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# Optimization of polysaccharides extraction from *Trametes robiniophila* and its antioxidant activities

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ABSTRACT

Based on a single-factor experiment, Box–Behnken design was used to optimize the ultrasound-assisted extraction process of *Trametes robiniophila* (Huaier) polysaccharides (HPs). The optimum conditions were predicted as follows: ratio of water to raw material, 46.0 mL/g; extraction temperature, 68.9 °C; ultrasonic power, 51.3 W; and extraction time, 36.8 min. Under these conditions, the highest yield of HPs obtained was 36.8 ± 0.12%, which was in good agreement with the predicted value 36.6%. Additionally, chemical analysis of HPs showed a high content (85.3 ± 1.3%) of carbohydrates containing fucose, arabinose, xylose, mannose, galactose and glucose, with molar percentages 5.82%, 13.11%, 16.88%, 15.85%, 11.40% and 36.94%, respectively. Besides, HPs demonstrated appreciable antioxidant potential on ABTS, superoxide anion and hydroxyl radicals *in vitro*. These may further provide theoretical basis for the widely application of HPs in medicine and health care products.



## 1. Introduction

*Trametes robiniophila* Murr, commonly called "Huaier" in Chinese, is a kind of officinal fungus and has been widely used as a traditional Chinese medicine (TCM) to treat a wide variety of diseases, being recorded for the first time in *Zhou Hou Fang* 1500 years ago (Li, Ye, Wang, & Tang, 2007; Ren et al., 2009; Zhang, Kong, Yan, Yuan, & Yang, 2010; Zhuang, 1998). In recent decades, Huaier extract has been reported to be effective in treatment of cancers including hepatocellular carcinoma and breast cancer (Ren et al., 2009; Xu et al., 2011; Zhang et al., 2010), displaying various biological activities, such as apoptosis, antiangiogenesis, drug resistance reversal, anti-metastasis and system immune activation (Wang, Zhang, Huo, & Yang, 2012; Zhang et al., 2010). Additionally, Huaier extract was also found to be effective against *Echinococcus granulosus* (Lv et al., 2013). As an anti-carcinogen medicine, Huaier mainly contains effective medicinal fungal substances, such as protein-bound polysaccharides, organic constituents and mineral elements, of which protein-bound polysaccharides have been proved to be the main active ingredient from Huaier aqueous extract in the respect of anti-cancer effects and immunity-enhancing activities (Cui, Goh, Archer, & Singh, 2007; Jia, Dong, Lu, Guo, & Wei, 2009; Zhuang, 1998). In recent years, as many polysaccharides extracted from fungi possessed a multitude of biological activities such as anti-tumor, anti-oxidant and anti-inflammatory effects, fungi polysaccharides with lower toxicity and fewer side effects than chemical drugs were thought to be an abundance of medicinal resources (Jin & Ning, 2013; Sun, Li, Yan, & Liu, 2010; Wang, Wang, & Quan, 2014; Yin, You, & Su, 2014). Therefore, a great deal of attention has been paid to both the crude and purified polysaccharides from Huaier due to their unique bioactivities such as anti-tumor, anti-invasive and anti-metastatic effects (Sun et al., 2013; Zheng et al., 2014). Unfortunately, little attention has been given to the extraction of HPs under optimal conditions, which may lead to low yields and high cost of HP production and hinder its further exploitation and utilization.

Nowadays, optimizing extraction process to get maximum polysaccharide yield is becoming a research focus (Chen et al., 2014; Gan & Latiff, 2011; Li et al., 2013; Li, Wang, & Li, 2014; Shen et al., 2014; Wang, Sun, et al., 2014; Yin et al., 2014; You, Yin, Zhang, & Jiang, 2014; Zhu et al., 2014). Box–Behnken design (BBD), being a spherical and revolving design, has been widely used in optimization of extraction processes because of its best possible combination of extraction conditions for maximum polysaccharide production (Box & Wilson, 1951; Han, Jiang, & Zhang, 2011; Li et al., 2013; Wang, Sun, et al., 2014; Xia et al., 2011). BBD is more efficient to conduct experiments compared with traditional

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methods, simplifying the complexity of the experimental trials needed to evaluate multiple variables and their interactions (Zhu et al., 2014). Current commonly used methods for polysaccharide extraction include hot water extraction (HWE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), of which UAE was selected because of its lower energy consumption, lower consumption of solvents, higher extraction efficiency and higher level of automation (Vilkhu, Mawson, Simons, & Bates, 2008; Xu, Zhang, & Hu, 2000).

