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Optimization of ultrasound extraction of *Alisma orientalis* polysaccharides by response surface methodology and their antioxidant activities

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ABSTRACT

Rhizoma alismatis (the rhizome of *Alisma orientalis*) polysaccharides (RAP) have been reported to have a variety of important biological activities. However, effective extraction of RAP has been an unsolved issue. In this study, we used an ultrasound method for high yield extraction of RAP and optimized the conditions using the response surface methodology (RSM). Following multiple regression analyses of the experimental results, we applied the 3-D response surface and the contour plots to determine the optimal conditions, which were found to be ultrasound treatment at 76.1 °C for 75.2 min, and water to material ratio at 30.1 ml/g. Under such conditions, the yield was 6.90% which was much higher than traditional hot water extraction yield (3.41%). The fractionated RAPs following stepwise ethanol precipitation showed strong antioxidant activities. The results indicated that ultrasound extraction was a very effective method for the extraction of RAP and the polysaccharides could be explored as a potential antioxidant agent for use in medicine or functional food.

1. Introduction

Rhizoma alismatis (RA) is the rhizome of *Alisma orientalis* (Sam.) Juzep, which belongs to the family *Alismataceae* and is mainly distributed in China, Japan, and India (Han et al., 2013; Wu, 2005). RA is recorded as a high-grade drug in Shennong Bencao Jing, the first drug monograph of ancient China (Gu, 2007). As a folk medicine, it has been prescribed for treatment of hypertension, hyperlipemia and diabetes (China, 2005; Li & Qu, 2012; Wu, 2005), and is included in the Pharmacopoeia of China as one of the traditional Chinese herbal medicines (China, 2005). Recently, interest in RA is growing, because many of its components, such as triterpene, alisol B, and especially polysaccharides, have been reported to have important bioactivities, including anti-hepatitis, anti-inflammatory, hypoglycemic and immunological effects (Han et al., 2013; Jiang et al., 2006; Lee et al., 2013; Li & Qu, 2012; Shimizu, Ohtsu, Tomoda, Gonda, & Ohara, 1994; Tomoda, Gonda, Shimizu, & Ohara, 1994).

The RA polysaccharides (RAP) have been documented for their effects of against lipid peroxidation (Li, Jin, & Zhang, 2008), which are potentially important for human health. However, there are few studies on effective extraction of RAP. Conventional methods, such as those by hot water, immersion or Soxhlet, are often time-consuming and expensive, with low yield of RAP or even loss of some of their pharmacological activities (Li, Ding, & Ding, 2007; Wang, Cheng, Mao, Fan, & Wu, 2009). Ultrasound extraction technology has several advantages, such as reasonably high extraction yield, moderate solvent requirement, relatively short extraction time, etc., and so has been used to extract high quality bioactive polysaccharides from plant materials (Hromadkova & Ebringerova, 2003; Hromadkova, Ebringerova, & Valachovic, 2002; Pan et al., 2010; Ying, Han, & Li, 2011). Hence, ultrasound extraction technique was used to improve the extraction yield of RAP with minimally impact on their bioactivity. To apply this method for RAP, we optimized the extraction conditions and procedure using the response surface methodology (RSM), which is an effective tool for optimizing the experimental process when many factors and interactions may affect extraction yield (Box & Wilson, 1951; Prakash Maran, Manikandan, Thirugnanasambandham, Vigna Nivetha, & Dinesh, 2013). The main advantage of RSM is the considerably reduced number of tests potentially needed for evaluating

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multiple variables and their interactions, making it less laborious than many other approaches for process optimization.

In this study, based on the results of single factor experiments, three major influence factors (ultrasound extraction time, ratio of water to material, ultrasound extraction temperature) were used to optimize the experimental conditions for extraction of RAP. Then, according to different solubility of different molecular weight polysaccharides in ethanol, two major polysaccharides were obtained using stepwise ethanol precipitation, and both of them exhibited strong antioxidant activities.

2. Materials and methods

2.1. Materials

The plant of *A. orientalis* was collected from Heilongjiang Province, China. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH•) was purchased from the Sigma Chemical Co. (St. Louis, MO, USA). D-Glucose was bought from Sigma-Aldrich (USA). Ultra-pure water was used throughout the experiments.

2.2. Extraction of RAP and determination of the yield

The dried RA was ground into fine powder by a disintegrator (WND-200, Weinengda Instrument Co., Ltd., Zhejiang, China), and then was extracted three times with 85% ethanol at 60 °C, each for 5 h, to remove lipids, pigments, amino acids, monosaccharide and oligosaccharides according to our previous work (Zhao, Xu, Ye, & Dong, 2013). The insoluble parts were separated from the solvent by filtration and dried (60 °C, 24 h). The polysaccharides were then extracted from the pretreated dry powder in a designated ultrasound extraction time, ratio of water to material, and ultrasound extraction temperature by an ultrasonic cleaner (KQ-100VDB, Kun Shan Ultrasound Instrument Co., Jiangsu, China, 45 kHz) using the methods of Zhao et al. (2013) and Prakash Maran et al. (2013) with minor modifications.

The supernatants were separated from insoluble residue by vacuum filtration and then were treated by the Sevag method (Jia et al., 2014; Sevag, Lackman, & Smolens, 1938) three times to remove protein and then intensively dialyzed for two days against distilled water (cut-off *Mw* 3500 Da). The resulting solutions were precipitated by addition of dehydrated ethanol to a final concentration of 80% (v/v) and kept at 4 °C overnight. The precipitates were collected by centrifugation and lyophilized to get the RAP. The sugar content was measured by phenol-sulfuric method using D-Glucose as a standard (Balavigneswaran, Sujin Jeba Kumar, Moses Packiaraj, Veeraraj, & Prakash, 2013; Dubois, Gilles, Hamilton, Rebers, & Smith, 1951). The yield (%) of RAP was then calculated using the following equation:

$$\text{Yield}\ \ (\%) = \frac{W_p}{W_r} \times 100 \tag{1}$$

where W_p is the weight of polysaccharides, and W_r is the weight of raw material.

