extraction solution were removed through filtration and centrifugation. The supernatant was left to stand for a period of time to allow the separation of phases. The upper phase, alcohol-rich phase, was enriched with juglone and other weakly polar components, and the bottom phase, the  $\text{NaH}_2\text{PO}_4$  phase, was enriched with proteins, polysaccharides, and other strongly polar components.<sup>26–28</sup> The two phases were then separated using a separating funnel and transferred individually. And a small volume of the upper phase supernatant was diluted to a certain multiple for subsequent HPLC analysis.

The extract in the upper phase was collected, and five times its volume of absolute ethanol was added to precipitate the dissolved salt.<sup>27,28</sup> The solution was then filtered and centrifuged. The supernatant was concentrated using a rotary evaporator (RE-52A, Shanghai Yarong Bio., China), and the dried extracts were freeze-dried and collected.

The yield, recovery and purity of juglone were used as indicators to evaluate the extraction effect, and their calculation formulas are as follows:

$$\text{Recovery (\%)} = \frac{x_U V_U}{x_U V_U + x_B V_B} \times 100\% \quad (1)$$

$$\text{Yield (mg g}^{-1}\text{)} = \frac{x_U V_U}{m} \times 100\% \quad (2)$$

$$\text{Purity (\%)} = \frac{x_U V_U}{m_D} \times 100\% \quad (3)$$

where,  $x_U$  ( $\text{mg mL}^{-1}$ ) and  $x_B$  ( $\text{mg mL}^{-1}$ ) are the concentrations of juglone in the upper and bottom phases, respectively;  $V_U$  (mL) and  $V_B$  (mL) are the volumes of the solution after dilution of the upper and bottom phases, respectively;  $m_D$  (mg) and  $m$  (g) represent the mass of dry extracts and raw materials, respectively.

### 2.3 HPLC analysis of juglone

The study by Ramezani *et al.* was referred to determine the content of juglone in the extracts using an HPLC system (LC-15C, Shimadtsu, Japan) equipped with an SPD-15C UV-detector.<sup>7</sup> The chromatographic conditions involved the use of a  $\text{C}_{18}$  column (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ). The mobile phase A consisted of 60% methanol solution, which was acidified with formic acid to achieve a pH of approximately 4.0. The mobile phase B was water, with the pH adjusted to around 4.0 using phosphoric acid. An isocratic elution method was employed with a flow rate of 0.8  $\text{mL min}^{-1}$ . The injection volume was 10  $\mu\text{L}$ , and the detection wavelength was set at 250 nm. A standard curve was plotted with mass concentration/internal standard concentration as the horizontal coordinate and the peak area/internal standard peak area as the vertical coordinate. The resulting standard curve is shown in eqn (4).

$$y = 1.3510x - 0.6686 \quad (R^2 = 0.992) \quad (4)$$

where  $y$  is the peak area/internal standard peak area;  $x$  is the concentration of juglone/internal standard concentration ( $\text{mg mL}^{-1}$ ).

### 2.4 Experimental design

Different salts ( $\text{NaH}_2\text{PO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and  $\text{K}_2\text{HPO}_4$ ),  $\text{NaH}_2\text{PO}_4$  concentrations (23.5%, 25%, 26.5%, 28%, and 29.5% w/w), ethanol concentrations (17%, 18.5%, 20%, 21.5%, and 23% w/w), milling speeds (300, 350, 400, 450, and 500 rpm), milling times (4, 8, 12, 16, and 20 min), and reagent–material ratios (20, 40, 60, 80, and 100  $\text{mL g}^{-1}$ ) were preliminarily optimized by single-factor experiments to study their influence on the extraction process. After that, the MAATPE process was further optimized by RSM (Design expert 12.0), which was based on a Box–Behnken design with three factors and three levels. The factors considered were  $X_1$ : reagent-material ratio,  $X_2$ : milling speed,  $X_3$ : milling time. The experimental conditions and results are presented in Table 1. A quadratic polynomial model was adopted to fit the data (eqn (5)).

$$Y = \gamma_0 + \gamma_1 X_1 + \gamma_2 X_2 + \gamma_3 X_3 + \gamma_{11} X_1^2 + \gamma_{22} X_2^2 + \gamma_{33} X_3^2 + \gamma_{12} X_1 X_2 + \gamma_{13} X_1 X_3 + \gamma_{23} X_2 X_3 \quad (5)$$

where  $Y$  is the respond value (juglone yield,  $\text{mg g}^{-1}$ );  $X_1$ ,  $X_2$  and  $X_3$  represent three variables. The different subscripts of  $\gamma$  represent the intercept coefficient ( $\gamma_0$ ), linear coefficient ( $\gamma_1$ ,  $\gamma_2$ ,  $\gamma_3$ ), interaction coefficient ( $\gamma_{12}$ ,  $\gamma_{13}$ ,  $\gamma_{23}$ ) and quadratic coefficient ( $\gamma_{11}$ ,  $\gamma_{22}$ ,  $\gamma_{33}$ ).

### 2.5 Control experiments

In this study, in order to investigate the extraction performance of MAATPE, comparative studies were performed using HRE, ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), and traditional ME for the extraction of juglone. Moreover, the extraction effect of ME followed by ATPS (ME + ATPS) was also studied.

**2.5.1 Heat reflux extraction.** According to the research of Aiello *et al.*, HRE was selected to extract juglone from BJM.<sup>30</sup> 8.0 g of raw material was added to 480 mL of 60% ethanol, followed by extraction for 90 min at 80  $^\circ\text{C}$ . The subsequent procedures were the same as MAATPE.

**2.5.2 Ultrasound-assisted extraction.** Referring to Xu's research, 8.0 g of raw material was uniformly mixed with 480 mL of 60% ethanol at room temperature, and ultrasonic treatment at 500 W was carried out for 30 min (HN99-IID, Shanghai Hanuo Instrument Co., Ltd, Shanghai, China).<sup>30</sup> The subsequent procedures were the same as MAATPE.

**2.5.3 Microwave assisted extraction.** According to the research of Xu *et al.*, 8.0 g of raw material was uniformly mixed with 480 mL of 60% ethanol and treated with 300 W microwave for 5 min (XO-SM200, Nanjing Xian'ou Bio-tech Co. Ltd, Nanjing, China). The subsequent procedures were the same as MAATPE.

