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Ultrasonic-assisted extraction optimized by response surface methodology, chemical composition and antioxidant activity of polysaccharides from *Tremella mesenterica*

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ABSTRACT

Box–Behnken design was employed to optimize the ratio of water to raw material (X_1: 20–40 mL/g), extraction time (X_2: 20–40 min) and extraction temperature (X_3: 40–60 °C) to obtain a high crude polysaccharides yield from *Tremella mesenterica* (TMP) by ultrasonic-assisted extraction technique (UAE). The optimum conditions were a ratio of water to raw material of 34 mL/g and extraction time of 30 min at 50 °C. Under these conditions, the experimental yield was 8.26%, well matched with the predicted models with the coefficients of determination (R^2) of 0.9918. The crude TMP was purified by Sephadex G-100 chromatography, giving two major fractions (TMPA and TMPB), which were composed of xylose, mannose and glucose in molar ratio of 1.00:4.74:0.91 and 1.00:6.63:2.34, respectively. The evaluation of antioxidant activity suggested that the polysaccharides exhibited significant protection against hydrogen peroxide induced oxidative damage in rat erythrocytes and could be explored as a nutraceutical agent.

1. Introduction

Polysaccharides have recently been widely studied as a new source of additives for the food and pharmaceutical industries due to their unique bioactivities and chemical structures (Forabosco et al., 2006; Schepetkin & Quinn, 2006). In particular, mushrooms polysaccharides are reported to possess a wide range of pharmacological properties such as anti-tumor and antioxidant, as well as anti-diabetic activity and immunity-modulation (Cho, Oh, Changand, & Yun, 2004; De & Vandamme, 2001; Kwon, Qiu, Hashimoto, Yamamoto, & Kimura, 2009).

Tremella mesenterica, which belongs to the Jelly Mushroom group of organisms that form gelatinous fruiting bodies, is widely used in East Asian Countries as a tonic and health food (Chen, Hsu, Lin, Lai, & Wu, 2006; Chen, Lo, et al., 2006). Other reported pharmacological activities of this mushroom include anti-diabetic, anti-inflammatory, cytokine-stimulating, and vascular-stimulating effects (Lo, Tsai, Wasser, Yang, & Huang, 2006). Unlike other mushroom polysaccharides, polysaccharides extracted from *Tremella* species consist of a (1 → 3)-α-mannan backbone with smaller amount of xylose- and glucuronic acid (Khondkar, Aidoo, & Tester, 2002). *Tremella* polysaccharides can affect cytokine (IL-6 and TNF-α) and nitric oxide production in RAW 264.7 macrophage cells (Chen, Hsu, et al., 2006; Chen, Lo, et al., 2006). Studies have shown that polysaccharides are the main bioactivital components of *T. mesenterica*. However, to the author's knowledge, there are almost no previous reports regarding the extraction process of *T. mesenterica* polysaccharides (TMP). Therefore, an efficient technique for the extraction of TMP is needed.Several conventional extraction techniques have been reported for the extraction of target compounds from raw materials. Hot water technology is the main and most conventional extraction method for polysaccharides mentioned in recent studies (Bendjeddou, Lalaoui, & Satta, 2003; Errea & Matulewicz, 1996). However, it is usually associated with longer extraction time and higher temperature but lower extraction efficiency. Recently, ultrasonic-assisted extraction (UAE) has been widely employed in the extraction of target compounds from different materials owing to its facilitated mass transfer between immiscible phases through super agitation at low frequency (Tsochatzidis, Guiraud, Wilhelm, & Delmas, 2001). It offers high reproducibility at shorter times, simplified manipulation, and lowered energy input, as well as solvent consumption (Vilkhu, Mawson, Simons, & Bates, 2008). By using conventional extraction under ultrasound irradiation (20–100 kHz), structural changes and degradation of polysaccharides can be avoided (Khan, Maryline, Fabiano-Tixier, Dangles, & Chemat, 2010; Zhou & Ma, 2006). Thus, UAE may be an effective and advisable technique for the extraction of polysaccharides.

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Recently, response surface methodology (RSM) has been used increasingly to optimize processing parameters because it allows more efficient and easier arrangement and interpretation of experiments compared to other methods (Box & Behnken, 1960; Gan, Manaf, & Latiff, 2010). In this study, a three-level, three-variable Box–Behnken design (BBD) is used to optimize the extraction parameters (ratio of water to raw material, extraction time, and extraction temperature) of TMP by UAE. Structural features of the fruit bodies of *T. mesenterica* after extraction were inspected using a scanning electron microscope (SEM). Then, the crude polysaccharide (TMP) was isolated and purified using gel filtration to afford two major polysaccharides, namely TMPA and TMPB, and the chemical composition and antioxidant activity of these polysaccharides were investigated.

