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Extraction, purification, and antioxidant activities of polysaccharides from *Tricholoma mongolicum Imai*

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ABSTRACT

Ultrasonic-microwave synergistic extraction (UMSE), purification, and characterization of *Tricholoma mongolicum Imai* polysaccharides (TMIPs) from fruit bodies were investigated in this study. Response surface methodology (RSM) was employed to optimize the UMSE conditions. The results indicated that the optimal extraction conditions were as follows: extraction time of 24.65 min, microwave power of 109.98 W, and water to raw material ratio of 21.62 ml/g. Under optimized conditions, the yield and purity of TMIPs were 35.41 ± 0.62% and 73.92 ± 0.83%, respectively. Crude TMIPs were purified by DEAE-Cellulose 52 chromatography and Sephadex G-100 chromatography to afford four fractions, namely, TMIP-1, TMIP-2, TMIP-3, and TMIP-4. Preliminary TMIP characterization was conducted by Fourier transform infrared spectroscopy. TMIP antioxidant activities were investigated by measuring its scavenging ability on 2,2-diphenyl-1-picrylhydrazyl, hydroxyl radicals, ferric reducing activity power, and reducing power assay. The results indicated that TMIPs have good antioxidant activity.

1. Introduction

Since the ancient times, many kinds of mushrooms have been widely used as food, medicine, and functional resources in many Asian countries because of their diverse biological activities (Hoshi et al., 2005). *Tricholoma mongolicum Imai* has a worldwide reputation as one of the notable edible-medicinal basidiomycete. Many Asian countries favored its taste, characteristic delicate flavor, and rich nutrients. Polysaccharide is one of the most important activity substances in *Tricholoma mongolicum Imai*, and has upper research value. *Tricholoma mongolicum Imai* polysaccharides (TMIPs) have several known bioactivities, such as potent anti-cancer, immunostimulatory, anti-mutagenic, antioxidant, and hematopoietic (Fang, Li, Ke, & Zhao, 2008; Sha et al., 2007; Wu & Tuli, 2007; Zeng, Chen, & Deng, 2010).

Extraction is a very important process for the application and further research and development of TMIPs. In recent years, several research papers have encouraged polysaccharide extraction technology from the plethora of fungi (Chen et al., 2010a). The polysaccharide extraction method includes hot-water treatment (HWE), ultrasonic assisted extraction (UAE), microwave assisted extraction (MAE), and so on. HWE requires high temperature and long extraction time, and has lower extraction efficiency. Meanwhile, UAE has been widely employed to extract polysaccharides from different plant materials because of its mass transfer intensification, cell disruption, improved penetration, and capillary effects (Chen et al., 2010b; Tsochatzidis, Guiraud, Wilhelm, & Delmas, 2001; Yan et al., 2011). However, the UAE temperature during the extraction process is difficult to control, which may lead to lower repeatability of the results.

MAE can accelerate the extracting process, which may improve the extraction efficiency of polysaccharide (Csiktusnádi Kiss et al., 2000; Salisova, Toma, & Masor, 1997; Zhang & Liu, 2008). The main advantage of applying microwave approaches is the efficiency of results, whereas its disadvantage is inhomogeneous heating (Zhang & Liu, 2008).

Ultrasonic-microwave synergistic extraction (UMSE) is a new technology which is a fusion of ultrasonic and microwave methods. UMSE is the organic combination of ultrasonic and microwave techniques, fully utilizing the high-energy effect of microwave and ultrasonic cavitation, and overcoming the shortcomings of conventional extraction, ultrasonic, and microwave extraction. UMSE realizes low temperature atmospheric environment, but fast and efficient extraction. (Chen et al., 2010b; Zhang & Liu, 2008).

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Response surface methodology (RSM) is an statistical method that uses quantitative data from appropriate experimental design to determine and simultaneously solve multivariate equations. Therefore, RSM is less strenuous and less time-consuming than other methods (Gan, Manaf, & Latiff, 2010; Gan & Latiff, 2011; Yin, You, & Jiang, 2011). In addition, RSM has been widely used in optimizing the polysaccharide extraction process variables (Zhong & Wang, 2010; Zou, Chen, Yang, & Liu, 2011). To the best of our knowledge, studies on RSM being applied to optimize the UMSE extraction process variables, especially the UMSE extraction of polysaccharides from *Tricholoma mongolicum Imai* are limited.

This study aims to investigate the effect of UMSE conditions on the polysaccharide extraction yield and purity. RSM was used to evaluate the effects of extraction time, microwave power, and water to raw material ratio on the recovery and purity to obtain the optimal extraction conditions. We purified the crude polysaccharides from *Tricholoma mongolicum Imai* by DEAE-52 and Sephadex G-100 chromatography, and characterized the purified fractions of crude TMIP. The preliminary structural characterization and antioxidant activities of four polysaccharides were estimated by chemical composition analysis, Fourier transform infrared (FTIR) spectroscopy, and antioxidant assays including 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, hydroxyl radicals assay, ferric reducing activity power (FRAP) assay, and reducing power assay.

2. Materials and methods

2.1. Materials and equipment

The fruiting bodies of *Tricholoma mongolicum Imai* were purchased from Inner Mongolia, China. Samples were soaked for 2 h with cold water, rinsed with flowing water, dried at 60 °C for 12 h, ground, and sieved through a 60 mesh sieve. All other chemical reagents were of analytical grade. A UMSE apparatus (CW-2000, Shanghai Xintuo Microwave Instrument Co. Ltd., China) with microwave power of 10–800 W at a frequency of 2450 MHz and an ultrasonic transducer with a fixed power of 50 W at a frequency of 40 kHz was used for polysaccharide extraction.

2.2. UMSE

Exactly 5 g of dried samples was defatted in a Soxhlet apparatus with petroleum ether (boiling point: 60–90 °C), and then pretreated twice with 80% ethanol to remove the colored materials, monosaccharides, oligosaccharides, and small molecules. The pretreated samples were subsequently separated from the organic solvent by centrifugation (5000 × *g* for 10 min) and dried at 60 °C for 12 h.

Polysaccharide extraction was carried out in a UMSE apparatus. Exactly 2 g of the pretreated dried powder was transferred into a 100 mL beaker, and properly distilled water was added into the beaker. The beaker was then transferred into a chamber of the UMSE apparatus connected to condensing tubes, held in the UMSE apparatus, and exposed to extract for different times under various microwave power. After the extraction using UMSE, the extracted mixture was centrifuged (5000 × *g* for 10 min) to collect the supernatant, and the insoluble residue was again treated twice as previously mentioned. The supernatants were incorporated and concentrated to one-fifth of the initial volume using a rotary evaporator at 50 °C under vacuum, and then precipitated by adding ethanol to a final concentration of 80% (v/v). The precipitates as crude extract were collected by centrifugation (5000 × *g*, 10 min). After washing for three times with anhydrous ethanol, the precipitate was air-dried at 50 °C a constant weight was achieved. The polysaccharide content was measured by phenol–sulfuric acid method (Masuko et al., 2005). Glucose was used to construct a standard curve, and the results were then expressed in terms of glucose.