2.3. Experimental design

2.3.1. Single factor experiments

According to the results of preliminary experiments (Fig. S1), three major influence factors (ultrasound extraction time, ratio of water to material, ultrasound extraction temperature) were selected for next experiments. The effects of the above three factors on the extraction yield of RAP were studied by single factor design. In detail, the single factor experiment was performed in a designed ultrasound extraction time (range from 30 to 90 min), ratio of water to material (range from 10 to 50 ml/g), and ultrasound extraction temperature (range from 40 to 80 °C). One factor was changed, while the other factors kept constant in each experiment. The effect of each factor was evaluated by determining the extraction yield of RAP.

Table 1
Independent variables and their levels in Box–Behnken design.

Independent variables	Symbol	Level		
		−1	0	1
Ultrasound extraction time (min)	X_1	60	75	90
Ratio of water to materials (ml/g)	X_2	20	30	40
Ultrasound extraction temperature (°C)	X_3	60	70	80

2.3.2. Optimization of extraction conditions by Box–Behnken design (BBD)

On the basis of the single factor experiment results, the ranges of each factor were confirmed, and then a three variables (X_1, ultrasound extraction time; X_2, ratio of water to material; X_3, ultrasound extraction temperature), three levels Box–Behnken design (Design Expert software, Trial Version 6.0.5, Stat-Ease Inc., Minneapolis, MN) was used to evaluate the best conditions for the extraction of RAP. For statistical calculation, the above three values were coded as the following equation:

$$\chi_i = \frac{(X_i - X_0)}{\Delta X} \quad i = 1, 2, 3 \tag{2}$$

where χ_i is the coded value of the variable, X_i is the actual value of variable, X_0 is the actual value of the X_i on centre point, and ΔX is the step change value. The range of independent variables and their levels were presented in Table 1. The experimental runs for BBD were shown in Table 2. Each experimental run was performed in triplicate except the five centre points and the averages of polysaccharide yield were taken as response.

In order to predict the optimized conditions, a second-order polynomial model (Eq. (3)) was fitted to correlate the relationship between the independent variables and the response (polysaccharides yield).

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \beta_{ii} X_i^2 + \sum_{i \neq j} \beta_{ij} X_i X_j \tag{3}$$

Table 2
Box–Behnken experimental design and the results for extraction yield of *Rhizoma alismatis* polysaccharides ($n = 3$).

Run	Coded variable levels			Yield of polysaccharides (%)[a]
	X_1	X_2	X_3	
1	−1	−1	0	3.98
2	1	−1	0	4.18
3	−1	1	0	4.80
4	1	1	0	3.55
5	−1	0	−1	4.75
6	1	0	−1	3.34
7	−1	0	1	5.09
8	1	0	1	5.49
9	0	−1	−1	3.42
10	0	1	−1	3.26
11	0	−1	1	5.93
12	0	1	1	5.94
13	0	0	0	6.82
14	0	0	0	6.70
15	0	0	0	6.77
16	0	0	0	6.73
17	0	0	0	6.76

[a] Each value is the mean of triplicate measurements.

where Y is the predicted response; X_i and X_j are the coded values of independent variables; and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively.

The responses obtained from each set of experimental design (Table 2) were subjected to multiple non-linear regressions using Design Expert software to plot response surface graphs. The analysis of variance tables was generated, and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The values of R^2, adjusted-R^2 of models were evaluated to check the model adequacies. Additional confirmation experiments at the optimized conditions were subsequently conducted to verify the validity of the statistical experimental design. The p-values of less than 0.05 were considered to be statistically significant.

2.4. Comparison with traditional hot water extraction (THWE)

Based on the results of preliminary experiments, the pretreated dry powder (10.0 g) was reflux extracted twice in a water bath at extraction time 120 min, ratio of water to material 30 ml/g, extraction temperature 75 °C. According to the procedure described in Section 2.2, the RAP was obtained and the extraction yield was determined.

In order to investigate the structural alteration during different extraction methods, the Fourier transform infrared (FTIR) spectra of samples extracted with two methods (ultrasound extraction and THWE) were recorded on an FTIR spectrophotometer (IRPresting-21, Shimadzu Co., Japan). The dried sample was ground with potassium bromide powder and pressed into pellet for spectrometric measurement in the frequency range of 4000–400 cm^{-1} at the resolution of 4 cm^{-1} and a maximum source aperture.

2.5. Preparation and fractionation of RAP

The pretreated dry powder of *R. alismatis* was extracted by the above mentioned method in Section 2.2. Stepwise ethanol precipitation method was used to fractionate RAP. The procedure of fractionation was described as follows: dehydrated ethanol was slowly added to the extraction solution to the ethanol concentration of 40% (v/v). Then the mixed solution was kept at 4 °C overnight and the precipitate (RAP-I) was collected by centrifugation and lyophilization. The supernatant was subsequently added with ethanol to a final concentration of 80% (v/v) to precipitate second fraction of polysaccharide (RAP-II).

2.6. Infrared spectroscopy and molecular weight of RAP

The characteristic absorption of crude RAP, RAP-I and RAP-II were identified by the FTIR spectrum. The powder of crude RAP and its purified fractions was mixed with KBr powder, grinded and pressed for FTIR measurement. The procedure was identical as described in Section 2.4. The molecular weights of RAP, RAP-I and RAP-II were determined by viscosimeter (Zhou, Guo, Cai, & Li, 2005).