2. Materials and methods

2.1. Materials

The fruit bodies of *T. mesenterica* in dried powder form was obtained from Jinyun, Zhejiang Province, China. Samples were ground and sieved using a grinder and were passed through a 40-mesh sieve.

2.2. Ultrasonic extraction

Pretreatment processing of raw material was carried out as reported previously (Hou & Wei, 2008) with modifications. Five grams of dried sample was defatted in a Soxhlet apparatus with petroleum ether (boiling point: 60–90 °C) and pretreated with 80% ethanol twice to remove some colored materials, monosaccharides, oligosaccharides, and small molecule materials. The organic solvent was volatilized and the pretreated dry powder was obtained and immersed in distilled water for 2 h. (No more than 1‰ polysaccharide was extracted. Compared to the content of the polysaccharide of 9.0%, the effect of immersing the dry sample in distilled water for the yield of the polysaccharide was very small.) The suspensions were extracted with a power of 200 W (ultrasound irradiation of 40 kHz) in an ultrasonic bath (KQ-400GKDV, Kunshan Ultrasonic Instrument Co., Ltd., China). The extraction process was performed at different ratios of water to raw material (X_1: 20–40 mL/g), extraction time (X_2: 20–40 min), and extraction temperature (X_3: 40–60 °C). The suspension was then centrifuged (2000 rpm, 20 min) and the supernatant precipitated by the addition of ethanol to a final concentration of 80% (v/v). Precipitates were collected by centrifugation (2000 rpm, 20 min), washed successively with ethanol, and solubilized in deionized water.

2.3. Quantitative analysis of TMP

Using glucose as a standard, polysaccharide content was measured by the phenol–sulfuric acid method after a conversion factor of 0.9 was multiplied to it (Hou & Wei, 2008). Next, 1.0 mL polysaccharide sample solution was mixed with 1.0 mL 5% phenol and 5 mL 98% H_2SO_4. The absorbance at 490 nm was recorded after standing for 10 min. The polysaccharide yield (%) was then calculated using the following equation:

$$\text{polysaccharide yield } (\%) = \frac{C \times N \times V}{W \times 1000} \times 100\% \quad (1)$$

where C is the concentration of polysaccharide calculated from the calibrated regression equation (mg/mL); N is the dilution factor; V is the total volume of extraction solution (mL); and W is the weight of raw material (g).

Table 1
Box–Behnken design and the response values for yields of polysaccharides.

Run	Coded variable levels			Yield of polysaccharides (%)	
	x_1 (ratio)	x_2 (time)	x_3 (temperature)	Experimental values	Predicted values
1	40	20	50	6.72	6.73
2	40	30	60	7.05	6.88
3	40	40	50	6.16	6.17
4	30	30	50	8.04	8.06
5	30	30	50	7.97	8.06
6	30	40	40	5.88	5.72
7	30	20	60	5.98	6.14
8	30	30	50	8.04	8.06
9	20	20	50	4.39	4.38
10	20	40	50	4.15	4.14
11	40	30	40	6.86	7.01
12	30	30	50	8.04	8.06
13	20	30	40	4.48	4.65
14	30	30	50	8.23	8.06
15	20	30	60	4.99	4.84
16	30	40	60	5.66	5.82
17	30	20	40	6.36	6.20

2.4. Design of experiments

Box–Behnken design (BBD) with three independent variables was used for the optimization. The parameters ratio of water to raw material, extraction time, and extraction temperature were chosen as key variables based on the results of preliminary experiments and designated as X_1, X_2, and X_3, respectively, as shown in Table 1. The complete quadratic equation used is as follows:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i Y_j, \quad (2)$$

where Y is the estimated response; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linearity, square, and interaction, respectively; and X_i and X_j are the independent variables.

The variables were coded according to the equation:

$$x_i = \frac{X_i - X_0}{\Delta X}, \quad (3)$$

where x_i is the (dimensionless) coded value of the variable X_i, X_0 is the value of X_i at the center point, and ΔX is the step change.

2.5. Comparison with other extraction procedures

The pretreated dry powders were immersed in distilled water overnight. Then, the suspensions were extracted with hot water in order to obtain a similar and optimized extraction procedure. Each process was repeated for 1 cycle. To further investigate structural changes after extraction, SEM images of the fruit bodies of *T. mesenterica* were obtained to provide visual evidence of the disruption effect.

2.5.1. Hot water extraction (HWE)

The suspensions were placed in a flask and extracted with 100 mL water for 2 h at 50 °C. After the extraction step, the filtered solutions were precipitated by the addition of ethanol to a final concentration of 80% (v/v). Precipitates were collected by centrifugation (2000 × *g*, 20 min), washed successively with ethanol, and then dissolved to a constant volume.