**2.5.4 Traditional ME.** Referring to the study of Wu and Xi,  $\text{Na}_2\text{CO}_3$  was selected as the solid reagent for ME to optimize the yield of juglone.<sup>31</sup> 8.0 g of raw material and 0.48 g of solid

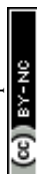


Table 1 The Box–Behnken design for optimizing extraction conditions

Run	Factor 1	Factor 2	Factor 3	Response
	$X_1$ : reagent–material ratio (mL g <sup>-1</sup> )	$X_2$ : milling speed (rpm)	$X_3$ : milling time (min)	Juglone yield (mg g <sup>-1</sup> )
1	60	500	8	11.77 ± 0.10
2	60	450	12	11.52 ± 0.12
3	40	450	16	13.09 ± 0.05
4	40	400	12	12.80 ± 0.16
5	40	450	8	12.79 ± 0.12
6	60	450	12	12.51 ± 0.15
7	60	450	12	14.09 ± 0.04
8	80	450	8	13.98 ± 0.13
9	60	400	8	10.99 ± 0.08
10	60	450	12	12.22 ± 0.16
11	60	450	12	12.37 ± 0.18
12	60	400	16	13.96 ± 0.14
13	80	450	16	15.19 ± 0.07
14	40	500	12	15.06 ± 0.05
15	80	500	12	15.17 ± 0.09
16	80	400	12	15.16 ± 0.11
17	60	500	16	15.07 ± 0.05

reagent were ground at 400 rpm for 12 min in a planetary ball mill. Then, the treated mixture was dissolved in 480 mL of water and extracted for 12 min at ambient temperature, and the pH of solution was adjusted to 4.0. The subsequent procedures were the same as MAATPE.

**2.5.5 ME + ATPS.** First, 8.0 g of raw material was ground in a planetary ball mill at a rotational speed of 400 rpm for 12 min. Subsequently, the treated raw material was dissolved in 480 mL of ethanol/NaH<sub>2</sub>PO<sub>4</sub> ATPS. After extraction at room temperature for 15 min, solid impurities were removed through filtration and centrifugation. The subsequent procedures were the same as MAATPE.

## 2.6 Recycling of solution

The ethanol solution from the ATPS upper phase can be recovered by rotary evaporation. Considering that the juglone content in the lower phase of the ATPS is extremely low, the lower phase solution can be reused. A new ATPS solution can be obtained simply by adding ethanol solution to the recycled NaH<sub>2</sub>PO<sub>4</sub> solution. It is worth noting that with the re-addition of ethanol solution, the proportion of the ATPS will change to some extent. Therefore, ethanol solutions with different volume fractions were added to the recycled sodium phosphate solution respectively to form new ATPSS, which were then reused for the extraction of juglone by MAATPE to explore the extraction performance after recycling.

## 2.7 Statistical analysis

Each experiment and measurement were performed in triplicate, and the results are presented as average value ± SD. The analysis encompassed various statistical tests, including the least significant difference test, ANOVA, and Tukey test ( $p < 0.05$ ), which were employed to discern the statistically significant disparities among the outcomes.

## 3. Results and discussion

### 3.1 Selection of the short-chain alcohol/salt ATPS

The types of short-chain alcohol and salt are important parameters that determine the distribution behavior of juglone.<sup>21</sup> The selection of short-chain alcohols should follow the principle of “like dissolves like,” meaning that solvents with similar polarity and chemical structure to the target compounds can enhance solubility. For example, polar target substances (*e.g.*, polyphenols) require alcohols with matching polarity indices (*e.g.*, ethanol). This perspective is further supported by Hansen’s solubility theory, which correlates solubility with similarities in dispersion forces, polar interactions, and hydrogen bonding.<sup>22</sup> The polarity of juglone is weak, so it is easily soluble in methanol, ethanol, ethyl acetate, chloroform, and benzene and other organic solvents.<sup>2</sup> Among these organic solvents, short-chain alcohols, *i.e.* ethanol and propyl alcohol can be used as ATPS components.<sup>21,27–29</sup> Compared with propyl alcohol, ethanol has the advantages of non-toxicity, better solubility, low cost and ease of recycling, making it more suitable for the extraction of juglone in the pharmaceutical and food industries.<sup>21,32</sup> Therefore, ethanol was chosen as a component of ATPS to extract juglone from BJM in this study.

The primary consideration in the selection of salt is whether it can form a stable ATPS with ethanol. In addition, in order to improve the stability of the target components, the acid–base properties of the selected salt should be consistent with those of the target components.<sup>21</sup> The salts that can quickly produce pellucid and stable ATPS with ethanol generally include NaH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub>, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.<sup>33</sup> Since juglone in BJM is a weakly acidic compound, the acid salts NaH<sub>2</sub>PO<sub>4</sub> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were selected to provide an acidic environment to improve the stability of juglone, and alkaline salt K<sub>2</sub>HPO<sub>4</sub> was used as a control for comparison with acidic salts.<sup>34</sup>



To select the appropriate salt, the effects of 25% (w/w)  $\text{NaH}_2\text{PO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and  $\text{K}_2\text{HPO}_4$  on the yield and recovery of juglone were investigated at room temperature under the operating parameters: 18.5% (w/w) ethanol, 400 rpm milling speed, 4 min milling time, and  $100 \text{ mL g}^{-1}$  reagent-material ratio. As shown in Fig. 2, both the juglone yield ( $7.47 \pm 0.17 \text{ mg g}^{-1}$ ) and recovery ( $82.38 \pm 0.67\%$ ) with  $\text{NaH}_2\text{PO}_4$  were significantly higher than those with  $(\text{NH}_4)_2\text{SO}_4$  ( $5.69 \pm 0.12 \text{ mg g}^{-1}$ ,  $71.34 \pm 0.64\%$ ) and  $\text{K}_2\text{HPO}_4$  ( $4.04 \pm 0.09 \text{ mg g}^{-1}$ ,  $67.37 \pm 0.90\%$ ). In the ATPS, an appropriate acid–base environment can affect the solubility and stability of the target product. In addition, due to differences in charge, size and shape of different inorganic salt ions, the interaction between the ATPS and the target product also varies, which can lead to different distribution behaviors of juglone. An ATPS composed of  $\text{NaH}_2\text{PO}_4$  can provide a weak acid environment which is conducive to the distribution of juglone in the upper phase, thereby increasing the yield and recovery of juglone. In contrast, other salts such as  $\text{K}_2\text{HPO}_4$  and  $(\text{NH}_4)_2\text{SO}_4$  cannot provide the same environment, resulting in lower extraction yield and recovery. This conclusion is consistent with those obtained by other researchers, such as Xie *et al.*, who extracted acid chlorogenic acid with the ethanol/ $\text{NaH}_2\text{PO}_4$  ATPS, Zhu *et al.*, who extracted basic solanine with an ATPS composed of alkali salts, and so on.<sup>35,36</sup> Therefore, ethanol/ $\text{NaH}_2\text{PO}_4$  ATPS was chosen as the biphasic system for subsequent experiments.