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

Independent variables	Factor level		
	−1	0	1
Extraction time (min)	20	25	30
Microwave power (W)	60	100	140
Water to raw material ratio (mL/g)	16:1	20:1	24:1

The percentage of polysaccharide yield (%) was calculated using Eq. (1):

$$\text{yield}\ (\%,\ w/w) = \frac{\text{weight of dried crude extraction}}{\text{weight of material}} \times 100 \quad (1)$$

The purity of the polysaccharides (%) was calculated using Eq. (2):

$$\text{purity}\ (\%,\ w/w) = \frac{\text{weight of polysaccharides}}{\text{weight of dried crude extraction}} \times 100 \quad (2)$$

2.3. Box–Behnken factorial design (BBD) for polysaccharide extraction

RSM was used to explore the effect of the independent variables on the response within the range of investigation. Based on the preliminary range of extraction variables, a three-level-three-factor BBD was adopted in this study. Three extraction variables were considered, namely: X_1 (extraction time), X_2 (microwave power), and X_3 (water to raw material ratio) (Table 1a).

The yield and purity of the polysaccharides from *Tricholoma mongolicum Imai* were used as dependent variables. The whole design consisted of 16 experimental points, including 12 factorial points and 4 axial points. The 4 axial points were used to allow for pure error sum estimation of squares. The experiment was performed at all design points in a standard order. The non-linear computer-generated quadratic model used in the response surface is shown in Eq. (3),

$$y = \beta_{k_0} + \sum_{i=1}^{3} \beta_{k_i} X_i + \sum_{i=1}^{3} \beta_{k_{ii}} X_i^{\ 2} + \sum_{i<j=2}^{3} \beta_{k_{ij}} X_i X_j \quad (3)$$

where y is the dependent variable, β_{k_0} is the constant, β_{k_i}, $\beta_{k_{ii}}$, and $\beta_{k_{ij}}$ represent the regression coefficients of variables for linear, quadratic, and interaction terms, respectively; and X_i and X_j are the levels of the independent variables. The fitted polynomial equation was expressed as surface and contour plots to visualize the relationship between the response and experiment levels of each factor and deduce the optimum conditions (Lu, Engelmann, Lila, & Erdman, 2008). The regression coefficients were then used for statistical calculation to generate dimensional and contour maps from the regression models. The analysis of the experimental design and the calculation of the predicted values were carried out using Statistica (Version8.0, USA) to estimate the response of the independent variables.

2.4. Purification of crude polysaccharides

Crude polysaccharides were purified sequentially by DEAE-52 and Sephadex G-100 chromatography according to a previous study (Qiao et al., 2009). Approximately 5 ml of crude polysaccharide solution (5 g/L) was applied to the DEAE-52 column, which was

then stepwise eluted at a flow rate of 1.0 mL/min with 0, 0.1, 0.3, and 0.5 M NaCl solutions. The 5 mL fractions were collected by an automatic fraction collector. The carbohydrates were determined by the phenol–sulfuric acid method using glucose as standard (Masuko et al., 2005). Four fractions of polysaccharides (a–d) were obtained (Fig. 4), concentrated, dialyzed against distilled water, and further purified through a column of Sephadex G-100 eluted with deioned water. The resulting elute was then collected. Each fraction generated a single elution peak representing TMIP-1, TMIP-2, TMIP-3, and TMIP-4, respectively, as shown in Fig. 5a–d. The four purified fractions were collected, concentrated, dialyzed, and freeze-dried for further study.

2.5. Preliminary characterization of TMIP

The average weight of molecular polysaccharides was determined by gel permeation chromatography, in combination with a high-performance liquid chromatography (Angilent 1100, USA). The sample (2.0 mg) was dissolved in distilled water (2 mL), passed through a 0.45 μm filter, and then applied to a gel-permeation chromatographic column of TSK-GEL G3000SWxl columns (5 μm, 7.8 mm × 300 mm). The sample was maintained at 40 °C, eluted with 0.05 mol L^{-1} Na_2SO_4, at a flow rate of 0.5 ml min^{-1}, and detected by a refractive index detector (RID-10A) (Li et al., 2012). The molecular weight was estimated by reference to the calibration curve made from a Pullulan standard of known molecular weight (P-400, P-100, P-50, P-10, and P-5). The contents of protein and uronic acid were determined according to the references (Bradford, 1976; Dubios, Gilles, Hamilton, Rebers, & Smith, 1956), using bovine serum albumin and glucuronic acid as the standards, respectively. The polysaccharide purity extracted was determined by soluble non-cellulose polysaccharides to the weight of crude polysaccharides. The monosaccharide compositions of crude TMIP and its purified fractions (TMIP-1, TMIP-2, TMIP-3, and TMIP-4) were determined by gas chromatography (GC, Agilent 6890, USA) according to the reference (Li, Nie, Yang, Qiu, & Xie, 2011). The monosaccharide standards, including glucose, galactose, xylose, mannose, ribose, fucose, arabinose, and rhamnose, were applied according to the reference (Gan, Ma, Jiang, Xu, & Zeng, 2011).

2.6. Infrared spectroscopy analysis of TMIP

Crude TMIP and its purified four fraction powders were mixed with KBr powder, and then ground and pressed for Fourier transform infrared (FT-IR) measurement. An FT-IR spectrum of the TMIP was determined using the Nicolet 5700 spectrometer (Thermo Electron, Madison, WI, USA) at the frequency range of 4000–400 cm^{-1}.

2.7. Antioxidant activities

2.7.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was determined by the method of Blois (1958) with some modification. DPPH radical was prepared by dissolving in ethanol (0.2 mM). A sample of each fraction (1 mL; 0.2, 0.4, 0.6, 0.8, and 1 mg/mL concentration) in distilled water was added to 1 mL of 0.2 mM DPPH in ethanol. The fluid was kept in dark at room temperature for 30 min after vibration, and the absorbance was measured at 517 nm. Vitamin C was used as a positive standard. The scavenging activity of the DPPH radicals was expressed in Eq. (4):

$$\text{DPPH radical scavenging activity } (\%) = \left(1 - \frac{A_1 - A_3}{A_2}\right) \times 100 \quad (4)$$

where A_1 is the absorbance of the reaction solution, A_3 is the absorbance of the solution including 1 mL of sample and 1 mL of ethanol, and A_2 is the absorbance of the solution including 1 mL of DPPH and 1 mL of ethanol.

2.7.2. FRAP

FRAP assay was measured using the method described by Vasco, Ruales, and Kamal-Eldin (2008), with some modifications. FRAP reagent was prepared by mixing 300 mmol/L acetate buffer (pH 3.6) with 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mmol/L $FeCl_3{\cdot}6H_2O$ in ratio of 10:1:1. The samples were dissolved in distilled water to form sample solutions with final concentrations of 0.1, 0.2, 0.4, 0.6, and 0.8. Subsequently, 1 mg/mL, 20 μL polysaccharide solution was added to 500 μL of FRAP reagent, and the mixture was warmed at 37 °C. After 8 min, the light absorption value of the mixture solution was at 593 nm. A standard curve was prepared using $FeSO_4{\cdot}7H_2O$ solution with several concentrations (100–1000 μM). The final results were expressed as the concentrations of $FeSO_4{\cdot}7H_2O$ with equivalent antioxidant activity.