2.5.2. Scanning electron microscope (SEM)

SEM images of *T. mesenterica* dried powder were obtained for untreated samples as well as for samples that underwent HWE and UAE. The powder was fixed on the specimen holder with aluminum tape and sputtered with gold using an ion sputter coater (E-1010,

Hitachi, Japan). All specimens were examined with a Hitachi S-3000 scanning electron microscope (Tokyo, Japan) under high vacuum condition at an accelerating voltage of 20.0 kV and a working distance of 8–9 mm.

2.6. Separation and purification of the polysaccharides

The protein was removed using the Savage method and then followed by papain digestion. The supernatant was separated from insoluble residue with a nylon cloth (pore diameter: 38 μm) according to previous reports (Xie et al., 2010). Then, the crude TMP was dissolved in 0.1 M NaCl solution and filtered through 0.45 μm Millipore filter, and then the solution was subjected to DEAE Sephadex A-50 column (5 cm × 60 cm) chromatography and eluted with deionized water at a flow rate of 2.0 mL/min. The elution fraction (5 mL) were collected and monitored for carbohydrate content based on phenol–sulfuric acid method at 490 nm absorbance. Finally, the eluted fractions were concentrated, dialyzed and lyophilized. The products were further purified on a Sephadex G-100 column (2.6 cm × 60 cm) with water and lyophilized to afford two major polysaccharides, namely TMPA and TMPB.

2.7. Carbohydrate, protein and uronic acid analysis

Phenol–sulfuric acid method was employed for the measurement of carbohydrate content of polysaccharide extracts before and after purification, with glucose as the standard. Protein content was measured according to Bradford's method with bovine serum albumin as the standard (Chen, Zhang, Qu, & Xie, 2008). Uronic acid content was determined by *m*-hydroxydiphenyl method with galacturonic acid as standard (Kimberley & Jock, 1992).

2.8. Molecular weight determination

The molecular weight of fractions were evaluated and determined by the gel permeation chromatography with a HPLC apparatus (LC-10AD, Shimadzu Co., Ltd., Japan) equipped with an ultrahydrogel column (30 cm × 7.5 mm), a model 10-A refractive index detector (RID). The detailed operation conditions were mobile phase: 0.7% (w/v) sodium sulfate; flow rate: 0.5 mL/min; column temperature: 35 °C; injection volume: 20 μl. The calibration curve for molecular weight determination was made using a series of Dextran standards and empower software was used for the calculation of average molecular weights.

2.9. Analysis of monosaccharide composition

GC–MS was used for identification and quantification of the monosaccharides. Fifty milligrams of polysaccharides were hydrolyzed with 5 mL of 1 M sulphuric acid at 100 °C for 6 h, and then evaporated continuously by a rotary evaporator at 45 °C for pH up to neutrality. The hydrolysate was dissolved in 0.5 mL of pyridine with 10.0 mg hydroxylamine hydrochloride, and allowed to react at 90 °C for 30 min. After cooled, 0.5 mL of acetic anhydride was added and the tube was sealed and incubated at 90 °C for 30 min. Its corresponding alditol acetates were analyzed by gas chromatography (GC) on a Hewlett–Packard model 6890 instrument equipped with a capillary column of HP-5MS phenyl methyl siloxane (30 m × 0.25 mm × 0.25 μm). The operation was performed in the following conditions – injection temperature: 100–240 °C; detector temperature: 230 °C; column temperature programmed: 160 °C holding for 2 min, then increasing to 230 °C at 10 °C/min and finally holding for 5 min at 230 °C. Nitrogen was used as the carrier gas and maintained at 1.0 mL/min.

2.10. Protection against oxidative hemolysis of rat erythrocytes

2.10.1. Preparation of erythrocytes

Blood obtained from anesthetized male SD rats (300 ± 20 g) was collected into heparinized tubes and centrifuged at 1500 rpm for 10 min. After removing the supernatant, the pellet was washed and centrifuged at 1500 rpm for 10 min with 10 mM phosphate buffered saline (pH 7.4; PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes were stored at 4 °C and used within 6 h for further studies.

2.10.2. Inhibition of rat erythrocyte hemolysis

The inhibition of rat erythrocyte hemolysis by TMP was evaluated according to the method previously reported (Tedesco et al., 2000) with slight modifications. The rat erythrocyte hemolysis was performed with H_2O_2 as free radical initiator. Briefly, 0.25 mL of test samples at different concentrations and 1.0 mL of 10 mM H_2O_2 were added in succession to 0.5 mL of 20% (v/v) erythrocyte suspension. The mixtures were incubated at 37 °C for 1 h with gentle shaking in dark. After incubation, an aliquot of the reaction mixture was diluted 20 times with phosphate buffered saline (PBS; pH 7.4) and centrifuged at 2000 rpm for 10 min. The absorbance of the resulting supernatant was measured at 415 nm by spectrophotometer (TU-1900, Beijing Perkinje General Instrument Co., Ltd., Peking, China) to determine the hemolysis. Likewise, the erythrocytes were treated with 100 mM H_2O_2 and 0.9% (w/v) saline to obtain a complete hemolysis. The absorbances of the supernatant treated with H_2O_2 and treated with 0.9% (w/v) saline were measured at the same condition. The inhibitory effect of the extract was compared with ascorbic acid. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