2.7. Determination of antioxidant activity

2.7.1. Assay of DPPH free radical scavenging activity

The scavenging activity on DPPH free radical (DPPH·) was measured as described previously (Zhao et al., 2013), with slight modification. Briefly, 0.1 mM solution of DPPH· in methanol was prepared and 1.0 ml of this solution was added to 3.0 ml of the polysaccharides at various concentrations (0.05, 0.1, 0.2, 0.4 and 0.8 mg/ml) in water. The mixture was shaken and incubated at 25 °C for 30 min in the dark. Then the absorbance was measured at 517 nm (UV-2550, Shimadzu Co., Japan). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The scavenging percentage was calculated by the following equation:

$$\text{Scavenging activity } (\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100 \quad (4)$$

where A_0 is the absorbance of the blank control (water instead of sample solution), A_1 is the absorbance of the sample and A_2 is the absorbance of the sample under identical conditions as A_1 with water instead of DPPH• solution.

2.7.2. Assay of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of polysaccharides was measured according to Fenton method described before (Halliwell, 1987; Li et al., 2013), with minor modifications. Samples were dissolved in distilled water to form final concentrations of 0.05, 0.1, 0.2, 0.4 and 0.8 mg/ml and incubated (37 °C, 30 min) with 9.0 mM $FeSO_4$ (1.0 ml), 0.3% H_2O_2 (1.0 ml) and 0.5 ml salicylic acid–ethanol solution (9.0 mM). The total volume of the mixture in each tube was made up to 10 ml by adding the required amount of distilled water. The absorbance of the mixture was read at 510 nm against a blank. The scavenging activity on •OH was calculated by the following equation:

$$\text{Scavenging activity } (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (5)$$

where A_0 and A_1 represent the absorbance of blank control group and sample group under 510 nm.

2.7.3. Assay of superoxide anion radical scavenging activity

Superoxide anion radical was generated in the system of pyrogallol's autoxidation in an alkalescent condition. The scavenging effect on superoxide anion radical ($O^{2\bullet}$) was measured as described previously (Balavigneswaran et al., 2013; Qi et al., 2006) with a minor modification.

Briefly, samples at different concentrations (0.05, 0.1, 0.2, 0.4 and 0.8 mg/ml) and 50 mM Tris–HCl buffer (pH 8.2) were incubated at 25 °C for 20 min, then 25 mM pyrogallol at the same temperature was added to the mixture and the reaction was proceed for 5 min. 1 ml of 8 mM HCl was added quickly to terminate reaction. The absorbance of the mixture was determined at 299 nm. The superoxide anion scavenging activity was calculated by using the equation:

$$\text{Scavenging activity } (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (6)$$

where A_0 is the absorbance without sample and A_1 is absorbance with sample.

2.7.4. Assay of erythrocyte hemolysis inhibitory activity

The experiment was conducted by the method (Ko, Hsiao, & Kuo, 1997) with a little modification. In the experiment, we followed the Guidelines for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Animal Ethical Committee of Harbin Medical University. Adult Male Wistar Rats with a mean weight of 150–200 g were from the Animal Research Center of Harbin Medical University accredited by the Institutional Animal Care and Use Committee (IACUC). Controlled ambient temperature of 22–24 °C, 50% relative humidity and a 12 h-light–dark cycle, food and water were provided to all rats. Briefly, about 2.0 ml blood from healthy rats was collected into centrifugal tubes containing heparin. Blood was immediately centrifugated at 2500 rpm for 5 min to get erythrocytes. Erythrocytes were washed three times with isotonic saline at 4 °C and resuspended in isotonic saline to obtain a mixture of

2% (v/v) red blood cell suspension (RBCs). Test samples with different concentrations (0.1, 0.5 mg/ml) were incubated with RBCs and hydrogen peroxide (7.5 mM) at 37 °C in the presence of 5% CO_2 for 3 h, the blank control (isotonic saline instead of sample solution) were conducted in the same way. After incubation, 0.2 ml incubation mixture was taken out, diluted with 4.0 ml of isotonic saline and centrifuged at 2500 rpm for 5 min. Absorption (A) of the supernatant was measured at 540 nm on a spectrophotometer. The same volume of mixture was treated with 4.0 ml ice cold distilled water in order to get a complete hemolysis, and the absorption (B) was read at the same wavelength. The ratio of erythrocytes hemolysis was calculated as (A/B) × 100%.

2.8. Statistical analysis

All the experiments were carried out in triplicate, and the data presented are the mean values of these independent experiments. Statistical analysis of RSM was performed using Design-Expert (Version 8.0, Stat-Ease). For multiple comparisons, analysis of variance (ANOVA) was used, and p values of <0.05 were considered statistically significant.

3. Results and discussion

3.1. Single factor experiments

In this part, ultrasound extraction time, ratio of water to material, and ultrasound extraction temperature were investigated, respectively. The results of single factor experiments are presented in Fig. 1.

3.1.1. Effect of ultrasound extraction time on polysaccharides yield

Ultrasound extraction time is an important parameter of polysaccharides extraction (Chen et al., 2012). In this work, ultrasound extraction time was set at 30, 45, 60, 75, and 90 min, respectively, to investigate its influence on the yield of polysaccharides extraction when other parameters were set as follows: ratio of water to material 30 ml/g and ultrasound extraction temperature 70 °C. According to Fig. 1a, the yield of polysaccharides increased with the increase of ultrasound extraction time. The results indicated that a longer extraction time presents a positive effect on the yield of RAP. This might be due to the time requirement of the exposure of the RAP to the release medium where the liquid penetrated into the raw materials, dissolved the RAP and subsequently diffused out from the raw materials (Samavati, 2013). After 75 min, a longer ultrasound extraction time resulted in a slightly increased yield of polysaccharides which indicated that the yield of polysaccharides started to maintain a dynamic equilibrium with increasing extraction time. This is probably because the polysaccharides in plant cells have been already sufficiently extracted up (Hou, Zhang, Xiong, Li, & Yang, 2008; Ray et al., 2004).