The phase diagram of the ethanol/ $\text{NaH}_2\text{PO}_4$  ATPS used by Huang *et al.* is shown in Fig. 3.<sup>31</sup> The single-phase region without phase separation is below the binodal curve. The two-phase region where the solution distinctly separates into two phases is above the binodal curve. In this region, ethanol dissolves in the upper phase, and  $\text{NaH}_2\text{PO}_4$  dissolves in the bottom phase. The experimental point should be selected from the area above the binodal curve. According to Fig. 3, a stable ATPS cannot form when the salt concentration is higher than 38% or the ethanol concentration is higher than 59%. Therefore, the salt concentration should not be higher than 38%

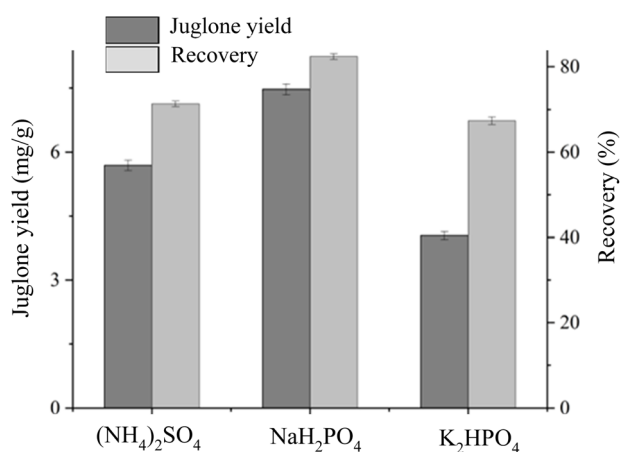


Fig. 2 The extraction yield and recovery of juglone using  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NaH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  under the conditions of 25% (w/w) salt and 18.5% (w/w) ethanol at room temperature by MAATPE. Data represent means  $\pm$  SD of three independent experiments.

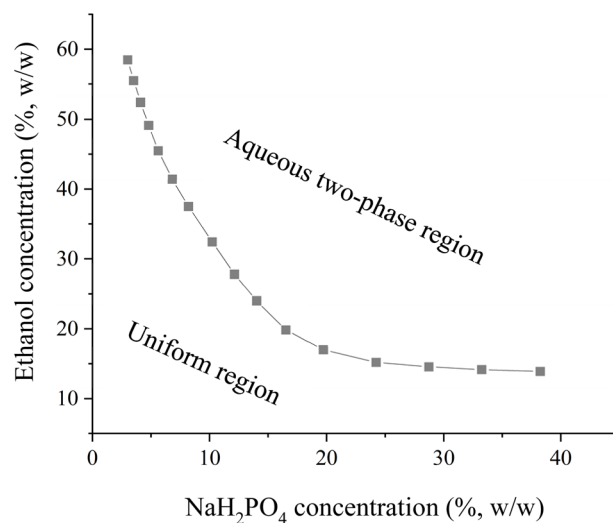


Fig. 3 The phase diagram of the ethanol/ $\text{NaH}_2\text{PO}_4$  ATPS at room temperature, as reported by Huang *et al.*

(w/w), and the ethanol concentration should not be higher than 59% (w/w).

### 3.2 Effect of concentration of $\text{NaH}_2\text{PO}_4$ and ethanol

According to the phase diagram and preliminary experiments, the concentration of  $\text{NaH}_2\text{PO}_4$  was selected in the range of 23.5% to 29.5% to investigate its effect on the yield and recovery of juglone at room temperature under the operating parameters: 17% (w/w) ethanol, 400 rpm milling speed, 4 min milling time, and  $100 \text{ mL g}^{-1}$  reagent-material ratio. Fig. 4(a) shows that both the yield ( $8.89 \pm 0.06 \text{ mg g}^{-1}$ ) and recovery ( $88.91 \pm 0.53\%$ ) of juglone reached their maximum values at a  $\text{NaH}_2\text{PO}_4$  concentration of 28%. This can be attributed to the increase in the concentration of  $\text{NaH}_2\text{PO}_4$ , which not only enhanced the hydrophilicity of the bottom phase, allowing more water to enter the bottom phase and thus increasing the ethanol concentration in the upper phase, but also significantly lowered the pH value of the solution, providing a more suitable pH environment for juglone.<sup>37</sup> However, an excessive amount of  $\text{NaH}_2\text{PO}_4$  will cause a significant decrease in the volume of the upper phase, preventing juglone from being fully dissolved.

According to the phase diagram and preliminary experiments, the effects of ethanol concentrations at 17%, 18.5%, 20%, 21.5%, and 23% on the yield and recovery of juglone were studied at room temperature under the conditions of 28% (w/w)  $\text{NaH}_2\text{PO}_4$  concentration, 400 rpm milling speed, 4 min milling time, and a  $100 \text{ mL g}^{-1}$  reagent-material ratio. As shown in Fig. 4(b), with the increase in ethanol concentration from 17% to 23%, both the yield and recovery of juglone first increased and then decreased. The maximum yield ( $9.32 \pm 0.10 \text{ mg g}^{-1}$ ) and recovery ( $94.16 \pm 0.45\%$ ) of juglone were obtained at an ethanol concentration of 21.5%. When the concentration of ethanol was increased, it not only increased the amount of juglone dissolved, but also enhanced the competitiveness of the upper phase for water, resulting in an increase in the volume of the top phase, so that more juglone can be enriched. However,



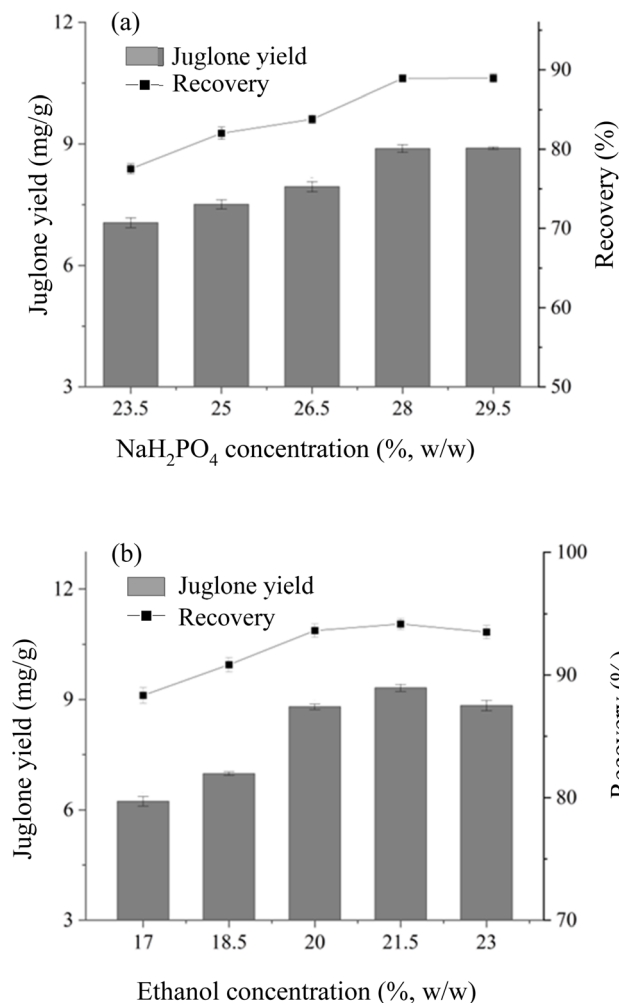


Fig. 4 Effect of  $\text{NaH}_2\text{PO}_4$  concentration (a) and ethanol concentration (b) on the extraction yield and recovery of juglone by MAATPE. Data represent means  $\pm$  SD of three independent experiments.