2.10.3. Inhibition of lipid peroxidation on erythrocyte ghost membrane

The erythrocyte ghost membrane lipid peroxidation was performed according to the method of Ajila and Rao (2008). Test samples at different concentrations were added to 1.0 mL of erythrocyte ghost membrane suspension (200 μg protein) and lipid peroxidation was induced in the membrane by the addition of 100 μl of 0.2 mM H_2O_2. After incubation for 60 min at 37 °C, trichloroacetic acid (TCA)–2-thiobarbituric acid (TBA)–HCl [2 mL, 15% (w/v) TCA, 0.375% (w/v) TBA, 0.25 M HCl] solution was added, and the mixture boiled at 100 °C for 10 min. Then after cooled, the absorbance of the supernatant at 532 nm was measured. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% lipid peroxidation.

2.11. Statistical analysis

All the data were shown as the mean of three replicate determinations within significance $P < 0.05$ after subjecting to an analysis of variance (ANOVA) and processed with SPSS 13.0. The "Design Expert" software trial version 7.0.0 (Stat-Ease, Minneapolis) was employed for the regression analysis and the graphical optimization.

3. Results and discussion

3.1. Optimization of extraction conditions by BBD

3.1.1. Extraction model and statistical analysis

At present, orthogonal testing is applied extensively in the extraction of polysaccharides. Orthogonal test design focuses on arranging reasonable experiments that can consider several factors simultaneously and find optimal factor levels, but it fails

Table 2
Regression coefficients estimate and their significance test for the quadratic polynomial model.

Source	Degrees of freedom	Sum of squares	Mean square	*F*-value	*P*-value
Model[a]	9	30.20	3.36	93.71	<0.01
X_1	1	9.64	9.64	269.14	<0.01
X_2	1	0.32	0.32	8.94	0.0202
X_3	1	1.250×10^{-3}	1.250×10^{-3}	0.034	0.8571
X_1X_2	1	0.026	0.026	0.72	0.4257
X_1X_3	1	0.026	0.026	0.72	0.4257
X_2X_3	1	6.400×10^{-3}	6.400×10^{-3}	0.20	0.6851
X_1^2	1	8.45	8.45	236.13	<0.01
X_2^2	1	7.03	7.03	196.31	<0.01
X_3^2	1	2.71	2.71	75.64	<0.01

[a] $R^2 = 0.9918$, $R^2_{Adj} = 0.9812$, C.V. = 2.95%.

to give a regression equation for the whole parameter space tested. Response surface optimization, on the other hand, establishes a high precision regression equation, details the interactions between several factors, and is more advantageous than the traditional orthogonal test design because of its highly efficient, time-saving design pattern (Qiao et al., 2009; Liang, 2008). A total of 17 runs for optimizing the three individual parameters in the current BBD are applied in the production of TMP. The values of the independent process variables (X_1, X_2, and X_3) considered, as well as the measured and predicted values for response (extraction yields of polysaccharides), are given analytically in Table 2. The TMP yields range from 4.15% to 8.23%. By applying multiple regression analysis on the experimental data, the response variable and the test variables can be related using the following polynomial equation:

$$Y = 8.96 + 1.22X_1 - 0.22X_2 + 0.014X_3 - 0.090X_1X_2 - 0.090X_1X_3 + 0.048X_2X_3 - 1.58X_1^2 - 1.44X_2^2 - 0.89X_3^2, \quad (4)$$

where Y is the polysaccharide yield and X_1, X_2, and X_3 are the values for the ratio of water to raw material, extraction time, and extraction temperature, respectively.

The ANOVA of the quadratic regression model shows the value of the determination coefficient ($R^2 = 0.9918$), while the value of the adjusted determination coefficient ($R^2_{Adj} = 0.9812$) is almost 1, indicating a high degree of correlation between the observed and predicted values. At the same time, the coefficient of variation (C.V. = 2.95%) has a low value, indicating a high degree of precision and reliability of the experimental values. All these results suggest that the model, as evident from the calculated F value (93.71) and the low probability value ($P < 0.01$), is adequate for predicting within the range of the variables employed. On the other hand, the coefficient values were also calculated and tested for their significance, as shown in Table 2. The calculated coefficient for the parameter optimization suggests that all the independent variables studied (X_1, X_2), along with the two quadratic terms (X_1^2, X_2^2), are significant ($P < 0.05$) according to Table 2. Results also show that the ratio of water to raw material is the most significant impact factor on the yields of crude polysaccharides due to it having the greatest P-value ($P < 0.01$) while those of the two other parameters are more than 0.01.