3.1.2. Effect of ratio of water to material on polysaccharides yield

Many studies indicated that different ratio of water to material will significantly affect the yield of polysaccharides (Chen et al., 2012; Li et al., 2013; Ying et al., 2011). In this work, ratio of water to material was set at 10, 20, 30, 40, and 50 ml/g when other parameters were set as follows: ultrasound extraction time 60 min and ultrasound extraction temperature 70 °C. As can be seen in Fig. 1b, the polysaccharides yield increased with increasing ratio of water to material, and the highest value was obtained at the ratio of 30 ml/g. This is because a larger ratio of water to material implied greater concentration difference between the interior plant cells and the exterior solvent, and leaded to the increase in the driving force for the mass transfer of polysaccharides. But after 30 ml/g the curve leveled off, meaning that further increase of ratio of water to material would not increase the extraction yield of polysaccharides. This might be due to the reason that a higher ratio of water to material prolonged the distance of diffusion towards the interior tissues and caused big loss during production collection (Ying et al., 2011).

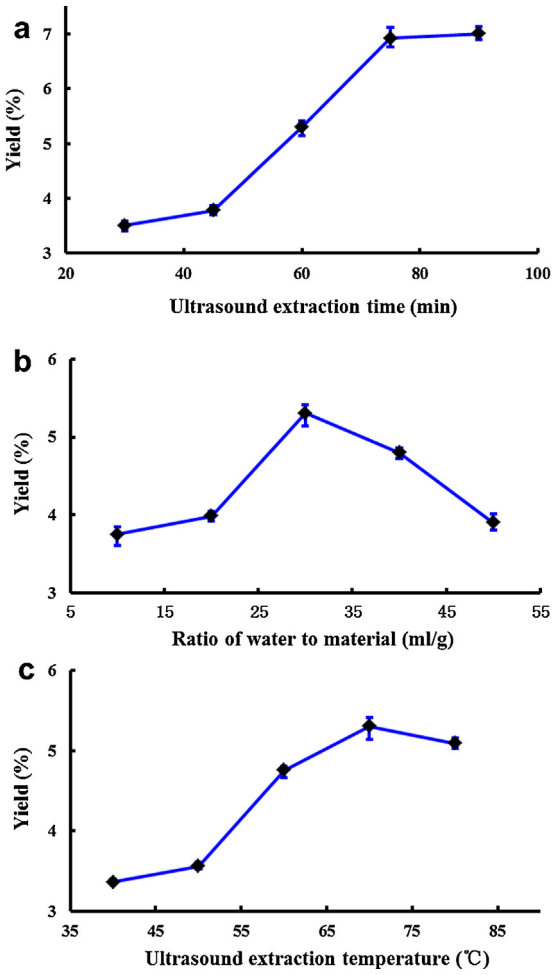

Fig. 1. Effect of ultrasound extraction time (a), ratio of water to material (b) and ultrasound extraction temperature (c) on the extraction yield of RAP (n = 3).

3.1.3. Effect of ultrasound extraction temperature on polysaccharides yield

As shown in Fig. 1c, the effect of ultrasound extraction temperature on extraction yield of polysaccharides was studied. The extraction process was carried out at 40, 50, 60, 70, and 80 °C, while other parameters were set as follows: ultrasound extraction time 60 min and ratio of water to material 30 ml/g. Fig. 1c indicated that the yield significantly increased when the temperature increased from 40 to 70 °C, and reached the peak value (5.30%). The positive effect of ultrasound extraction temperature could be explained by the higher solubility of polysaccharides in the solvent, the higher diffusivities of polysaccharides and the improved mass transfer at higher temperature. And then the yield of RAP started to decrease when ultrasound extraction temperature continued to rise. A possible explanation is that the polysaccharides may be hydrolyzed at a high temperature. This tendency is in good agreement with other previous studies (Li et al., 2013; Ying et al., 2011).

According to the results of single factor experiments, we adopted an ultrasound extraction time of 60–90 min, ratio of water

Table 3
Analysis of variance of the experimental results of the BBD.

Variables	Sum of squares	DF	Mean square	*F* value	*p* Value Prob > *F*
Model	28.38	9	3.15	23.64	0.0002[a]
X_1	0.53	1	0.53	3.98	0.0864
X_2	0.02	1	0.02	0.15	0.9702
X_3	7.37	1	7.37	55.27	0.0001[a]
$X_1 \times X_2$	0.53	1	0.53	3.94	0.0875
$X_1 \times X_3$	0.82	1	0.82	6.14	0.0423[a]
$X_2 \times X_3$	0.07	1	0.07	0.54	0.8226
$X_1 \times X_1$	7.13	1	7.13	53.45	0.0002[a]
$X_2 \times X_2$	7.46	1	7.46	55.94	0.0001[a]
$X_3 \times X_3$	2.64	1	2.64	19.76	0.0030[a]
Residual	0.93	7	0.13		
Cor total	29.31	16			
R^2	0.9966				
Adj R^2	0.9922				
Pred R^2	0.9455				
Adeq Precision	36.839				
C.V. %	3.53				

[a] Means significance (significance level 0.05).

to material of 20–40 ml/g, and ultrasound extraction temperature of 60–80 °C for the RSM experiments.