when the ethanol concentration was further increased, the polarity of the solution became inconsistent with that of the juglone, leading to a decrease in the amount of dissolved juglone. As a result, the yield and recovery of juglone decreased slightly. In addition, if the ethanol concentration continues to increase, a salting-out effect would occur, and the ATPS would become unstable.

In summary, the concentrations of  $\text{NaH}_2\text{PO}_4$  and ethanol selected for subsequent experiments were 28% and 21.5%, respectively.

### 3.3 Effect of operating parameters of MAATPE

**3.3.1 Effect of milling speed.** The effects of milling speed on the extraction process were investigated at 300, 350, 400, 450 and 500 rpm. Other parameters were set as follows: 21.5% (w/w) ethanol concentration, 28% (w/w)  $\text{NaH}_2\text{PO}_4$  concentration, 4 min milling time, and 100  $\text{mL g}^{-1}$  reagent-material ratio. As shown in Fig. 5(a), the juglone yield reached its maximum value ( $10.86 \pm 0.07 \text{ mg g}^{-1}$ ) at 450 rpm. The higher milling speed

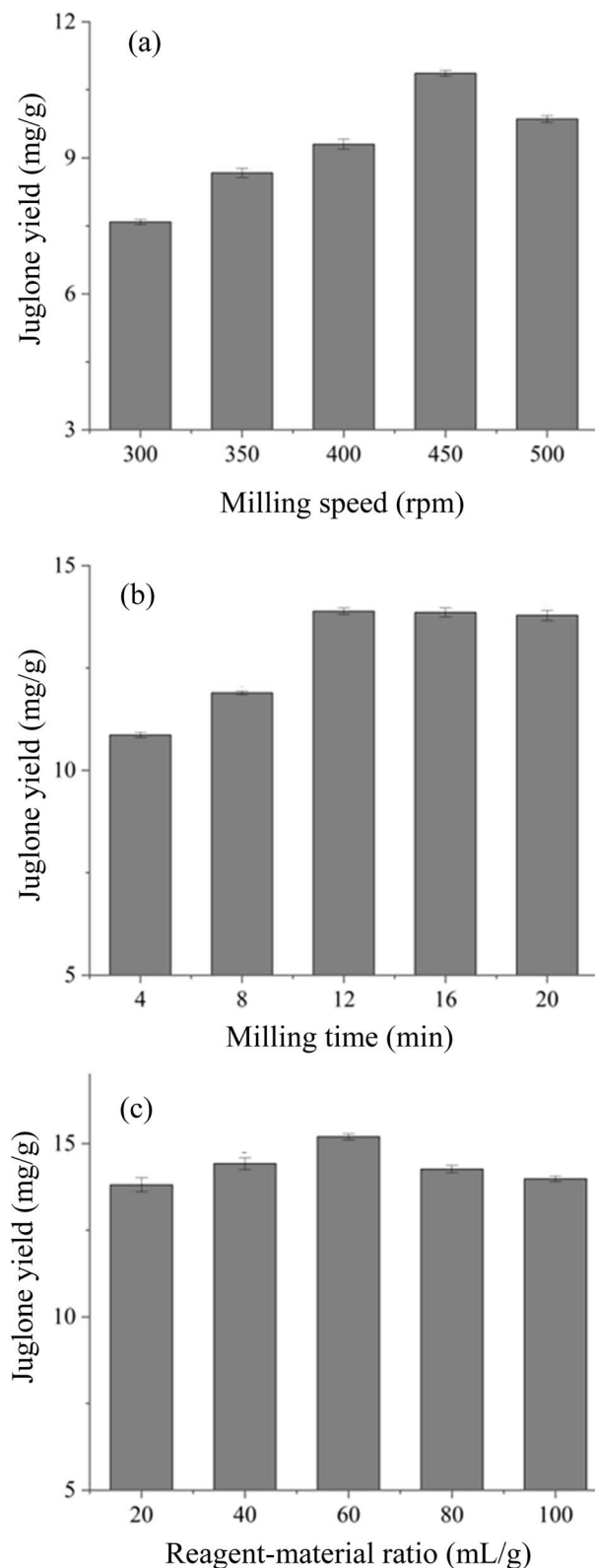


Fig. 5 Effect of milling speed (a), milling time (b) and reagent-material ratio (c) on the juglone yield determined by MAATPE. Data represent means  $\pm$  SD of three independent experiments.

intensifies the collision between grinding balls and raw materials, thereby enhancing the grinding ability and causing more severe damage to the cell wall of raw materials, thus reducing



the resistance to juglone diffusion. In addition, the higher milling speed can exacerbate the turbulent mixing of the liquid reagents and accelerate the diffusion of juglone. When milling speed exceeded 450 rpm, the juglone yield reduced slightly, which may be due to the fact that higher milling speed generates a lot of heat, causing degradation of juglone. Therefore, 450 rpm milling speed was chosen for the following experiment.

**3.3.2 Effect of milling time.** In the MAATPE process, milling time can influence the degree of cell damage, particle size distribution, juglone and liquid reagent dissolution. The effects of milling times of 4, 8, 12, 16, 20 min on MAATPE process were studied under the conditions of 21.5% (w/w) ethanol concentration, 28% (w/w)  $\text{NaH}_2\text{PO}_4$  concentration, 450 rpm milling speed, and 100  $\text{mL g}^{-1}$  reagent–material ratio. It can be observed from Fig. 5(b) that the juglone yield reached its maximum value ( $13.88 \pm 0.08 \text{ mg g}^{-1}$ ) when the milling time was 12 min. With a further increase in milling time, the juglone yield remained unchanged, indicating that the amounts of juglone extracted had reached saturation at this time. Therefore, 12 min milling time was chosen for subsequent experiments.