2.7.3. Assay of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined based on a reported method (Wu, Zhu, Zhang, Yang, & Zhou, 2012). The four polysaccharides were dissolved in water with various concentrations (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mg/mL), yielding a series of sample solutions. A mixture solution was prepared by mixing several solutions into 2 mL of PBS phosphate buffer (pH 7.4) in the following order: 1 mL of water, 1 ml of 1,10-phenanthroline ethanol solution (0.75 mM), 1 mL of $FeSO_4$ (0.75 mM), and 1 mL of H_2O_2 (0.01%). The final mixture was incubated at 37 °C for 60 min, and was used as the blank solution. A similar procedure was used to prepare for the control solution, wherein 1 mL of water instead of H_2O_2 was added. The absorbance of the blank (B_{blank}), control ($B_{control}$), and sample solutions (B_{sample}) was determined at 510 nm. Hydroxyl radical scavenging activity was calculated using Eq. (5):

$$\text{hydroxyl radical scavenging activity } (\%) = \frac{B_{sample} - B_{blank}}{B_{control} - B_{blank}} \times 100 \quad (5)$$

2.7.4. Assay of reducing power

The reducing power of the polysaccharides was determined according to the method of Oyaizu (1986). Each sample in 2.0 mL of double distilled water was added to 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated for 20 min at 50 °C, and then 2.5 mL of 10% (w/v) TCA was added to the reaction mixture and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of double distilled water and 0.5 mL of 0.1% (w/v) ferric chloride in test tube. After 10 min of reaction, the absorbance of the resulting solution was measured at 700 nm. The increased absorbance of the reaction mixture indicated an increased reducing power. Ascorbic acid was used to compare the reducing power.

3. Results and discussion

3.1. Optimization of the extraction parameters of the polysaccharides

3.1.1. Statistical analysis and model fitting

Sixteen experimental points were run regularly according to the UMSE experiment planning (Table 1b). The percentage yield and purity extraction of the polysaccharides in the extracts ranged from 31.26% to 35.07% and 66.46% to 75.51%, respectively.

Table 1b
BBD and the yield and purity of polysaccharides from *Tricholoma mongolicum Imai*.

Run	X_1/extraction time (min)	X_2/microwave power (w)	X_3/water to raw material ratio (mL/g)	polysaccharides yield (%)	polysaccharides purity (%)
1	20	60	20	32.50	68.69
2	30	60	20	34.88	69.09
3	20	140	20	34.43	68.86
4	30	140	20	33.53	68.38
5	20	100	14	31.26	69.30
6	30	100	14	34.86	70.13
7	20	100	26	34.49	71.60
8	30	100	26	33.30	68.15
9	25	60	14	31.49	66.59
10	25	140	14	32.93	69.21
11	25	60	26	33.18	69.93
12	25	140	26	34.21	66.46
13	25	100	20	34.92	75.51
14	25	100	20	34.96	75.41
15	25	100	20	35.07	74.37
16	25	100	20	34.88	74.69

The results of the analysis of variance and the adequacy of the models were summarized. The maximum extract yield (35.07%) was found under the following conditions: X_1 = 24.65 min, X_2 = 109.98 W, and X_3 = 21.62 mL/g. The fitted model for polysaccharide yield (Y_1) and polysaccharide purity (Y_2) to predict the relationships between the independent variables and the dependent variables can be expressed as follows:

$$Y_1 = -27.1649 + 1.9031X_1 + 0.2235X_2 + 2.4498X_3 - 0.012X_1^2 - 0.0005X_2^2 - 0.0328X_3^2 - 0.0041X_1X_2 - 0.0399X_1X_3 - 0.0004X_2X_3 \quad (6)$$

$$Y_2 = -70.4698 + 5.2483X_1 + 0.6493X_2 + 4.8269X_3 - 0.0899X_2^1 - 0.0025X_2^2 - 0.0820X_3^2 - 0.0011X_1X_2 - 0.0357X_1X_3 - 0.0063X_2X_3 \quad (7)$$

where Y_1 is the TMIP yield (%), and X_1, X_2, and X_3 are the coded variables for the extraction time, microwave power, and water to raw material ratio, respectively.

The results of the analysis of variance (ANOVA), adequacy, and fitness of the models for the response surface quadratic model are summarized in Table 1c. The yield and purity coefficients of determination, which are $R_1^2 = 0.9668$ and $R_2^2 = 0.9871$, respectively, is shown in the ANOVA of the quadratic regression model, indicating that only 3.32% and 1.29% of the total variations are not explained by the model, respectively. This result is in close agreement between the experimental results and the theoretical values predicted by the polynomial model. The *p*-values were used as a tool to verify the significance of each coefficient, which in turn may indicate interaction patterns among the variables. The smaller the *p*-value is, the more significant is the corresponding coefficient.

Table 1c
Regression coefficients and analysis of the model for two response variables.

Coefficient	Yield (%)	Purity (%)
β_{k_0}	−27.1649	−70.4698
β_{k_1}	1.9031[b]	5.2483[b]
β_{k_2}	0.2235[b]	0.6493[b]
β_{k_3}	2.4498[b]	4.8269[b]
$\beta_{k_{11}}$	−0.012[b]	−0.0899[b]
$\beta_{k_{12}}$	−0.0041[b]	−0.0011[c]
$\beta_{k_{13}}$	−0.0399[b]	−0.0357[a]
$\beta_{k_{22}}$	−0.0005[b]	−0.0025[b]
$\beta_{k_{23}}$	−0.0004[c]	−0.0063[a]
$\beta_{k_{33}}$	−0.0328[b]	−0.0820[b]
R^2	0.9668	0.9871

[a] Means $P < 0.05$.
[b] Means $P < 0.01$.
[c] Means $P > 0.05$.

From the yield and purity models, the linear coefficients (X_1, X_2, and X_3) and quadratic term coefficients (X_1^2, X_2^2 and X_3^2) are very significant with very small *p*-values ($p < 0.01$). From the yield model, the interaction coefficient, $X_2{}^*X_3$, was not significant ($P > 0.05$). From the purity model, interaction coefficients, $X_1{}^*X_3$ and $X_2{}^*X_3$, were significant ($0.01 < P < 0.05$), whereas interaction coefficient, $X_1{}^*X_2$, was not significant ($P > 0.05$). From the ANOVA of the yield and purity, all the independent variables are important factors that have effects on yield and purity of crude polysaccharides.

3.1.2. Analysis of the response surface and verification of the predictive model

The 3D response surface and 2D contour plots are the graphical representations of the regression equations. These types of plots show the effects of the two factors on the response at a given time point. In all the figures presented, one factor was held constant at level zero (Figs. 1–3). The maximum value predicted by the surface was confined in the smallest ellipse in the contour diagram. Elliptical contours were obtained when a perfect interaction between the independent variables exists.