3.2. Optimization of extraction conditions by BBD

As shown in Table 2, each experiment in the design matrix was performed and the experimental data were obtained. The data were analyzed by multiple regression analysis using the Design Expert software to get the following polynomial equation:

$$Y = 6.760 - 0.258X_1 + 0.005X_2 + 0.960X_3 - 0.363X_1X_2 + 0.453X_1X_3 + 0.043X_2X_3 - 1.301X_1^2 - 1.331X_2^2 - 0.791X_3^2 \quad (7)$$

The *F*-test and *p*-value were used to measure the significance of the coefficients of the model and results were shown in Table 3. The corresponding variables would be more significant if the absolute *F*-value becomes greater and the *p*-value becomes smaller. The analysis of variance (ANOVA) of the quadratic regression model demonstrated that the model was highly significant ($p = 0.0002$) and the result suggested that the model is adequate for predicting within the range of the variables employed. It also can be seen that the variables with the significant effects on the yield of RAP were the linear terms (X_3), the quadratic terms ($X_1 \times X_1$, $X_2 \times X_2$ and $X_3 \times X_3$) and the interaction between X_1 and X_3. As can be seen in Table 3, the determinant coefficient (R^2) and the coefficient of variation were 0.9966 and 3.53% which indicated that the polynomial model equation had a high quality fit, a good precision and reliability.

Response surfaces were plotted by Design Expert software to explain the interactions of the variables and to determine the optimal level of each variable for the maximum response. Three-dimensional response surfaces and two-dimensional contours were shown in Figs. 2 and 3. Each figure showed the effects of two factors on the polysaccharidesyield while the other one was kept at zero level. The 3-D plot and the contour plot in Figs. 2a and 3a, which set the ultrasound extraction temperature at zero level, showed that the yield of polysaccharides increased with increasing of ultrasound extraction time and ratio of water to material at the initial stage and then slightly decreased. Figs. 2b and 3b showed the 3-D plot and the contour plot at varying ultrasound extraction time and ultrasound extraction temperature. From the figures, it can be seen that the yield of polysaccharides increased with increasing of ultrasound extraction time and ultrasound extraction temperature. But further increasing of ultrasound extraction temperature would not increase the extraction yield of polysaccharides. The results are in accordance with single factor test and the ANOVA analysis. The 3-D plot and the contour plot based on independent variables ratio of water to material and ultrasound extraction temperature were shown in Figs. 2c and 3c, while the ultrasound extraction time was kept at zero level. The variation trends of polysaccharides yields of ratio of water to material and ultrasound extraction temperature were similar as mentioned above.

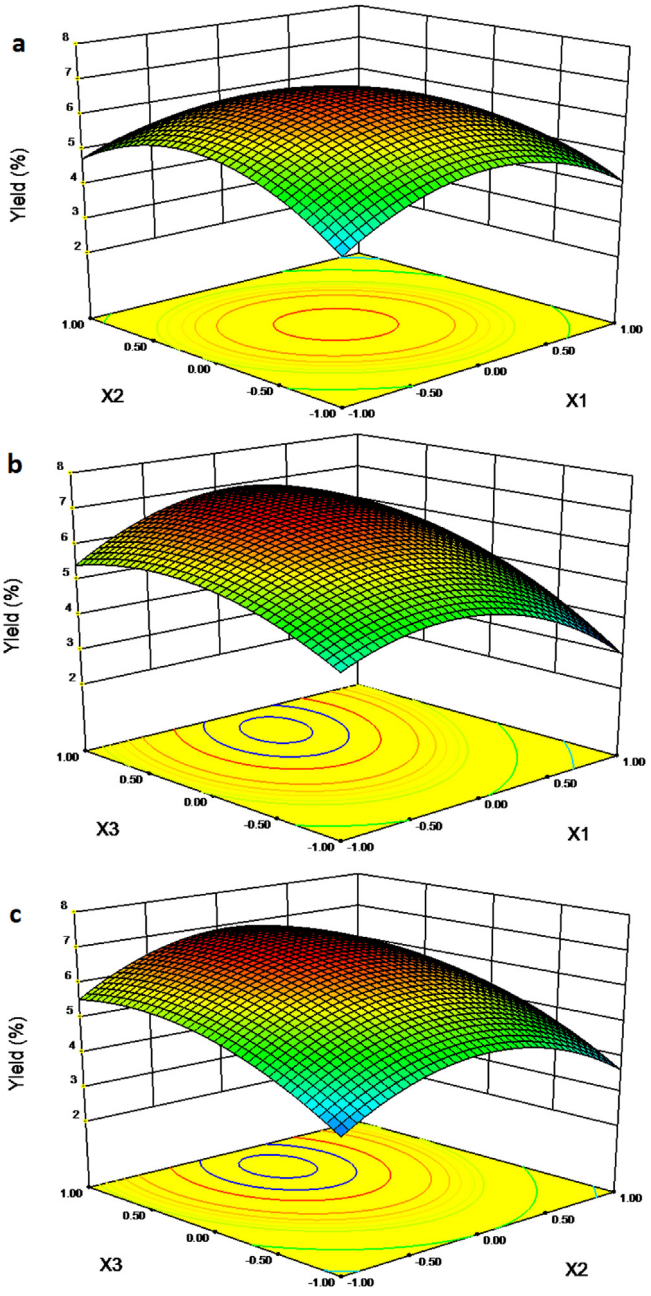

Fig. 2. Response surface polts showing the effects of variables (X_1: ultrasound extraction time, min; X_2: ratio of water to material, ml/g; and X_3: ultrasound extraction temperature, °C) on the extraction yield of RAP.

The analysis of response surface was performed by Design Expert software to determine the optimal extraction conditions. The optimal extracting conditions were ultrasound extraction time 75.2 min, ratio of water to material 30.1 ml/g, and ultrasound extraction temperature 76.1 °C, respectively. The maximum predicted yield of RAP was 7.05%.