**3.3.3 Effect of the reagent–material ratio.** The reagent–material ratio is a key factor that significantly affects the extraction efficiency in the MAATPE process. In this study, under the experimental conditions of 21.5% (w/w) ethanol concentration, 28% (w/w)  $\text{NaH}_2\text{PO}_4$  concentration, 450 rpm milling speed, and 12 min milling time, the juglone yield was investigated at different reagent–material ratios (20, 40, 60, 80, and 100  $\text{mL g}^{-1}$ ). Fig. 5(c) shows that the juglone yield gradually increased from  $13.81 \pm 0.28 \text{ mg g}^{-1}$  to  $15.19 \pm 0.08 \text{ mg g}^{-1}$  as the reagent–material ratio increased from 20 to 60  $\text{mL g}^{-1}$ . After that, the juglone yield started to decrease as the reagent–material ratio increased further. When the reagent–material ratio was below 60  $\text{mL g}^{-1}$ , an increase in the amount of ATPS solvent enabled more juglone to be fully dissolved, thereby improving the juglone yield. However, when the reagent–material ratio continued to increase, the excess solvent absorbed more energy from the extraction system, which led to incomplete processing of the raw materials and reduced the

juglone yield. Consequently, the reagent–material ratio of 60  $\text{mL g}^{-1}$  was selected for the subsequent experiment.

### 3.4 Response surface methodology analysis

Based on the optimized conditions (21.5% (w/w) ethanol concentration, 28% (w/w)  $\text{NaH}_2\text{PO}_4$  concentration, 450 rpm milling speed, 12 min milling time, and 60  $\text{mL g}^{-1}$  reagent–material ratio) obtained from single-factor experiments, three important operating parameters of MAATPE ( $X_1$ : reagent–material ratio,  $X_2$ : milling speed, and  $X_3$ : milling time) were selected for optimization by RSM. The response regression equation obtained through the quadratic model analysis and fitting for the juglone yield is as follows (eqn (6)).

$$Y = 15.1300 - 0.0950X_1 + 0.6788X_2 + 0.7838X_3 + 0.0050X_1X_2 - 0.0250X_1X_3 + 0.1375X_2X_3 - 0.9637X_1^2 - 1.8500X_2^2 - 0.9063X_3^2 \quad (6)$$

The *F*-value and *p*-value of the model were 1300.00 and <0.0001, respectively, indicating that the model was statistically significant (Table 2). In addition, the larger *p*-value (0.7312) for the “lack of fit” term and the higher *R*<sup>2</sup>-value (0.9994) of the correlation coefficient indicated small errors and high reliability of the model data. From eqn (6), it can be concluded that milling time has the greatest effect on the yield of juglone, followed by milling speed and finally the reagent–material ratio. Similarly, it can be observed from Table 2 that the significant model terms for juglone yields are  $X_2$ ,  $X_3$ ,  $X_1^2$ ,  $X_2^2$  and  $X_3^2$ . 3D response surface plots and corresponding contour plots in Fig. 6 illustrate the effects of each variable and its interaction on the juglone yields. When two variables were held constant, the effects of the other variables on the juglone yields were consistent with the previous single-factor experimental analysis. It can be seen from the contour plots that the effect of interaction between milling speed and milling time on the yield of juglone was significant. The optimal conditions for obtaining the highest juglone yield were predicted as follows: 13.718 min milling time, 58.730  $\text{mL g}^{-1}$  reagent–material ratio and 457.939 rpm milling speed, and the corresponding optimal

Table 2 ANOVA of the quadratic response surface regression model

Source	Sum of squares	df	Mean square	<i>F</i> -Value	<i>p</i> -Value	
Model	32.63	9	3.63	1300.00	<0.0001	Significant
$X_1$ -Reagent–material ratio	0.0722	1	0.0722	25.88	0.0014	
$X_2$ -Milling speed	3.69	1	3.69	1321.35	<0.0001	
$X_3$ -Milling time	4.91	1	4.91	1761.78	<0.0001	
$X_1X_2$	0.0001	1	0.0001	0.0359	0.8552	
$X_1X_3$	0.0025	1	0.0025	0.8963	0.3753	
$X_2X_3$	0.0756	1	0.0756	27.11	0.0012	
$X_1^2$	3.91	1	3.91	1402.08	<0.0001	
$X_2^2$	14.35	1	14.35	5145.46	<0.0001	
$X_3^2$	3.46	1	3.46	1239.77	<0.0001	
Residual	0.0195	7	0.0028			
Lack of fit	0.0049	3	0.0016	0.4498	0.7312	Not significant
Pure error	0.0146	4	0.0036			
Cor total	32.65	16				