3.1.2. Optimization of the procedure

The relationship between independent and dependent variables is illustrated by the three-dimensional representation of the response surfaces and the two-dimensional contours generated by the model. Two variables within the experimental range are depicted in three-dimensional surface plots when the third variable is kept constant at zero level. The shapes of the contour plots, elliptical or circular, indicate whether the interactions between the corresponding variables are significant or not (Luo, 2008). An elliptical contour plot means the interactions between the variables are significant while a circular contour plot means otherwise. As shown in Fig. 1, the polysaccharide yields are not affected significantly by alterations of the test variables through three independent response surface plots and their respective contour plots in the experimental range. In the present study, the ratio of water to raw material (X_1) and extraction time (X_2) both have a positive impact on the yields. There is an increase in the TMP yield when the yield reaches its maximum at a fixed extraction temperature, with no significant further improvement thereafter (Fig. 1A). The ratio of water to raw material and extraction temperature also has a similar effect on the yields when the extraction time is fixed (Fig. 1B). Nevertheless, longer or shorter times lead to a decrease in the TMP yield, as shown in Fig. 1C. However, results showed that interactions between the variables are insignificant ($P > 0.05$) in Table 2. It was in agreement with previous investigation (Li, Ding, & Ding, 2007; Zhong & Wang, 2010), which had been reported that the interactions between ultrasonic power and extraction time, and ultrasonic power and ratio of water to material caused no significant effect on the extraction yield.

Through these three-dimensional plots and their respective contour plots (Fig. 1), the optimal values of the tested variables for obtaining a TMP yield of 8.29% can be predicted as follows: ratio of water to raw material, 33.9; extraction time, 29.11 min; and extraction temperature, 49.86 °C. However, considering the production cost generated by electrical power charges and the operability in actual production, the optimal conditions can be modified as follows: ratio of water to raw material, 34 mL/g; extraction time, 30 min; and extraction temperature, 50 °C.

To validate the adequacy of the model equations, a verification experiment is carried out under the optimal conditions mentioned above. Under those conditions, the experimental yield is 8.26% ($n = 3$), which is well-matched with the predicted value obtained from real experiments (Table 3). The result demonstrates the validation of the extraction model.

3.2. Comparison with HWE

The extraction efficiency using UAE is mainly attributed to its greatly facilitated mass transfer between immiscible phases through super agitation, as well as to the important mechanical effects of microjetting and microstreaming (Tsochatzidis et al., 2001; Velickovic, Milenovic, Ristic, & Veljkovic, 2006). Through SEM, the surface and structural features of *T. mesenterica* fruit bodies before and after extraction is captured, resulting in an image that has a three-dimensional impression due to the depth of field provided by SEM. The SEM images provide a visual evidence of the structural changes in the samples. The hymenium of the untreated raw material is shown to be covered by a thick mist-like mucous layer full of basidiospores and yeast-like conidia, which presumably is why the surface of the fruit bodies appears matte and often pruinose (Fig. 2A). When the fruit bodies is immersed in an aqueous media, the hyphae in the hymenium swells, forming a markedly