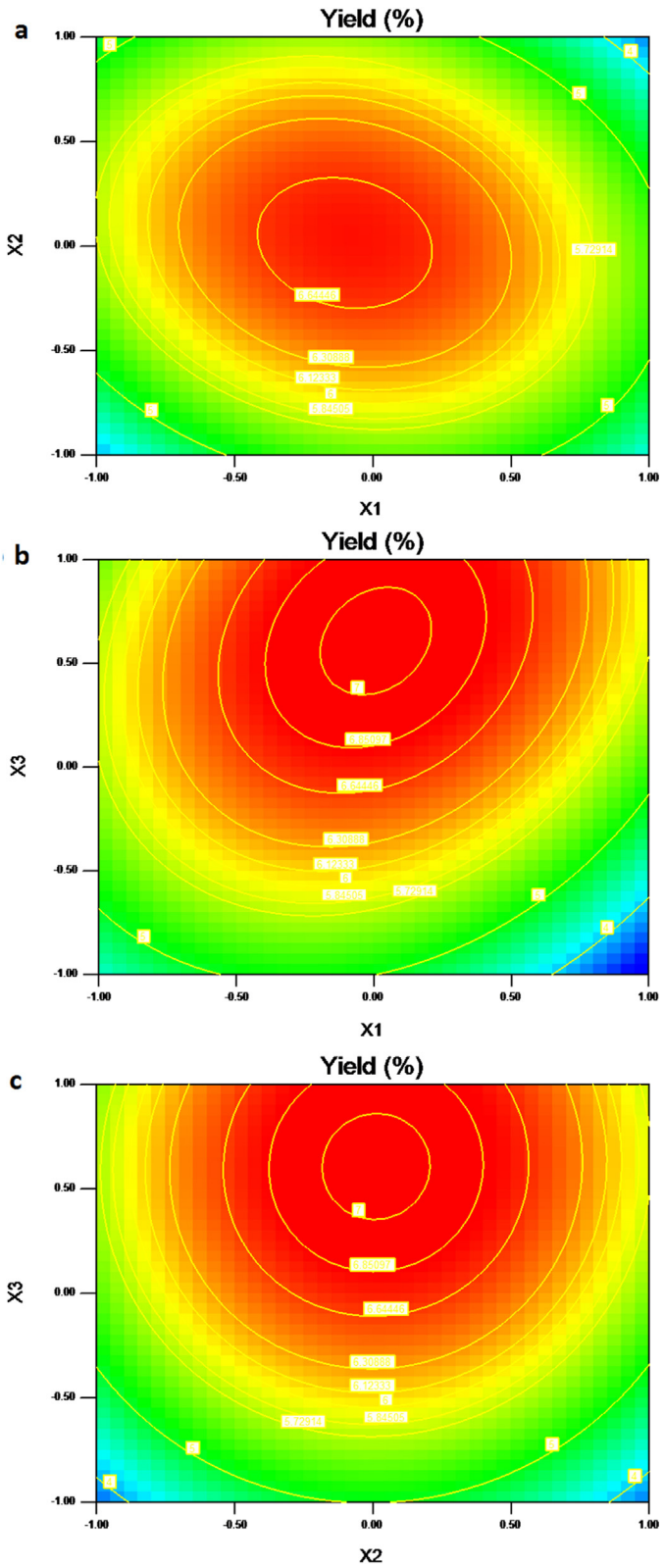

Fig. 3. Contour polts showing the effects of variables (X_1: ultrasound extraction time, min; X_2: ratio of water to material, ml/g; and X_3: ultrasound extraction temperature, °C) on the extraction yield of RAP.

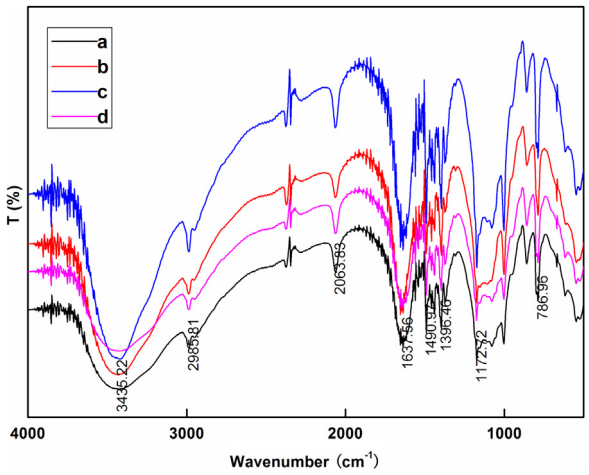

Fig. 4. FTIR spectra of polysaccharides from *Rhizoma alismatis* ((a) RAP; (b) RAP-I; (c) RAP-II; (d) RAP extracted by THWE).

3.3. Validation of the model

The suitability of the model equation for predicting the optimum response values was tested by using the selected optimal conditions. Under the optimal conditions, a mean value of 6.90%, which was obtained from real experiments, demonstrated the validation of the extraction model. The good correlation between these results confirmed that the model was adequate for reflecting the expected optimization.

3.4. Comparison with traditional hot water extraction (THWE)

Compared with traditional hot water extraction (THWE), the application of ultrasound extraction positively affected the extraction yield of RAP (Table 4). The extraction time was shortened greatly compared with THWE. This is because ultrasound radiation could accelerate the extracting process and may improve extraction of bioactive compounds. It was confirmed that ultrasound extraction should be an appropriate and effective extraction technique for polysaccharides from *R. alismatis*.

In order to prove the effect of ultrasound extraction and THWE on functional groups of polysaccharides, IR spectra of RAP extracted with two methods (ultrasound extraction and THWE) were recorded. As shown in Fig. 4a and d, there was a broad and strong absorption peak of polysaccharides extracted by two different methods at 3435 cm^{-1}, which attributed to hydroxyl groups. A weak —CH stretching peak near 2900–3000 cm cm^{-1} was displayed. A strong absorption peak at 1600–1700 cm^{-1} attributed to carbonyl group. A peak at 1400 cm^{-1} was an indication of the presence of carboxyl group. According to the IR spectra analysis, the functional groups of polysaccharides extracted by the two methods are fundamentally identical.