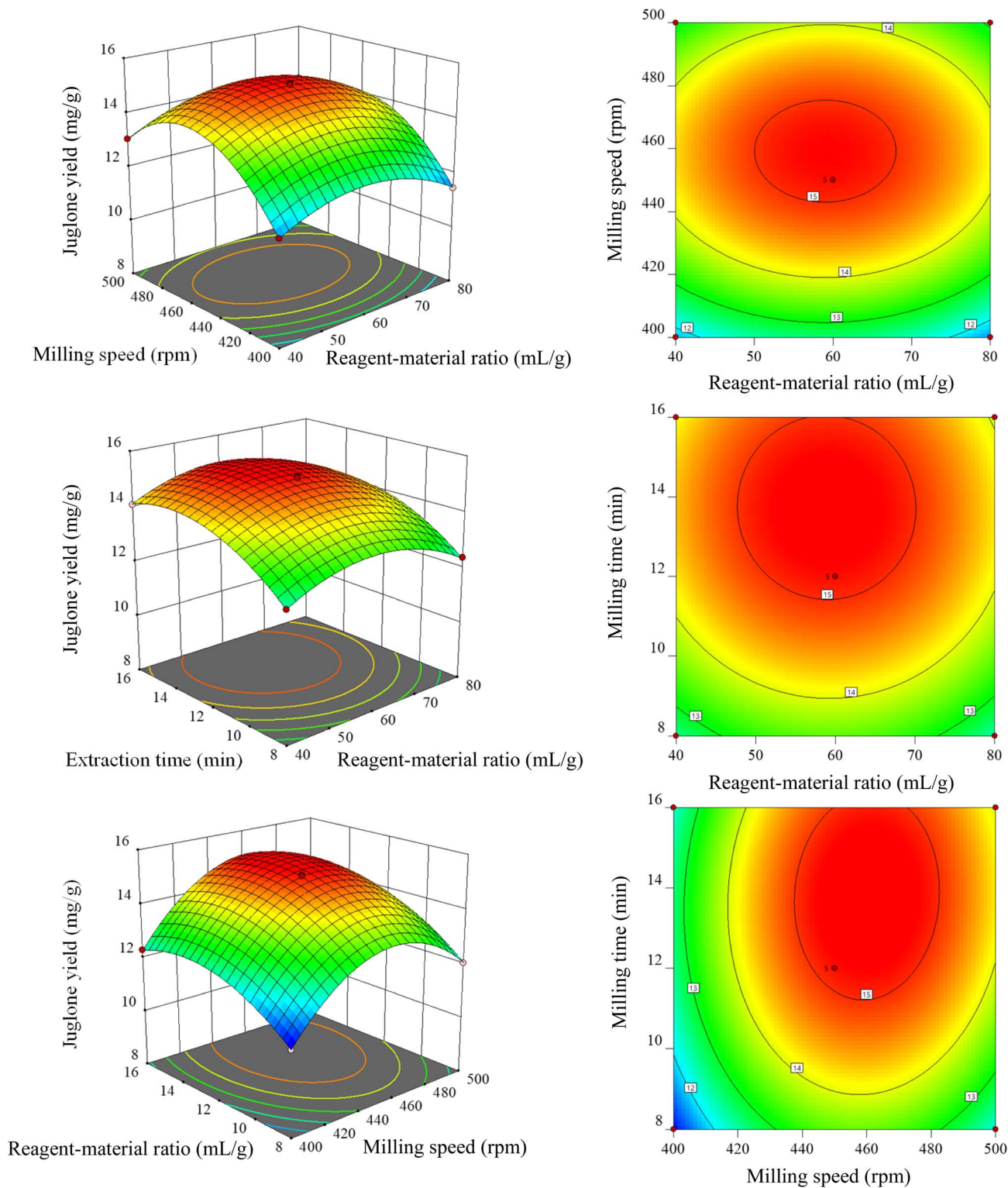


Fig. 6 The three-dimensional response surface plots and contour plots showing the effects of the reagent–material ratio ( $X_1$ ), milling speed ( $X_2$ ), milling time ( $X_3$ ) on the juglone yields by MAATPE.

juglone yield was  $15.373 \text{ mg g}^{-1}$ . The predicted optimization conditions were rounded to 14 min milling time, 59  $\text{mL g}^{-1}$  reagent–material ratio and 458 rpm milling speed. The actual juglone yield was found to be  $15.36 \pm 0.16 \text{ mg g}^{-1}$  through

experimental verification, which was close to the predicted value. Meanwhile, the purity of juglone in the dried extracts was  $9.82 \pm 0.12\%$ .



### 3.5 Comparison with control experiments

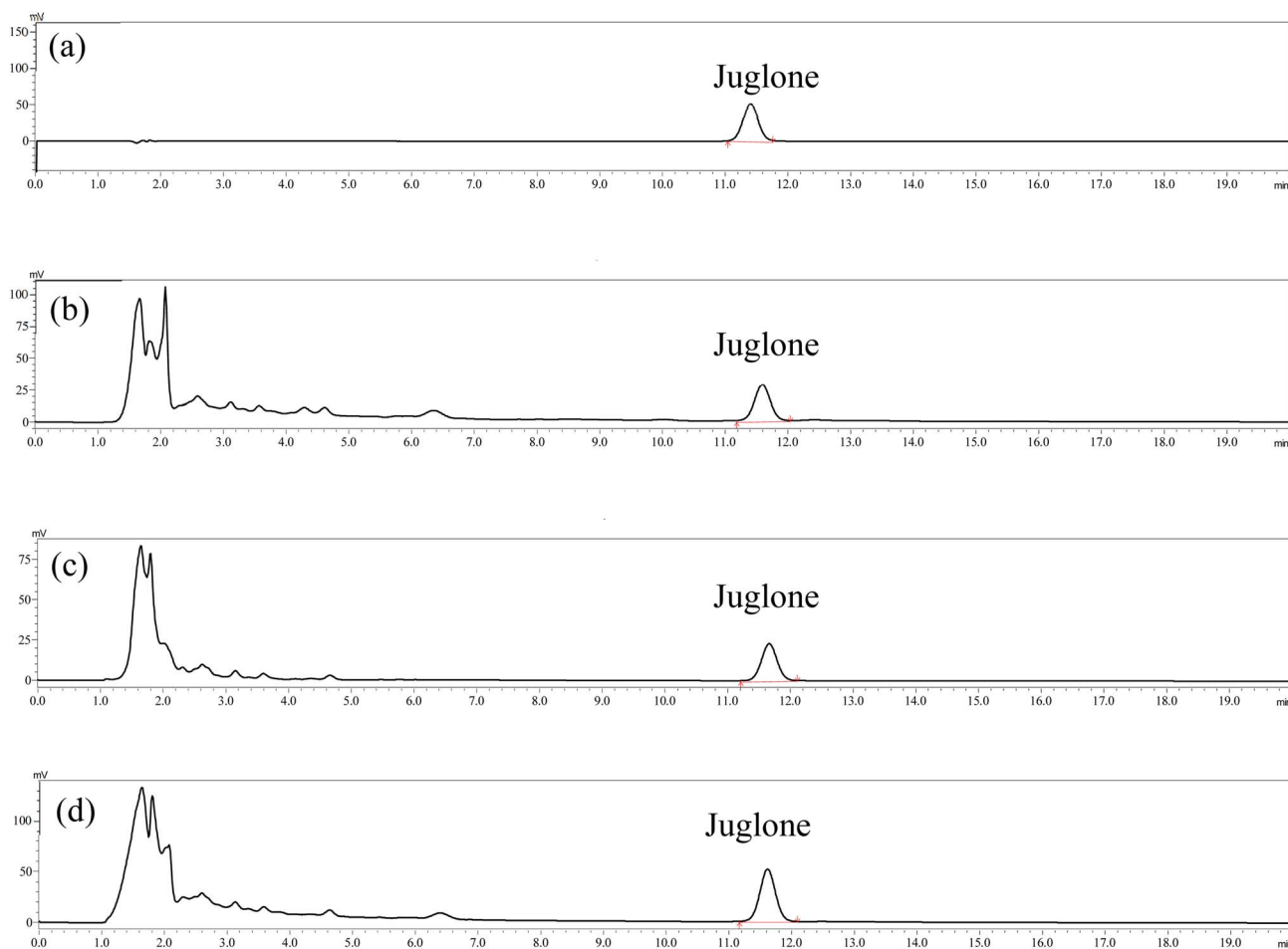
Fig. 7 shows the HPLC chromatograms of extracts obtained by three optimized extraction methods (HRE, traditional ME and MAATPE) and the juglone standard (among them, (a) measured using a  $0.10 \text{ mg mL}^{-1}$  juglone standard; (b) the HRE extract was diluted 200 times, and the measured concentration was  $0.056 \text{ mg mL}^{-1}$ ; (c) the ME extract was diluted 400 times, and the measured concentration was  $0.042 \text{ mg mL}^{-1}$ ; (d) the MAATPE extract was diluted 150 times, and the measured concentration was  $0.102 \text{ mg mL}^{-1}$ ). It can be found that the retention time of the juglone standard and the juglone obtained by the three extraction methods was about 11.5 min. There was no obvious impurity peak around the separated juglone, which indicated that the three methods could successfully extract juglone and that it was well separated in the chromatograms.