Therefore, the present study aimed to optimize UAE parameters using BBD to study the effects of extraction conditions on the extraction yield of HPs. Furthermore, its chemical composition characteristics, such as carbohydrate and protein contents and monosaccharide composition analysis, were determined. Besides, antioxidant activities of HPs, such as ABTS, superoxide anion and hydroxyl radicals, were evaluated *in vitro*.

## 2. Materials and methods

### 2.1. Materials

The dried fruiting bodies of Huaier were purchased from a local market (Tianjin city, China). 2-2-Azino-bis-(3-ethyl-benzthia-zoline-6-sulfonic acid) (ABTS), nitroblue tetrazolium (NBT), potassium persulfate, phosphate buffer, methionine (MET), riboflavin (RF), nitroblue tetrazolium (NBT), EDTA, ferrous sulfate ($FeSO_4$), $H_2O_2$ and sodium salicylate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemical reagents were of analytical grade.

### 2.2. Extraction procedure

The UAE experiments were performed according to the method described by Wang, Sun, et al. (2014) using an ultrasonic device (AS3120A, Tianjin Automatic Science Instrument Co., Ltd., China). Each sample of Huaier fruiting bodies (25 g) was ground (40 mesh) and extracted with distilled water in a 1000 mL beaker with a selected ratio of water to raw material, extraction temperature, ultrasonic power and extraction time. After extraction, the extract solutions were filtered through a filter paper and the filtrate was concentrated to 100 mL with a rotary evaporator (BÜCHI, Switzerland) at 45 °C under vacuum. Then 95% ethanol was added to a final concentration of 80% (v/v). It was kept overnight at 4 °C. The precipitates were obtained by centrifugation (4000 r/min, 15 min) and lyophilized to get HPs. Each HP sample was weighted with an analytical balance (BS2202S, SARTORIUS, Germany), and the extraction yield ($Y$) was calculated by the following equation:

$$Y\ (\%, w/w) = \frac{weight\ of\ the\ HPs\,(g)}{weight\ of\ each\ Huaier\ sample\,(g)} \times 100 \quad (1)$$

### 2.3. Single-factor design for HP extraction

The single-factor design was carried out to determine the preliminary range of the extraction variables including $X_1$ (ratio of water to raw material), $X_2$ (extraction temperature), $X_3$ (ultrasonic power) and $X_4$ (extraction time). In detail, each Huaier sample mentioned above was extracted with designed ratio of water to raw material (10, 20, 30, 40 and 50 mL/g, respectively), extraction temperature (40, 50, 60, 70 and 80 °C, respectively), ultrasonic power (40, 50, 60, 70 and 80 W, respectively), and extraction time (10, 20, 30, 40 and 50 min, respectively). Each experiment was conducted in triplicates.

**Table 1**
Levels and code of variable used for Box–Behnken design (BBD).

| Independent variables | Symbol | Range and level | | |
|---|---|---|---|---|
| | | −1 | 0 | 1 |
| Ratio of water to raw material (mL/g) | $X_1$ | 30 | 40 | 50 |
| Extraction temperature (°C) | $X_2$ | 50 | 60 | 70 |
| Ultrasonic power (W) | $X_3$ | 50 | 60 | 70 |
| Extraction time (min) | $X_4$ | 20 | 30 | 40 |

### 2.4. BBD for HP extraction

After the preliminary range of each extraction variable was confirmed by the single-factor design, a Box–Behnken design (BBD) was employed in this optimization study. As shown in Table 1, four variables were prescribed into three levels, coded +1, 0 and −1 for high, intermediate and low value, respectively, which were calculated by the following equation:

$$X_i = \frac{x_i - x_0}{\Delta x_i},\ i = 1, 2, 3, \quad (2)$$

where $X_i$ is the coded value of the independent variable; $x_i$ is the actual value of the independent variable; $x_0$ is the actual value of the independent variable at the center point; and $\Delta x_i$ is the step change of variable.