3.5. Infrared spectroscopy and molecular weight of RAP

As can be seen in Fig. 4a–c, the IR spectra of RAP, RAP-I and RAP-II were basically indistinguishable only with some difference in the intensity of bands. The absorption bands within the ranges of 3600–3200, 3000–2800 and 1250–1000 cm^{-1} were the characteristic absorption peaks of polysaccharides which were also can be found in the IR spectra of glucan (Xia, Dai, Fang, & Chen, 2007). Those results indicated that the RAP has the same main structure and similar typical functional groups with glucan. The strong and broad peak around the 3435 cm^{-1}, which was belong to the hydroxyl groups stretching vibration (Wang et al., 2012b; Xia

Table 4
Comparison of ultrasound extraction and THWE in extraction conditions and yield of polysaccharides from *Rhizoma alismatis* ($n = 3$).

Methods	Extraction time (min)	Ratio of water to material (ml/g)	Extraction temperature (°C)	Yield (%)[a]
Ultrasound extraction	75.2	30.1	76.1	6.90
THWE	120	30	75	3.41

[a] Each value is the mean of triplicate measurements.

et al., 2007). The weak peaks around 2980 cm^{-1} and 1390 cm^{-1} were assigned to the C—H asymmetric stretching vibration (Xia et al., 2007). The band around 1630 cm^{-1} was carbon-oxygen double bond (C=O) asymmetric stretching vibration absorption peak (Černá et al., 2003). A strong extensive absorption in the region of 1000–1200 cm^{-1} due to stretching vibrations of C—O—C and C—OH side groups were observed in the spectra (Coimbra, Goncalves, Barros, & Delgadillo, 2002). Furthermore, a characteristic band at 850 cm^{-1} was due to α-type glycosidic linkages (Xia et al., 2007). The molecular weights of RAP, RAP-I and RAP-II were 118.92 kDa, 140.39 kDa and 103.13 kDa, respectively.

3.6. Antioxidant activity of RAP

3.6.1. DPPH free radical scavenging activity

The DPPH free radical is a stable free radical and can be reduced by accepting an electron or hydrogen in the presence of an antioxidant. It has been widely used to estimate the free radical scavenging activities of antioxidants (Li et al., 2013; Ye & Huang, 2012). Fig. 5a depicted the DPPH scavenging abilities of different concentrations of polysaccharides samples. All the samples showed obvious scavenging activity on DPPH radical in a concentration-dependent manner. But the scavenging activity did not increase obviously after the concentration of the samples was higher than 0.4 mg/ml. RAP-II showed stronger DPPH scavenging activity than other samples and there is no obvious difference scavenging activity between RAP and RAP-I. The effect of antioxidants on DPPH scavenging was influenced by many factors and some references have reported that hydroxyl group of monosaccharide unit can donate proton to combine with unpaired electron of DPPH to form non-radical DPPH-H (Chen et al., 2012; Zhang, Yu, Zhang, Zhao, & Dong, 2014).

3.6.2. Hydroxyl radical scavenging activity

Hydroxyl radical which is one of the most harmful ROS can easily cross cell membranes and react with most biomolecules including carbohydrates, proteins, lipids, and DNA leading cell death (Vrchovská et al., 2006). Thus, removing hydroxyl radical is important for the protection of living systems. The results of hydroxyl radical scavenging activities were given in Fig. 5b. The scavenging effects of all samples increased with the increase of sample concentration at relatively lower concentration (<0.2 mg/ml). This result proved that all RAP samples had potential antioxidant ability of scavenging hydroxyl radical. When scavenging activity reached the plateau, the RAP-I showed the lowest scavenging activity, and scavenging activities of RAP and RAP-II were similar. The effect of antioxidants on hydroxyl radical scavenging might due to hydroxyl