As shown in Table 3, compared with other extraction methods, MAATPE offers significant advantages in terms of yield, extraction time, and purity. Although HRE is simple to operate, the extraction time is too long and the yield is low. In addition, due to the prolonged high-temperature decoction involved in HRE, more impurities are dissolved.<sup>38</sup> UAE and MAE, as novel extraction methods, can improve the extraction efficiency by disrupting the cell wall. However, this will cause

**Table 3** The comparison of MAATPE with other methods for juglone extraction

Method	Extraction time	Juglone yield ( $\text{mg g}^{-1}$ )	Purity (%)
MAATPE	14 min	$15.36 \pm 0.16$	$9.82 \pm 0.12$
HRE	90 min	$11.26 \pm 0.12$	$3.73 \pm 0.06$
UAE	30 min	$12.47 \pm 0.34$	$3.67 \pm 0.15$
MAE	5 min	$11.39 \pm 0.21$	$3.81 \pm 0.05$
Traditional ME	12 min + 15 min	$16.93 \pm 0.19$	$3.14 \pm 0.09$
ME + ATPS	12 min + 15 min	$14.02 \pm 0.15$	$8.90 \pm 0.18$

more impurities to dissolve in the solvent, reducing the purity of juglone. In addition, the thermal effect caused by MAE can destroy juglone and reduce the extraction yield.<sup>36</sup> Similarly, traditional ME can destroy the cell wall and has a higher extraction efficiency; however, when using acidified aqueous solution to extract, a large number of water-soluble impurities such as proteins and polysaccharides are also extracted, so it often fails to achieve the desired product purity.<sup>19</sup> When ME and ATPS are operated separately, it can be observed that both the juglone yield and purity in the extract show significant improvement, which is attributed to the destruction of cell wall



**Fig. 7** HPLC chromatograms of the standard (a), extracts from HRE (b), extracts from traditional ME (c) and extracts from MAATPE (d).



by ME and the selective enrichment effect of the ATPS, respectively. However, compared to MAATPE, the extraction time of ME followed by ATPS is longer, almost twice as long as that of MAATPE, due to the intermittent operation of ME followed by ATPS. In short, it can be seen that MAATPE can not only improve the extraction efficiency, but also selectively enrich the target ingredients to obtain high-purity products.

### 3.6 Mechanism analysis

Based on the above research, the mechanism of MAATPE can be explained as shown in Fig. 8. When the raw materials are ground at high speed in a ball mill, the plant tissues are destructively damaged so that the active components in the BJM could be released and come into full and rapid contact with ATPS.<sup>16</sup> After the mechanochemical treatment, various compounds partition into the upper phase or the bottom phase under the combined effects of polarity and acidity. The whole process involves the following strengthening and purification stages. First, in most extraction processes, the solvent needs to penetrate plant cells by surface tension to dissolve the target product. In MAATPE, since plant cells are destroyed, the solvent can directly come in contact with the active substance, which greatly improves the extraction efficiency. Due to the action of mechanical external force, the structure of the cell wall changes greatly, which decreases the bond energy of chemical bonds such as cellulose and lignin, which constitute the cell wall, thus promoting the fracture of chemical bonds. In addition, the effect of strong mechanical force can also reduce the particle size of plant cells, increase the specific surface area, and make the chemical bonds in cells such as glycosidic bonds and acetal bonds in cellulose, ester bonds and ether bonds in lignin more exposed on the surface of particles. These chemical bonds can

interact with acidic ATPS solution and break. For example, glycosidic bonds in cellulose can be hydrolyzed into glucose under acidic conditions, and acetal bonds can be hydrolyzed to form the corresponding carbonyl structures under acidic conditions. The ester bond in lignin can be hydrolyzed under acidic conditions to form alcohol and acid, and the ether bond can be broken under acidic conditions to form alcohol. The breaking of these chemical bonds can make the plant tissue looser, allowing the active substances to be released further. Second, according to Fick's law, active compounds always diffuse from a region of high concentration to a region of low concentration, and the greater the concentration difference between the two regions, the faster the diffusion. In MAATPE, the high-speed rotation of the ball mill can quickly renew the solvent around the material, so that the solvent concentration outside the material is always maintained at a low level, thus increasing the external diffusion rate of the material. Third, most extraction methods often extract many non-target products while extracting the target compound, thus affecting the purity of the products. In MAATPE, after non-target products are extracted, they are selectively distributed in the upper and lower phases by ATPS. Juglone, polyphenols, flavonoids and other weak polar compounds were enriched in the upper phase, while proteins, polysaccharides and other strong polar and hydrophilic compounds entered the bottom phase. Due to the selective distribution of compounds in the ATPS, the purity of the target product enriched in the upper phase is significantly improved.

Moreover, the mechanical forces generated by the high-speed rotation of the ball mill, including shear force and compressive force, are exerted on the molecules, causing some changes in the density and viscosity of the solution system. And