A design matrix comprising of 29 experimental runs was constructed, as shown in Table 2. All trials were performed in triplicate. The experimental data were fitted to the following second-order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii} X_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij} X_i X_j \quad (3)$$

where $Y$ is the response function; $\beta_0$ is an intercept; $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are the regression coefficients for the linear, quadratic, and

**Table 2**
BBD with the observed responses for the extraction yield of HPs.

| Run | Coded variable levels | | | | Extraction yield (%) | |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Experimental | Predicted |
| 1 | −1 | −1 | 0 | 0 | 28.85 | 28.34 |
| 2 | 1 | −1 | 0 | 0 | 22.77 | 22.36 |
| 3 | −1 | 1 | 0 | 0 | 25.62 | 26.04 |
| 4 | 1 | 1 | 0 | 0 | 30.83 | 31.25 |
| 5 | 0 | 0 | −1 | −1 | 24.79 | 25.49 |
| 6 | 0 | 0 | 1 | −1 | 28.00 | 28.50 |
| 7 | 0 | 0 | −1 | 1 | 34.78 | 34.49 |
| 8 | 0 | 0 | 1 | 1 | 25.12 | 24.83 |
| 9 | −1 | 0 | 0 | −1 | 27.33 | 27.66 |
| 10 | 1 | 0 | 0 | −1 | 24.64 | 25.00 |
| 11 | −1 | 0 | 0 | 1 | 27.87 | 28.04 |
| 12 | 1 | 0 | 0 | 1 | 29.35 | 29.95 |
| 13 | 0 | −1 | −1 | 0 | 22.95 | 23.69 |
| 14 | 0 | 1 | −1 | 0 | 34.34 | 34.95 |
| 15 | 0 | −1 | 1 | 0 | 28.01 | 28.33 |
| 16 | 0 | 1 | 1 | 0 | 23.27 | 23.66 |
| 17 | −1 | 0 | −1 | 0 | 30.56 | 29.99 |
| 18 | 1 | 0 | −1 | 0 | 29.05 | 28.55 |
| 19 | −1 | 0 | 1 | 0 | 25.35 | 25.61 |
| 20 | 1 | 0 | 1 | 0 | 26.15 | 26.28 |
| 21 | 0 | −1 | 0 | −1 | 24.30 | 24.10 |
| 22 | 0 | 1 | 0 | −1 | 28.96 | 28.67 |
| 23 | 0 | −1 | 0 | 1 | 27.89 | 28.04 |
| 24 | 0 | 1 | 0 | 1 | 30.30 | 30.06 |
| 25 | 0 | 0 | 0 | 0 | 32.42 | 32.77 |
| 26 | 0 | 0 | 0 | 0 | 32.42 | 32.77 |
| 27 | 0 | 0 | 0 | 0 | 33.33 | 32.77 |
| 28 | 0 | 0 | 0 | 0 | 33.58 | 32.77 |
| 29 | 0 | 0 | 0 | 0 | 31.11 | 32.77 |

interactive terms, respectively; $X_i$ and $X_j$ are the coded independent variables.

Analysis of the experimental data and calculation of predicted data were carried out using Design-Expert software (Version 8.0, Stat-Ease Inc., Minneapolis, USA). The regression coefficients of individual linear, quadratic, and interaction terms were determined according to the analysis of variance (ANOVA). The three-dimensional (3D) surface plots and contour plots were also generated from the fitted polynomial equation to visualize the relationship of each independent variable and the response (Triveni, Shamala, & Rastogi, 2001). *F*-value and *p*-value were used to check the significances of the regression coefficient, and the values of $R^2$, adjusted-$R^2$ of models were evaluated to check the model adequacies. Finally, the optimum condition was verified by performing three additional confirmation experiments.

### 2.5. Chemical analysis

HPs were obtained under the optimum extraction condition, and the chemical analysis was carried out using the following methods: phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and Bradford method (Bradford, 1976). Briefly, total carbohydrate content of HPs was measured by the phenol-sulfuric acid method using D-glucose as the standard, and protein content of HPs was determined by the Bradford method using bovine serum albumin as the standard.