The 3-D and the contour plots in Fig. 1, which provides the water to raw material ratio (fixed), shows that polysaccharide yield increased with increasing extraction time from 18 min to 27 min. Beyond 27 min, the polysaccharide yield decreased gradually with the increase in extraction time, and the polysaccharide yield increases rapidly with the increase of microwave power from 50 W to 135 W, and then decreased rapidly from 135 W to 150 W. Fig. 1 indicates that the maximum purity of polysaccharides can be achieved when the extraction time and the water to raw material ratio were 21.50 min and 100 mL/g, respectively.

The 3-D response surface plot and the contour plot at varying extraction times and water to raw material ratio at fixed microwave power 100 W is shown in Fig. 2. The yield of the polysaccharides increased gradually with the increase of extraction time and water to raw material ratio. Moreover, when the ratio of water to microwave power was constant (100 W), the purity increased substantially with the increase in extraction time (18–26 min) and water to raw material ratio (12–20.30 mL/g). The purity decreased gradually with the increase of extraction time (26–32 min) and water to raw material ratio (20.30–28 mL/g).

Fig. 3 shows that the 3-D response surface and the contour plots were developed for polysaccharide yield and purity with varying microwave power and water to raw material ratio at a fixed extraction time (25 min). An increase in polysaccharide yield and purity can be significantly achieved with the increases of water to raw material ratio. The polysaccharides yield increased

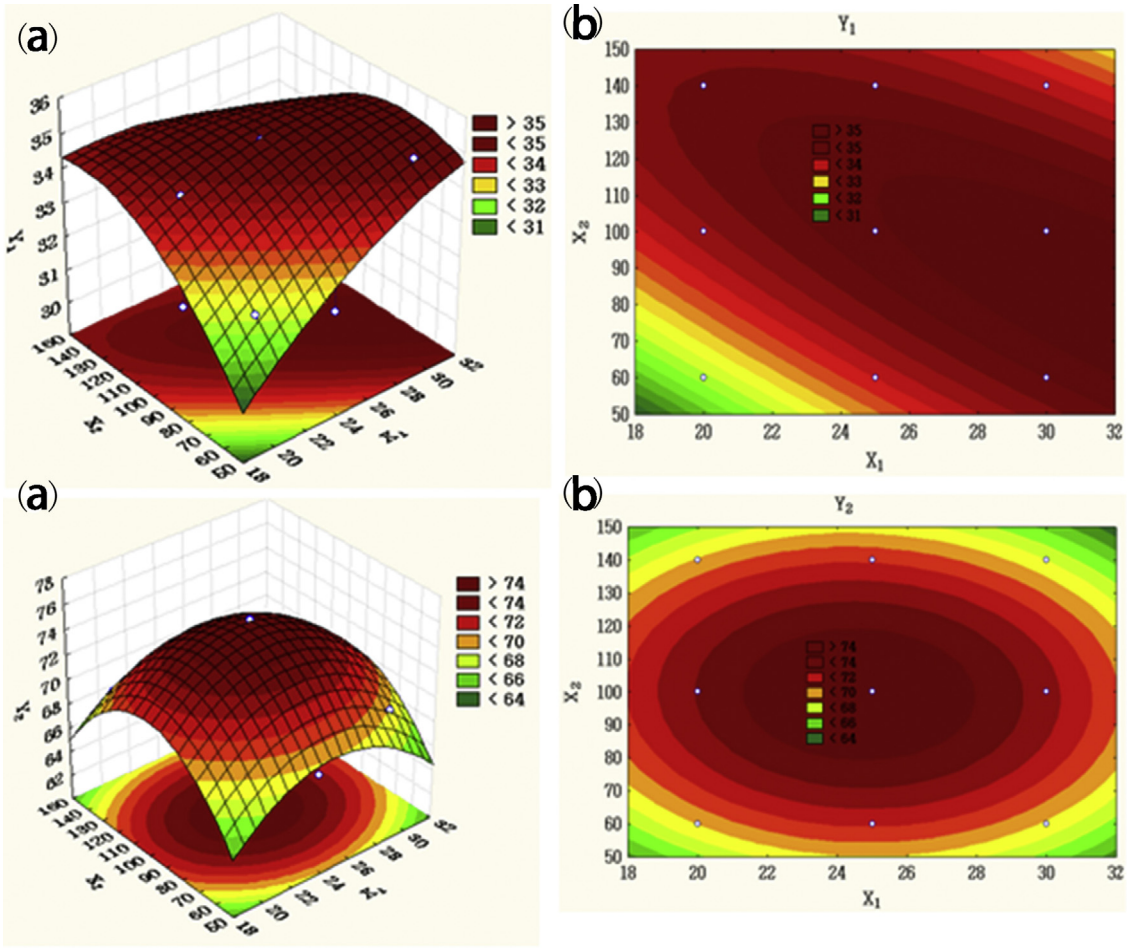

Fig. 1. Response surface (a) and contour (b) plots showing the effect of extraction time and microwave power (water to raw material ratio = 20) on the yield (Y_1) and purity (Y_2) of TMIP.

with increasing of microwave power from 50 W to 110 W, and then dropped slightly from 110 W to 150 W. This result indicates that further increase of microwave power would not increase the extraction yield of polysaccharides.

According to the fitted surface graphs (Figs. 1–3), the maximal polysaccharide yield can be achieved at higher microwave power and higher water to raw material ratio at relatively longer extraction time. The extraction conditions with optimal polysaccharide yield (35.07%) of UMSE were given by the RSM optimization approach as follows: extracting time was 24.65 min, microwave power was 109.98 W, and water to raw material ratio was 21.62 mL/g. In addition, the optimal extraction condition with high polysaccharide purity (75.01%), including an extraction time of 24.58 min, microwave power of 98.91 W, and water to raw material ratio 20.25 mL/g. Among the three effective parameters, the water to raw material ratio was the most significant factor affecting the extraction yield of polysaccharides, followed by microwave power and extraction time according to the regression coefficient significance of the quadratic polynomial model and gradient of slope in the 3-D response surface plot (Figs. 1–3).

3.1.3. Verification of predictive model

To validate the adequacy of the model equations, verification experiment was carried out under optimal conditions: extracting time 24.60 min, microwave power 110 W, and water to raw material ratio 21.62 mL/g. The mean yield value and purity of 35.41 ± 0.62% and 73.92 ± 0.83% (n = 3) obtained from real experiments demonstrated the validation of the RSM model, and indicated that the model was adequate for the extraction process (Table 1d). The analysis results indicated that the three groups of experimental values are in good agreement with the predicted one, and also suggested that the models of Eqs. (4) and (5) are satisfactory and accurate.

3.2. Purification and preliminary characterization of TMIP

3.2.1. Purification of crude TMIP

Crude TMIP was prepared using optimal extraction conditions. The crude TMIP was loaded first into an anion-exchange column of DEAE-52 cellulose, and the column was stepwise eluted with 0, 0.1, 0.3 and 0.5 M sodium chloride solutions. Four independent

Table 1d
Predicted and experimental values of the responses at optimum conditions.