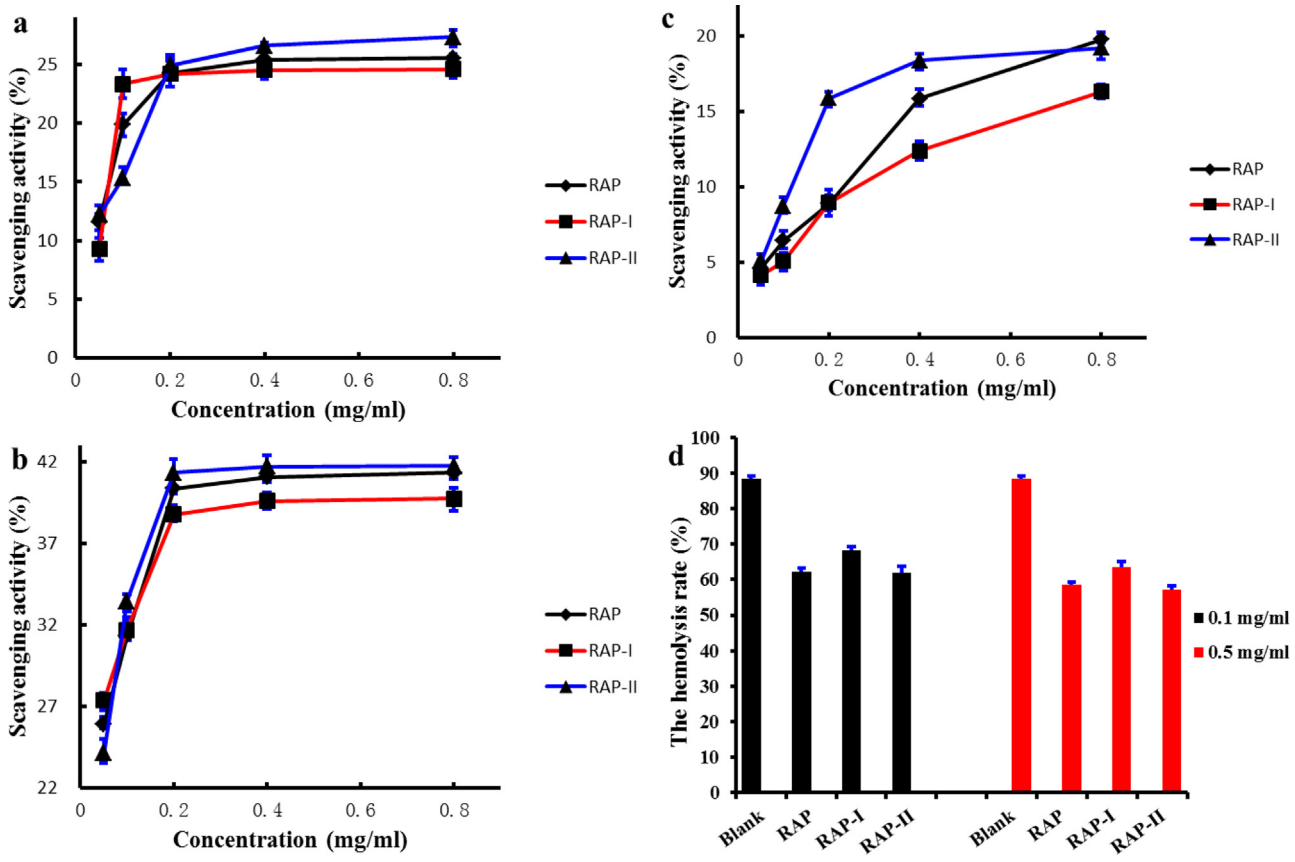

Fig. 5. Antioxidant activities ((a) scavenging effects on DPPH free radical; (b) scavenging effects on hydroxyl radical; (c) scavenging effects on superoxide radical; (d) effects on erythrocyte hemolysis) of RAP, RAP-I and RAP-II ($n = 3$).

group in polysaccharide which can donate hydrogen for binding with hydroxyl radical to achieve the scavenging effect (Lin, Wang, Chang, Inbaraj, & Chen, 2009; Tsiapali et al., 2001; Wang, Zhang, Zhang, & Li, 2008).

3.6.3. Superoxide anion radical scavenging activity

Superoxide anion radical is a weak oxidant but it could degrade continuously to form other active ROS, and then induce pathological incidents (Halliwell, Aeschbach, Loliger, & Aruoma, 1995). Therefore, it is very important to remove superoxide anion radical. Superoxide anion radical can be generated by auto oxidation of pyrogallol and a colored compound (tangerine phenol) can be produced (Chen, Xie, Nie, Li, & Wang, 2008). In this study, superoxide anion radical scavenging activities of different fractions of polysaccharides at different concentrations (0.05–0.8 mg/ml) were shown in Fig. 5c. For all the samples, the scavenging activities increased with increasing concentration, and this result is similar with the scavenging activities of all the samples on DPPH and hydroxyl radicals. The RAP-II had stronger scavenging activities for superoxide anion radical than RAP and RAP-I at a relative lower concentration (0.2 mg/ml). But at higher concentration, RAP-II showed the similar scavenging activity with RAP. Previous studies had reported the mechanism of scavenging superoxide anion may be associated with the presence of some types of electrophilic groups like keto or aldehyde in the former, facilitating liberation of hydrogen from O—H bond and thus stabilized superoxide anion (Lin et al., 2009).

3.6.4. Erythrocyte hemolysis inhibitory activity

Hemolysis of erythrocyte was selected as a simplified metabolism model system to evaluate antioxidant activity of RAP. If a large number of ROS is produced or endogenous antioxidative defenses are damaged, oxidative stress will be developed (Fernandes et al., 2010). Hydrogen peroxide crosses blood cell membrane and reacts with hemoglobin (Hb) generating highly active hydroxyl radicals, which may ultimately lead to erythrocyte hemolysis (Ko et al., 1997). The results of erythrocyte hemolysis of RAP were shown in (Fig. 5d). The hemolysis rates of all the test samples were much lower than that of blank control, indicating that all the samples could significantly protect erythrocyte from H_2O_2-induced hemolysis in the order of RAP-II > RAP > RAP-I. On the other hand, the hemolysis rates of all samples decreased with the increase of sample concentration, which indicated that it was in a concentration dependent way. The antihemolysis activity of RAP mainly relates to their active hydroxyl groups, which can scavenge ROS (Zhang et al., 2014).

Previous reports indicated that the antioxidant activity may be related to monosaccharide composition, molecular weight and structure of polysaccharides. So the antioxidant activities of polysaccharides were not a function of a single factor but a combination of several factors (Wang et al., 2012a). The exact mechanism underlying the antioxidant activity exerted by RAP is still not fully understood and the related in-depth works are in progress by our team.

4. Conclusion

An ultrasound extraction process has been optimized for effective extraction of RAP. The yield of RAP under the optimal extraction condition (ultrasound extraction time 75.2 min, ratio of water to material 30.1 ml/g, and ultrasound extraction temperature 76.1 °C) was 6.90%. Compared to THWE, the extraction yield was greatly increased. Furthermore, RAP were further fractionated by stepwise ethanol precipitation. The FTIR spectra and antioxidant activities of the three samples (RAP, RAP-I and RAP-II) were evaluated and all the polysaccharides possessed antioxidant activities. Hence, these polysaccharides may be explored as potential functional food ingredient or medicine. Further experiments on their structure-biological activity relationship are in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.11.052.

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