The monosaccharide composition of the HP sample was analyzed by gas chromatography–mass spectrometry (GC–MS) according to the reported method with slight modifications (Peng et al., 2013). In brief, the HPs sample (10 mg) obtained under the optimum condition was hydrolysed in 2 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 6 h. The hydrolysate was then diluted with anhydrous ethanol, and TFA was removed using a rotary vacuum evaporator at 45 °C, which process was repeated several times until the hydrolysate obtained were neutral. The obtained neutral hydrolysate was reduced to alditol using 25 mg of sodium borohydride with 3 mL of distilled water at room temperature for 12 h. The reaction products were analyzed by GC–MS (Shimadzu 2010 instrument) with a fused silica capillary column HP-5MS (0.25 μm × 0.25 mm × 30 m). The operation conditions of GC–MS were as follows: the column temperature was increased from 170 to 210 °C in a rate of 2 °C/min and then 8 °C/min to 250 °C (Dong, Yao, & Fang, 2003).

### 2.6. Antioxidant activity assays of HPs in vitro

#### 2.6.1. ABTS radical scavenging activity

The ABTS radical scavenging assay of HPs was performed using the method of Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993) with some modifications. The ABTS radical cation (ABTS+) was generated by mixing an ABTS (7 mM) solution with a potassium persulfate (2.45 mM, final concentration) aqueous solution and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. In the moment of use, the ABTS+ solution was diluted to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C for 30 min. Then, the ABTS+ solution (2.0 mL) was added to the HP sample solution (0.2 mL) with various concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). After reacting for 20 min at room temperature, the absorbance was immediately measured at 734 nm. BHT was used as standard. The scavenging effect (%) of HPs on the ABTS radical was calculated according to the following equation:

$$\text{ABTS scavenging effect}\,(\%) = \frac{A_{\text{blank}\,734} - A_{\text{sample}\,734}}{A_{\text{blank}\,734}} \times 100, \quad (4)$$

where $A_{\text{blank}\,734}$ and $A_{\text{sample}\,734}$ are the absorbance of blank control group (without sample) and sample group, respectively, at 734 nm.

#### 2.6.2. Superoxide radical scavenging activity

The superoxide radical scavenging activity of HPs was determined according to the method of Stewar and Beewley (1980) with slight modifications. The sample solution was treated with 3.0 mL of 50 mM phosphate buffer (pH 7.8) containing the following reagents (final concentrations): 13 mM methionine (MET), 10 mM riboflavin (RF), 75 M nitroblue tetrazolium (NBT), 100 mM EDTA and the HP sample (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). After illuminating the reaction mixture with a fluorescent lamp at 25 °C for 30 min, the absorbance of the HPs was measured at 560 nm using BHT for a positive control. The scavenging effect (%) of HPs on the superoxide radical was calculated using the following equation:

$$\text{superoxide radical scavenging effect}\,(\%) = \frac{A_{\text{blank}\,560} - A_{\text{sample}\,560}}{A_{\text{blank}\,560}} \times 100, \quad (5)$$

where $A_{\text{blank}\,560}$ and $A_{\text{sample}\,560}$ are the absorbance of blank control group (without sample) and sample group, respectively, at 560 nm.

#### 2.6.3. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of HPs was determined according to Smirnoff and Cumbes's work (1989) with minor modifications. Briefly, the reaction mixture contained 0.5 mL $FeSO_4$ (1.5 mM), 0.35 mL $H_2O_2$ (6 mM), 0.15 mL sodium salicylate (20 mM) and 1.0 mL HP sample (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). After incubation at 37 °C for 30 min, the absorbance of the mixture was measured at 562 nm, and BHT was used as a positive control. The scavenging effect (%) of HPs on the hydroxyl radical was calculated as follows:

$$\text{hydroxyl radical scavenging effect}\,(\%) = \frac{A_{\text{blank}\,562} - A_{\text{sample}\,562}}{A_{\text{blank}\,562}} \times 100, \quad (6)$$

where $A_{\text{blank}\,562}$ and $A_{\text{sample}\,562}$ are the absorbance of blank control group (without sample) and sample group, respectively, at 562 nm.