	Extracting time	Microwave power	Water to raw material ratio	Yield of polysaccharides (%)	Polysaccharides purity (%)
Optimum conditions	24.65 min	109.98 W	21.62	35.07% (predicted)	75.01% (predicted)
Optimum conditions	24.58 min	98.91 W	20.25		74.46% (predicted)
Modified conditions	24.60 min	110	21.62	35.41 ± 0.62% (actual)[a]	73.92 ± 0.83% (actual)[a]

[a] Mean ± standard deviation (n = 3).

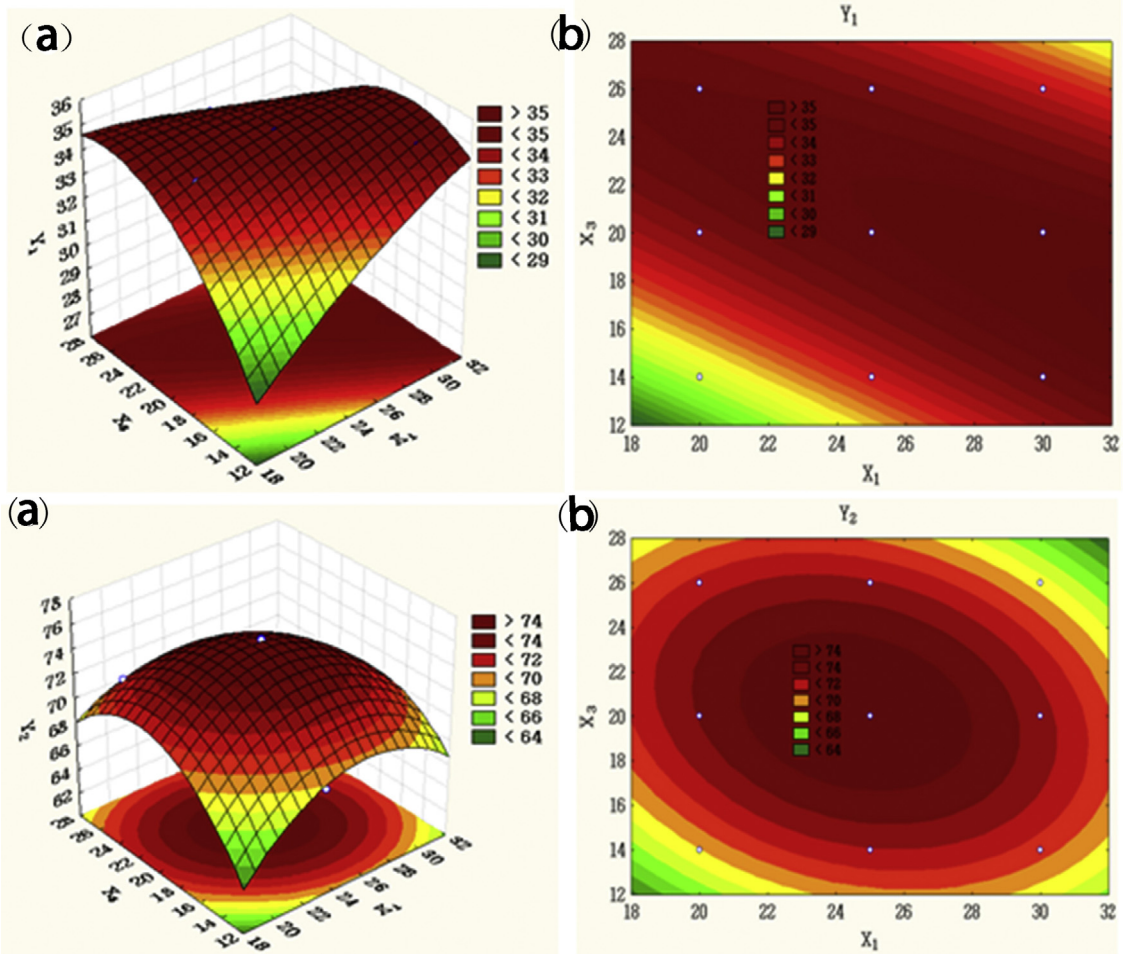

Fig. 2. Response surface (a) and contour (b) plots showing the effect of extraction time and water to raw material ratio (microwave power = 100 W) on the yield (Y_1) and purity (Y_2) of TMIP.

elution peaks (a–d) detected by the phenol–sulfuric acid assay were obtained (Fig. 4). The four fractions were then collected, concentrated, dialyzed, and loaded into a column of Sephadex G-100, respectively. The column was eluted with deionized water, and the resulting elute was collected. As a result, each fraction afforded one single elution peak (Fig. 5a–d).

3.2.2. *Contents of carbohydrate, protein, uronic acid, and monosaccharide composition of TMIP*

Table 1e shows the contents of carbohydrate, protein, and uronic acid in crude TMIP and its purified fractions. The carbohydrate contents in crude TMIP, TMIP-1, TMIP-2, TMIP-3, and TMIP-4 were 73.92%, 87.63%, 88.13%, 84.58%, and 83.26%, respectively. The uronic acid content of the TMIP, TMIP-1, TMIP-2, TMIP-3, and TMIP-4 are low. The protein contents of the TMIP-1 and TMIP-2 after purification was lower than crude TMIP, indicating that the purification of crude TMIP may be useful and efficient.

Table 1e
Chemical composition and content of carbohydrate, protein, and uronic acid for TMIP, TMIP-1, TMIP-2, TMIP-3, and TMIP-4.

Samples	TMIP	TMIP-1	TMIP-2	TMIP-3	TMIP-4
Carbohydrate (%)	73.92	87.63	88.13	84.58	83.26
Protein (%)	1.13	0.11	0.16	0.82	1.75
Uronic acid (%)	1.92	–[a]	1.66	1.96	2.34
Sugar components (%)					
Glucose	32.82	36.62	33.86	43.60	26.63
Xylose	8.93	11.52	12.62	7.65	6.88
Mannose	15.54	13.35	9.13	19.16	18.72
Arabinose	25.73	23.65	27.37	16.83	27.69
Rhamnose	16.98	14.86	17.02	12.76	20.08

[a] Not detected.

Table 1e shows the monosaccharide compositions of crude TMIP and its purified fractions (TMIP-1 TMIP-2 TMIP-3, and TMIP-4). The glucose contents were found to be at maximum in a ratio of 32.82%, 36.62%, 33.86%, 43.60%, and 26.63% for crude TMIP, TMIP-1, TMIP-2, TMIP-3, and TMIP-4, respectively. For various TMIPs, it is composed of glucose, arabinose, xylose, mannose, and rhamnose. However, other sugars, such as fucose, ribose, and galactose have not been found in TMIP. In addition, protein and uronic acid contents in TMIP-3 and TMIP-4 were higher than those in TMIP-1 or TMIP-2. The contents of various kinds of monosaccharide in TMIP-1, TMIP-2, TMIP-3, and TMIP-4 varied.