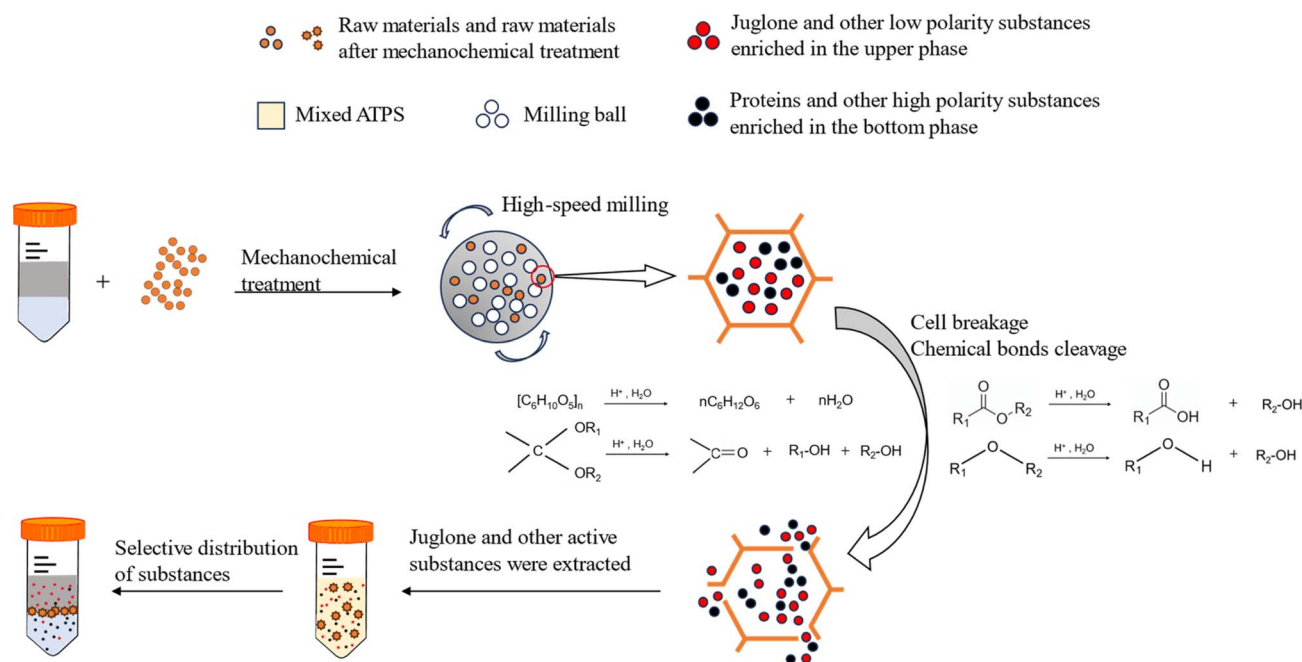


Fig. 8 Mechanism diagram of MAATPE.



as more substances gradually dissolve, the differences in density and viscosity between the two phases also increase, which is more conducive to the phase separation in the ATPS.<sup>39</sup> During the grinding process, the additional energy generated by friction enables the solution system to reach a new steady-state temperature, which further promotes the diffusion of molecules.<sup>40</sup> From a thermodynamic perspective, the free energy of the distribution process of juglone in the ATPS is  $\Delta G = -RT \ln K < 0$  (where  $K$  is the distribution coefficient of juglone in the upper phase under optimized conditions, and  $K = 24.18$ ), indicating that the selective distribution of juglone in ATPS occurs spontaneously. When the steady-state temperature rises, the driving force of the diffusion process also increases, which is more conducive to the enrichment of juglone.<sup>39</sup> Therefore, the MAATPE technique can achieve higher extraction efficiency in a relatively short time while ensuring higher purity of the extracted substances.

### 3.7 Solvent recycling

Recycling the inorganic salt solution in the bottom phase can not only significantly reduce the consumption of inorganic salts, but also eliminate the step of dissolving inorganic salts during the preparation of ATPS. Fig. 9 shows the performance of juglone extraction by MEATPE with the ATPS prepared using different concentrations of ethanol and different recovery times of the inorganic salt solution. When a relatively high-concentration ethanol solution ( $\geq 80\%$ ) is added to the recycled inorganic salt solution, the prepared ATPS can still maintain a high extraction efficiency in MEATPE. In particular, when the ATPS is formed by adding an 80% ethanol solution to the lower-phase solution, the extraction performance of MEATPE can still remain at 76.5% after 5 cycles of extraction. However, it can be observed that the extraction efficiency of juglone gradually decreases as the number of cycles increases. This is because the recycled bottom phase solution contains some impurities and a small amount of juglone remaining from the previous extraction, which decreases the concentration

difference and thus leads to a reduction in the amount of newly dissolved juglone.

### 3.8 Scalability of MEATPE

The scalability of the proposed MAATPE method is a critical factor for its practical application and industrial implementation. There are several key aspects that support the scalability of this method. The mechanochemical pretreatment is a simple and efficient process that can be easily scaled up using industrial-grade ball mills or grinding equipment.<sup>41</sup> The ATPS, which relies on water-soluble short-chain alcohol and salts, is also inherently scalable due to its simplicity and the availability of inexpensive, environmentally friendly materials. Compared to traditional extraction methods (*e.g.*, heat reflux extraction or organic solvent-based methods), the MAATPE method requires less energy because it operates at room temperature, avoiding the need for extensive heating or evaporation processes. Besides, MAATPE not only minimizes organic solvent consumption by 93%, thereby significantly reducing costs associated with solvent handling, storage, and disposal, but also cuts energy consumption by 80% and the carbon footprint per kg of extracted product decreases by 76% *versus* organic solvent-based methods. These advantages align the method with green chemistry principles, enhancing its appeal for large-scale industrial applications.<sup>42</sup> In this study, mechanochemical and aqueous two-phase systems were integrated into one system for extraction, which greatly improved the extraction efficiency.<sup>42</sup> We optimized the effects of key factors such as the short-chain alcohol/salt ATPS type, sodium dihydrogen phosphate concentration, ethanol concentration, solvent-to-material ratio, ball milling speed, and ball milling time on the extraction yield of the target product, providing a reference for large-scale industrial applications. In conclusion, the MAATPE method is highly scalable and holds great potential for industrial applications.

## 4. Conclusions

In this study, MAATPE was successfully applied to the extraction of juglone from BJM, which significantly improved the purity of the product while maintaining a high extraction yield by replacing the solid grinding agent in traditional ME with ATPS. The key factors affecting the extraction effect were systematically explored through single factor experiments and RSM analysis, and the optimal extraction conditions were determined as follows: 21.5% (w/w) ethanol concentration, 28% (w/w)  $\text{NaH}_2\text{PO}_4$  concentration, 458 rpm milling speed, 14 min milling time and 59  $\text{mL g}^{-1}$  reagent–material ratio. Under these conditions, the yield of juglone was  $15.36 \pm 0.16 \text{ mg g}^{-1}$ , and the purity was  $9.82 \pm 0.12\%$ . Compared with other extraction methods, MAATPE not only maintains high extraction efficiency but also significantly improves the product purity. Moreover, MAATPE can effectively recycle solvents, making it more environmentally friendly and cost-saving, and it has great potential for industrial applications. Therefore, MAATPE is a suitable method for the efficient preparation of high-purity products from different plant materials and is worthy of further investigation.

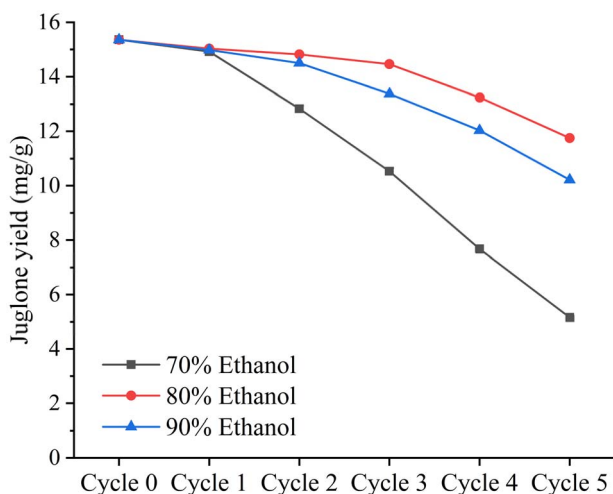


Fig. 9 Determination of the recovery performance of ATPS.



## Data availability

The data that support the findings of this study are available on request from the corresponding author.

## Conflicts of 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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