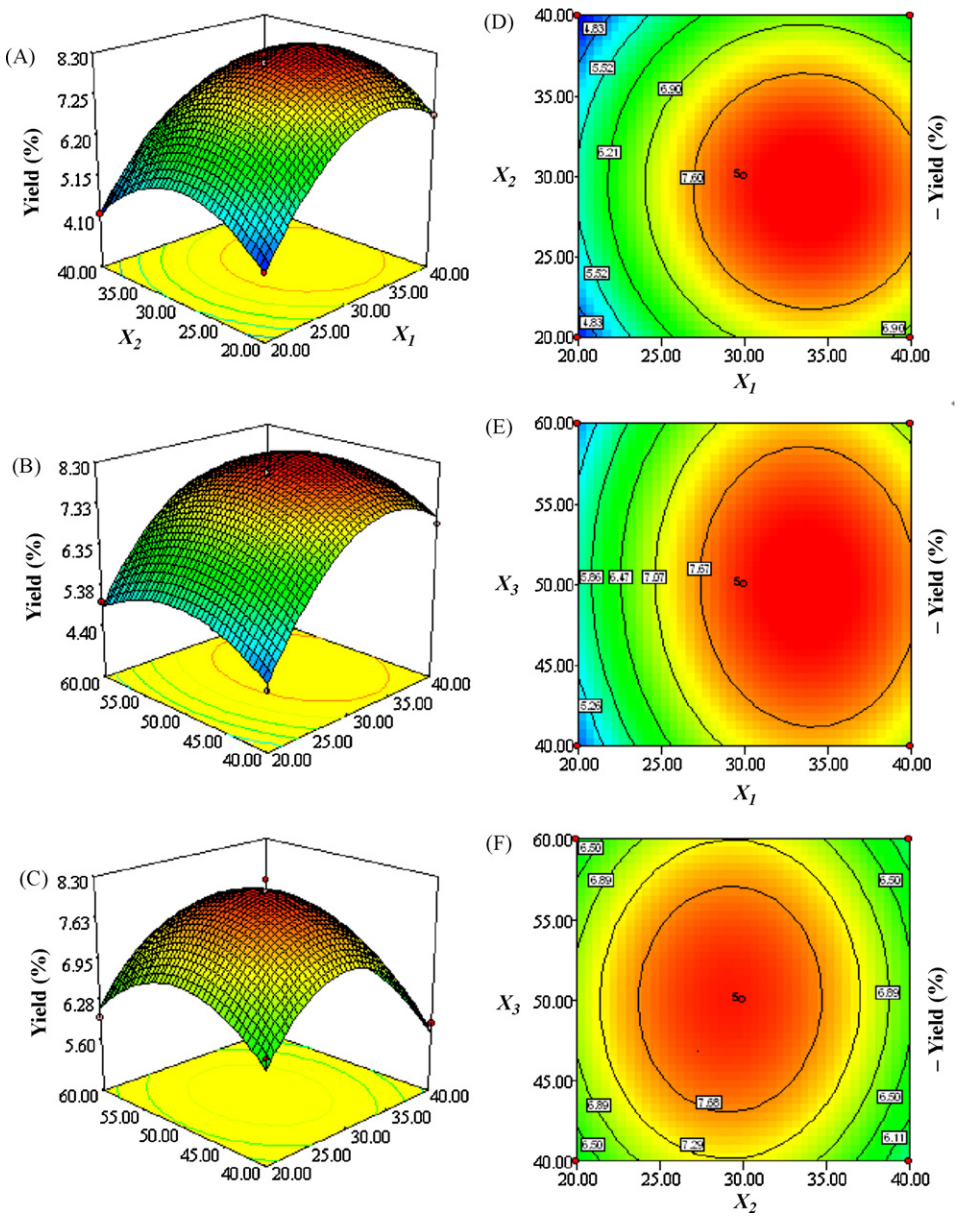

Fig. 1. Response surface plots (A–C) and contour plots (D–F) showing the effects of the variables on the yield of polysaccharide.

thickened membrane and the mucous layer starts to dissolve, so that little or no mucus can be seen after HWT (Fig. 2B). After UAE, a more significant impact on the structural changes caused by ultrasonic cavitation is observed. A spongy structure with a defined fracture on the thread-like hyphae can also be seen more clearly, suggesting that ultrasonic vibration increases the number of cavitation formed as well as the mass transfer rates to prompt the migration of target compounds from the material to the surroundings and to bring about a higher extraction efficiency of polysaccharides (Fig. 2C).

3.3. Composition analysis of TMP

The crude polysaccharides from the fruit bodies of *T. mesenterica* were further isolated and purified by DEAE-Sephadex A-50 and Sephrose 6B gel filtration chromatogram into the two major polysaccharides, TMPA and TMPB (Fig. 3). These polysaccharides showed negative Fehling's reagent and iodine–potassium iodide reactions, indicating that they didn't contain reducing sugar and starch-type polysaccharide, and both had negative responses to the Bradford test and no absorption at 280 nm in the UV spec-

Table 3
Predicted and experimental values of the responses under optimal conditions.

Extraction method	Sample of quantity (g)	Ratio of water to raw material (mL/g)	Extraction time (min)	Extraction temperature (°C)	Yield of polysaccharides (%)
UAE (predicted)	10.0	33.90	29.11	49.86	8.29
UAE (experimental)	10.0	34	30	50	8.26
HWE	10.0	34	120	50	2.31