3.3. *Molecular weight of polysaccharide fractions*

The linearity of the method was determined using Pullulan standards of different molecular weights. The calibration curve of pullulan was plotted as the molecular weights on a log scale versus the retention time. The regression equation was log $M_w = 8.21 - 0.233t_R$ (M_w is weight-average molar mass, Da; t was the retention time, min) with a high correlation of $R^2 = 0.9988$ obtained for the standard curve. The average molecular weight of TMIP-1, TMIP-2, TMIP-3, and TMIP-4 was 4218, 27,237, 61,336, and 272,643 Da, respectively.

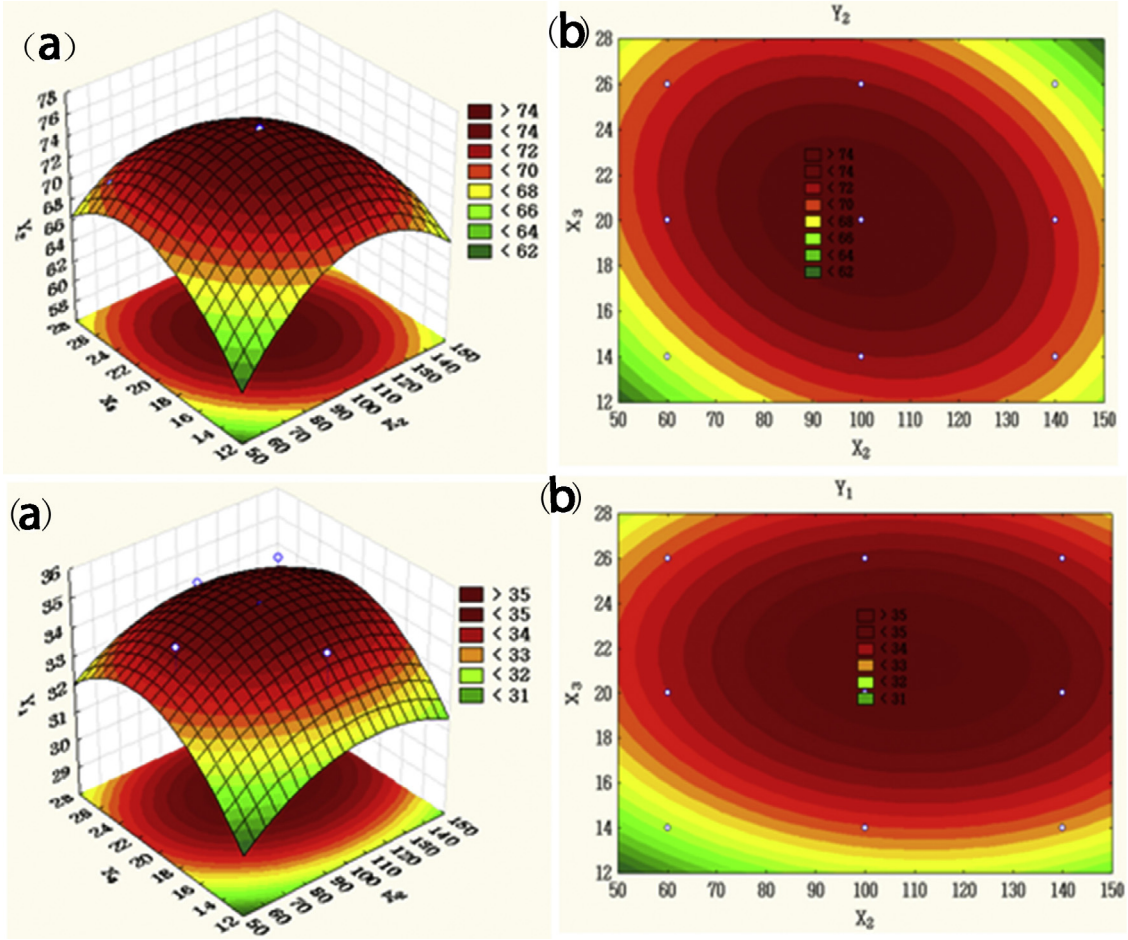

Fig. 3. Response surface (a) and contour (b) plots showing the effect of microwave power and water to raw material ratio (extraction time = 25 min) on the yield (Y_1) and purity (Y_2) of TMIP.

3.4. Antioxidant activity in vitro of TMIP

3.4.1. Ferric reducing activity power

FRAP is usually used to evaluate the antioxidant ability of many polysaccharides. Fig. 6a shows that all polysaccharides possessed reducing power in the order of TMIP-4, TMIP-3, TMIP-2, and TMIP-1. The reducing power of all four polysaccharides was notable at all tested concentrations, and positively correlated with increased concentration up to 1.20 mg/mL. The FRAP values of TMIP-4, TMIP-3, TMIP-2, and TMIP-1 were 1.1, 0.8, 0.62, and 0.41 mmol/L at the concentration of 1.20 mg/mL, respectively. Apparently, the ferric reducing power of TMIP-4 was distinctly higher than that of the other polysaccharide fractions.

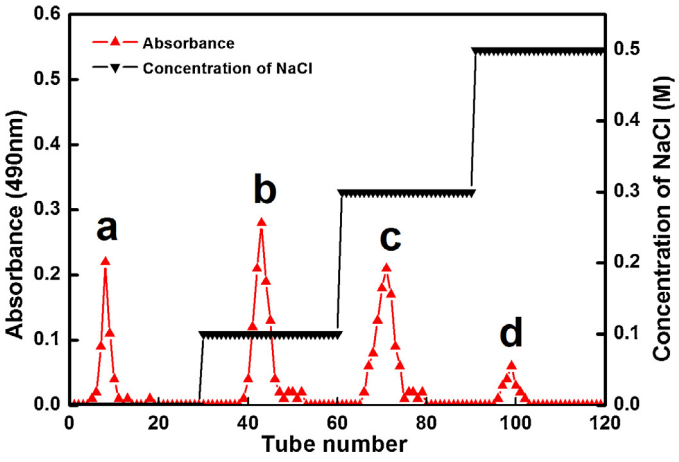

Fig. 4. 0–0.5 M NaCl stepwise elution curve of crude TMIP on DEAE-52 column.

3.4.2. DPPH• radical scavenging activity of TMIP

The antioxidant activities of purified TMIP-1, TMIP-2, TMIP-3, and TMIP-4 were determined by DPPH radical scavenging activity. The DPPH scavenging activities of the purified polysaccharides are presented in Fig. 6b. The polysaccharides showed good antioxidant capacities. A concentration-dependent DPPH radical scavenging activity was observed. The scavenging effect increased with the increase of concentration up to 1.2 mg/mL. An increase in DPPH free radical-scavenging occurred, in proportional line with the increase seen in the amount of polysaccharides. At the concentrations of 0.2–1.2 mg/mL, the scavenging ability of the polysaccharides on DPPH radical was in ranges of 3–81%. At the concentration of 1.2 mg/mL, TMIP-4 was observed to possess free radical scavenging activity with a scavenging value of about 80.1%, which indicated that the scavenging effects of TMIP-4 were much higher than that of TMIP-1, TMIP-2 and TMIP-3. The mechanism of DPPH radical scavenging activity is based on the reduction of DPPH to DPPH-H in the presence of a hydrogen-donating antioxidant, leading to the fading of purple color and therefore to inhibit the propagation of oxidizing reaction. It has been reported that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds and aromatic amines could reduce and decolorize DPPH by their hydrogen donating ability (Li, Zhou, & Li, 2007). The results mentioned

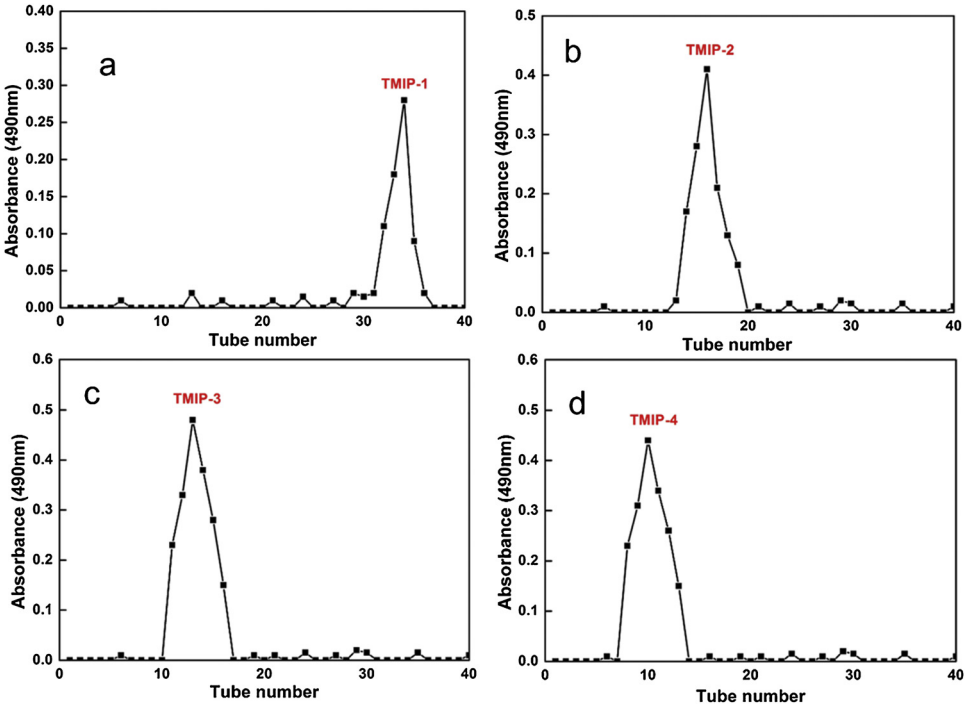

Fig. 5. Elution profiles of fraction on Sephadex G-100 gel-permeation chromatography column with distilled water elute (a) TMIP-1, (b) TMIP-2, (c) TMIP-3, and (d) TMIP-4.

above implied that purified polysaccharides might act as electron or hydrogen donator to scavenge DPPH.

3.4.3. Assay of hydroxyl radical scavenging activity

Hydroxyl radical is one of the most harmful reactive free radicals, hence, cleaning of hydroxyl radical is very important for the body under oxidized injury. The hydroxyl radical, generated by the Fenton reaction in the system, was scavenged by TMIP-1, TMIP-2, TMIP-3, TMIP-4 and ascorbic acid. The hydroxyl radical scavenging effects of TMIP-1, TMIP-2, TMIP-3, TMIP-4, and ascorbic acid increased with the increase of concentration up to 1.2 mg/mL (Fig. 6c). The scavenging effect of TMIP-4 was higher than that of TMIP-1, TMIP-2, and TMIP-3. At a concentration of 1.2 mg/mL, the hydroxyl radical scavenging activities were 89%, 65.6%, 49.6%, and 32.8% for TMIP-1, TMIP-2, TMIP-3, and TMIP-4, respectively. The results demonstrated that TMIP-3 and TMIP-4 possessed stronger

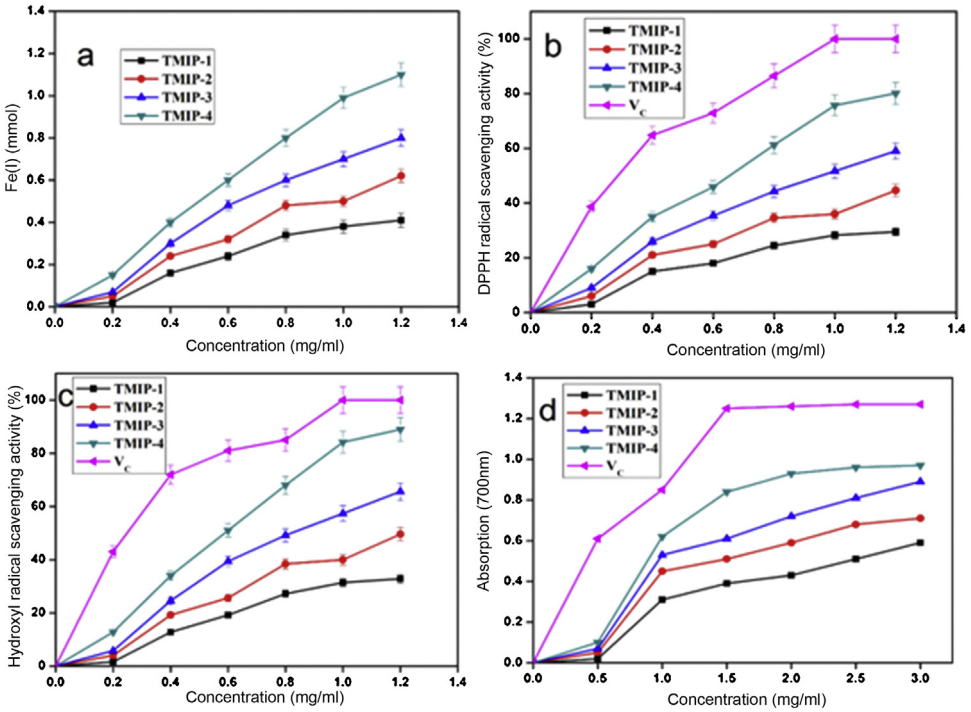

Fig. 6. Scavenging effects on (a) ferric reducing activity power, (b) DPPH assay, (c) hydroxyl radicals assay, and (d) reducing power assay of TMIP-1, TMIP-2, TMIP-3, and TMIP-4 at different concentrations. Values are presented as means ± SD ($n = 3$).