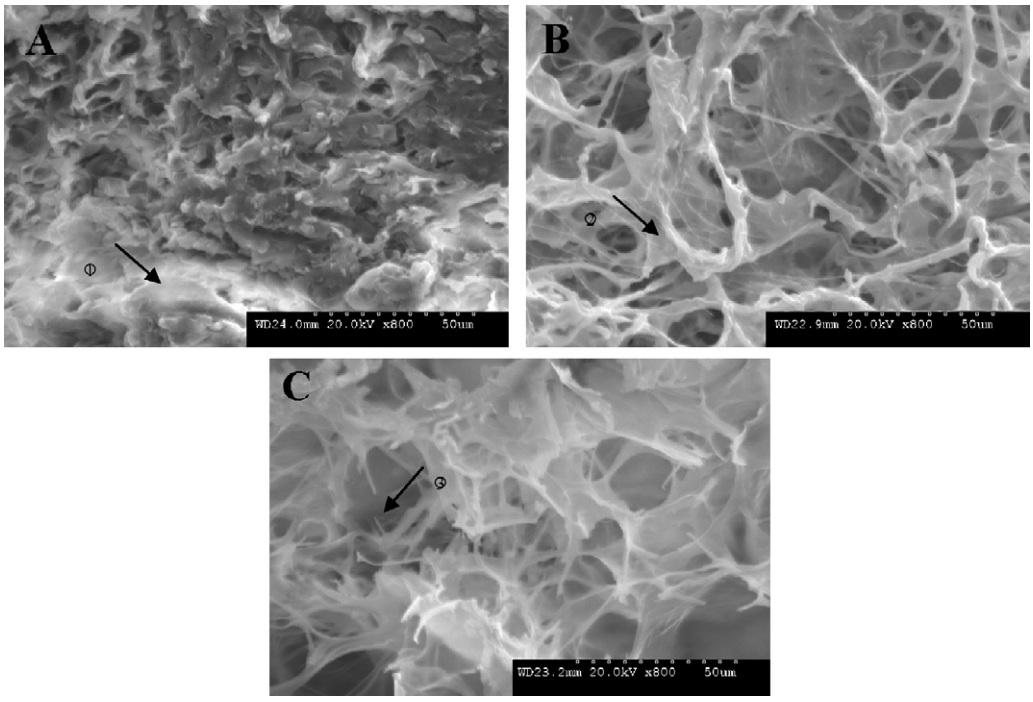

Fig. 2. SEM images of *T. mesenterica* fruit bodies.

trum, indicating the absence of protein. The average molecular weights were ca. 4.32×10^5 Da and 2.38×10^5 Da by HPPGC for TMPA and TMPB, respectively. For the facts that natural polysaccharides always conjugate with other components, such as amino acid, protein and nucleic acids, and molecular mass of crude polysaccharides detected by GC–MS was affected by impurities, which resulted in big errors compared to actual value, we did not detect molecular mass of crude TMP in this study. The results of GC quantitative analysis of the acetylated of monosaccharides revealed that TMPA and TMPB were composed of xylose, mannose and glucose with molar ratios of 1.00:4.74:0.91 and 1.00:6.63:2.34, respectively (Table 4). Furthermore, total uronic acid content was measured by carbazole–sulfuric acid method using glucuronic acid as the standard. The uronic acid contents in TMPA and TMPB was 14.29%, while TMPB was not detectable for uronic acid, indicated the difference between TMPA and TMPB.

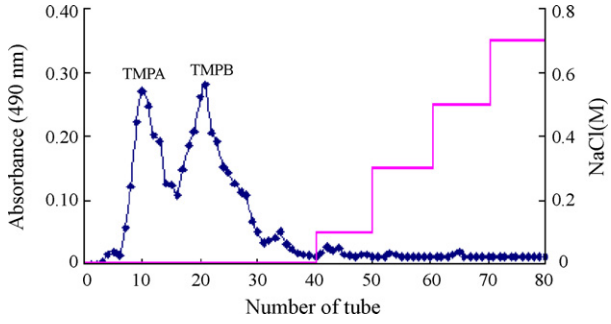

Fig. 3. Isolation and purification of polysaccharides.

3.4. Protection against oxidative hemolysis of rat erythrocytes

3.4.1. Inhibition of rat erythrocyte hemolysis

The in vitro oxidative hemolysis of rat erythrocytes was used here as a model to study the hydrogen peroxide-induced damage of biological membranes and the protective effects of TMP. When the erythrocytes were incubated with TMP alone (without H_2O_2) at the highest concentration tested (2.5 mg/mL), hemolysis is maintained at a background level similar to that in the normal control samples (H_2O_2 untreated samples) (data not shown). As shown in Fig. 4, the effects of crude TMP, TMPA and TMPB (0.5–2.5 mg/mL) on rat erythrocytes exposed to the radical initiator H_2O_2. When H_2O_2 was added to the suspension of erythrocytes, hemolysis induction was lagged, indicating that endogenous antioxidants in the erythrocytes could efficiently quench radicals to protect them against hydrogen peroxide-induced hemolysis as described previously (Zou, Agar, & Jones, 2001). It was clearly evident that crude TMP, TMPA and TMPB significantly protected the erythrocyte membrane from hemolysis induced by H_2O_2 in a concentration- dependent manner (Fig. 4). The IC_{50} value of crude TMP after 1 h of incubation was 1.01 mg/mL, and that of TMPA was 1.17 mg/mL, while that of TMPB was 0.52 mg/mL,

Table 4
Chemical composition and molecular weight of polysaccharides from the fruit bodies of *T. mesenterica*.

Sample	Monosaccharide composition (mol.%)[a]			Uronic acids (wt.%)[b]	Protein (wt.%)[b]	Molecular weight (Da)
	Xylose	Mannose	Glucose			
Crude TMP	18.51	70.72	10.37	10.05	9.43	ND[c]
TMPA	15.01	71.14	13.66	14.29	ND	4.32×10^5
TMPB	10.01	66.37	23.42	ND	ND	2.38×10^5

[a] The quantities of the neutral sugars was given in mol.%.
[b] The uronic acid content was calculated as wt.%.
[c] ND, not detected.