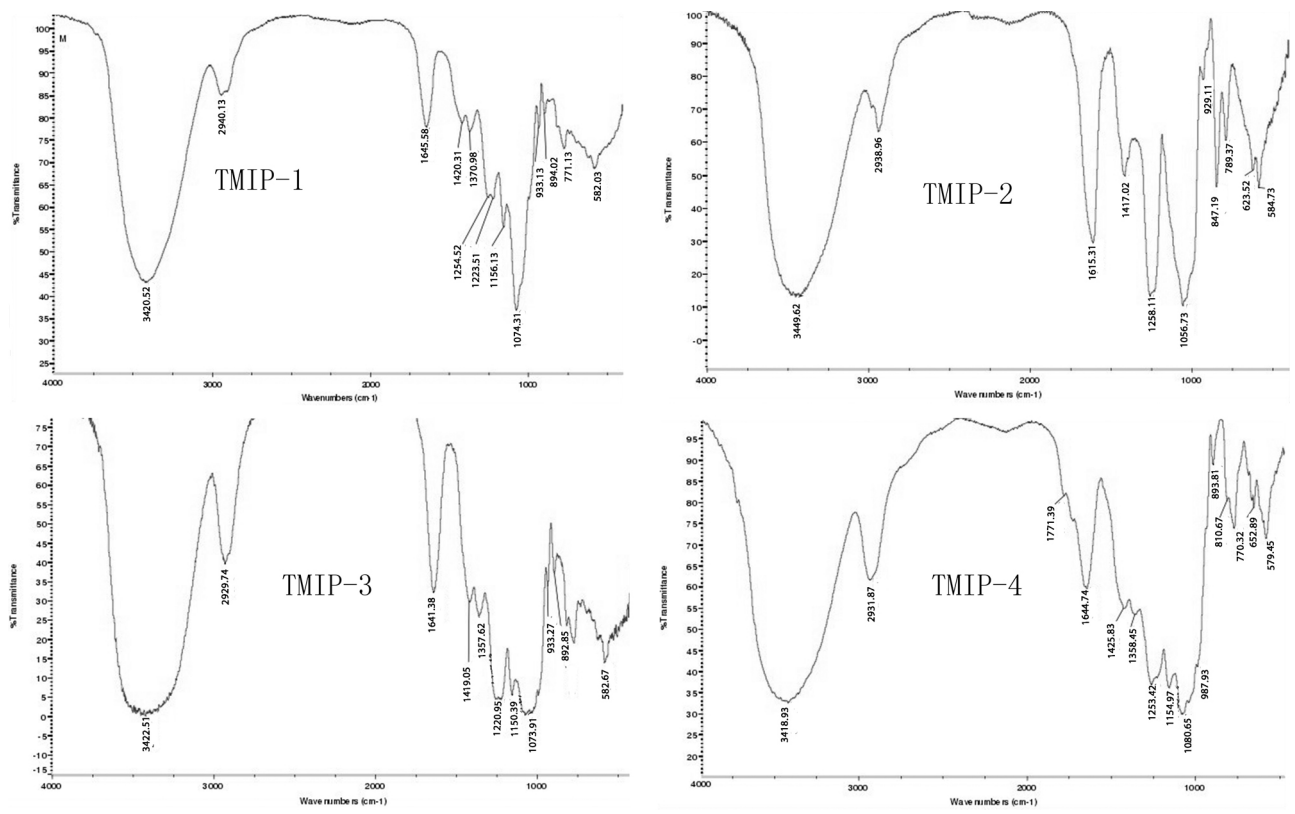

Fig. 7. FT-IR spectra of TMIP-1, TMIP-2, TMIP-3, and TMIP-4.

scavenging activities on hydroxyl radical than TMIP-1 and TMIP-2.

3.4.4. Assay of reducing power

Direct correlation between antioxidant activities and reducing power of certain plant extracts was previously reported. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Meir, Kanner, Akiri, & Hadas, 1995). Measuring the reducing power may directly reflect the production condition of the electron donor. The reducing powers of TMIP-1, TMIP-2, TMIP-3, TMIP-4, and ascorbic acid determined at 700 nm are depicted in Fig. 6d. The figure shows that the reducing power of ascorbic acid increased quickly at the concentration from 0 mg/mL to 1.0 mg/mL, and it showed the highest reducing power at the concentration of 1.0 mg/mL. The reducing power of all the polysaccharides increased gradually with the increasing concentration. Among the four polysaccharides, TMIP-4 showed obviously higher ability than TMIP-3, TMIP-2 and TMIP-1, but less than the ascorbic acid.

3.5. Infrared spectroscopy analysis of TMIP

The IR spectra of TMIP-1, TMIP-2, TMIP-3, and TMIP-4 were basically indistinguishable only with some difference in the intensity of bands (Fig. 7a–d). The absorption bands within the ranges of 3600–3200; 3000–2800; and 1250–1000 cm^{-1} were the characteristic absorption peaks of polysaccharides. The intensity of bands within the range of 3419–3451 cm^{-1} was assigned to hydroxyl stretching frequency, and as expected it was broad (Wang, Chen, Jia, Tang, & Ma, 2012). The weak intensity of band attributed to C—H group stretching (2929–2940 cm^{-1}) can also be observed (Lai, Wen, Li, Wu, & Li, 2010). New bands in the region of 1612–1644 cm^{-1} appeared, corresponding to carbon-oxygen double bond asymmetric stretching vibration absorption peak (Cerna et al., 2003). A strong extensive absorption in the region of 1000–1200 cm^{-1} due to C—O—C stretching vibration and stretching vibrations of C—OH side groups can be observed (Barros et al., 2002).

4. Conclusion

The performance of the ultrasonic–microwave synergistic extraction of TMIP was studied using a statistical method based on RSM to identify and quantify the variables that can maximize the polysaccharide yield and purity. Extraction time, microwave power, and water to raw material ratio were chosen as independent variables that might have an influence on the polysaccharide yield and purity extracted using the UMSE method. The linear coefficients (X_1, X_2 and X_3), quadratic term coefficients (X_1^2, X_2^2 and X_3^2), and interaction coefficients (X_1*X_3) were significant ($p < 0.05$). The optimum conditions obtained using RSM for polysaccharide production were extraction time of 24.65 min, microwave power of 109.98 W, and water to raw material ratio of 21.62 mL/g. Under these conditions, the yield and purity of TMIP were at 35.41 ± 0.62% and 73.92 ± 0.83%, respectively, almost equal to the predicted yield value (35.07%) and purity value (74.46%). Four purified fractions, namely, TMIP-1, TMIP-2, TMIP-3, and TMIP-4, were obtained from crude TMIP through the sequential purification step of DEAE-52 and Sephadex G-100 chromatography. The average molecular weight of the polysaccharides (TMIP-1, TMIP-2, TMIP-3, and TMIP-4) was 4218, 27,237, 61,336, and 272,643 Da, respectively. The chemical compositions and antioxidant activities of TMIP-1, TMIP-2, TMIP-3, and TMIP-4 were studied. Crude TMIP contained 73.92% carbohydrates, 1.13% proteins, and 1.92% glucuronic acid. The component monosaccharides were glucose, xylose, mannose, arabinose, and rhamnose. Monosaccharide content of polysaccharide is different in various TMIP. The FT-IR spectra of TMIP-1, TMIP-2, TMIP-3, and TMIP-4 were almost similar, with small differences. Moreover, TMIP-1, TMIP-2, TMIP-3, and

TMIP-4 showed significant free radical scavenging activities in a dose-dependent manner, whereas TMIP-3 and TMIP-4 showed significantly higher scavenging activity than TMIP-1 and TMIP-2.

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