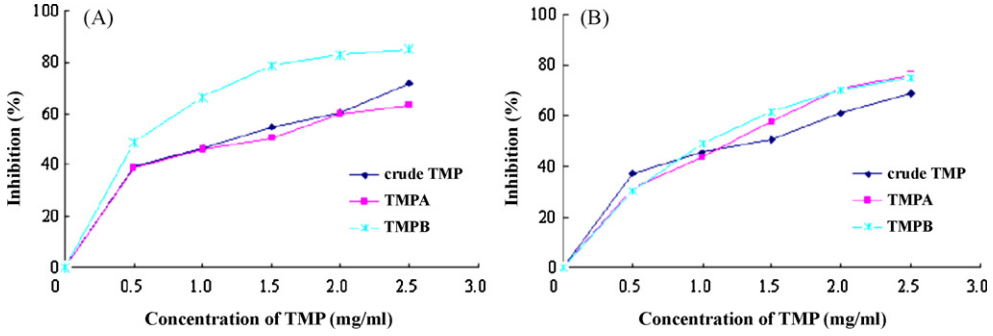

Fig. 4. Concentration-dependent antioxidant activities of TMP.

showed significantly higher protection against oxidative hemolysis of rat erythrocytes. It indicated that TMP might act as electron or hydrogen donator to scavenge OH• as described previously (Jing, Quanbin, Zhang, & Zhien, 2008).

3.4.2. Anti-LPO effects of TMP on erythrocyte ghost membrane

It had been reported that the hydroxyl radical reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids to produce lipid hydroperoxide, which can be decomposed into alkoxy and peroxyl radical. The two radicals further produce numerous carbonyl products such as malondialdehyde, which is responsible for DNA damage, generation of cancer and aging related disease (Ajila & Rao, 2008). Therefore, inhibition of lipid peroxides was chosen as the parameter to directly evaluate anti-LPO activities of TMP in vitro. Lipid peroxidation of erythrocyte ghost membrane was triggered by H_2O_2 and the end-products of the process were measured in terms of the thiobarbituric acid-reactive substances (TBARS) formed (Rathee, Hassarajani, & Chattopadhyay, 2006). As shown in Fig. 4, in lipid peroxidation system of rat erythrocyte ghost membrane, crude TMP, TMPA and TMPB showed antioxidant activities at concentrations of 0.5–2.5 mg/mL. Compared with the crude TMP (IC_{50}: 1.12 mg/mL) and TMPA (IC_{50}: 1.05 mg/mL), TMPB (IC_{50}: 1.01 mg/mL) showed litter higher inhibitory activities on lipid peroxidation of erythrocyte ghost membrane. These results were in accordance with antihemolytic activity determined above.

Many studies have suggested that polysaccharide- protein conjugates exhibit different antioxidant abilities depending on the protein content (Chaudhuri, Banerjee, Basu, Sengupta, & Sengupta, 2007; Singh & Rajini, 2008). However, in this study, the crude TMP with slightly higher protein content did not show significantly higher antioxidant activities than the purified TMP (TMPA and TMPB). Instead, TMPB with lower molecule weight did well. The putative interactions of TMP and protein have not yet been studied and thus further investigations about the structural analysis and evaluation of the bioactivities of TMP should be done in order to determine their application in both the food and medicinal field.

4. Conclusion

An efficient ultrasonic-assisted extraction (UAE) technique was employed to extract polysaccharides from the fruit bodies of *T. mesenterica* by RSM. Box–Behnken design (BBD) was used to determine the optimum process parameters that could give a high extraction yield. ANOVA showed that the effects of the variables (ratio of water to raw material and extraction time) are significant and quadratic models are obtained for predicting responses. The optimal conditions determined are as follows: ratio of water to raw material, 34 mL/g; extraction time, 30 min; and extraction temperature, 50 °C. Under these optimal conditions, a maximum polysaccharide yield of 8.26% can be achieved. Compared to other conventional extraction techniques, UAE requires less extraction time and provides higher extraction efficiency due to its ultrasonic cavitation.

Furthermore, two major fractions of polysaccharides (TMPA and TMPB) were obtained through purification of the crude TMP, while monosaccharide component analysis indicated that TMPA and TMPB were composed of xylose, mannose and glucose in molar ratio of 1.00:4.74:0.91 and 1.00:6.63:2.34. Compared to the crude TMP and TMPA, TMPB with lower molecule weight exhibited significantly higher protective activities on oxidative damage of rat erthrocytes. The mechanism might include, at least in part, reacting with free radicals of H_2O_2 (by donating electron or hydrogen), then preventing OH• production and lipid peroxidation. Further study should be carried out to elucidate bioactivity through animal experiments with the purpose of applying the polysaccharides as a potential natural antioxidant functional ingredient in the food industry.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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