### 2.7. Statistical analyses

All data are expressed as means ± standard deviations (SD), and all analyses were performed in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) for multiple comparisons using Design-Expert (Version 8.0, Stat-Ease). Data were analyzed using Student's *t*-test and *F*-test, and $p < 0.05$ were considered statistically significant.

## 3. Results and discussion

### 3.1. Single-factor experiments for HP extraction

In this part, ratio of water to raw material, extraction temperature, ultrasonic power and extraction time were studied, respectively, and the results of single factor experiments are presented in Fig. 1. Four factors were found to have a significant impact on the yield of HPs.

#### 3.1.1. Effect of ratio of water to raw material on extraction yield of HPs

Ratio of water to raw material is a routine parameter that significantly affects the extraction efficiency. In the present study, extraction was carried out at different ratios of water to raw material (10, 20, 30, 40 and 50 mL/g, respectively), when other

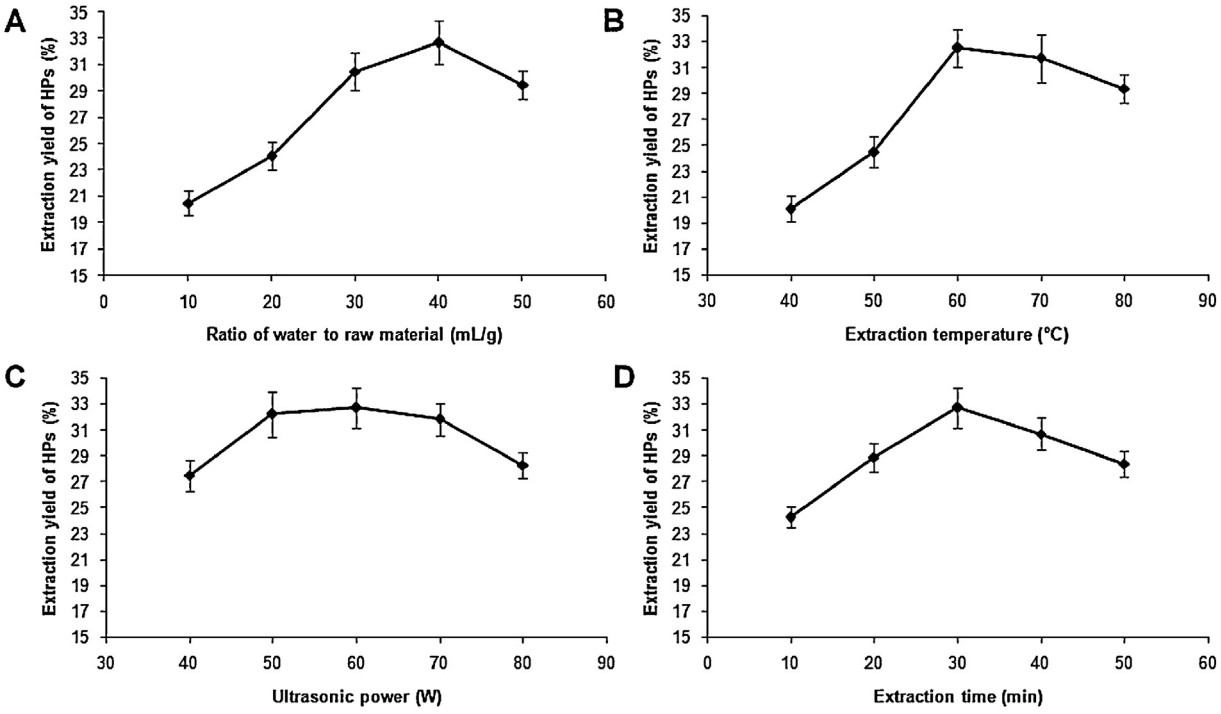


**Fig. 1.** Effect of ratio of water to raw material (A), extraction temperature (B), ultrasonic power (C) and extraction time (D) on the extraction yield of HPs.

parameters were as follows: extraction temperature 60 °C, ultrasonic power 60 W and extraction time 30 min. As shown in Fig. 1A, the extraction yield increased with the increase of ratio of water to raw material from 10 to 40 mL/g, while above 30 mL/g, the yield increased slowly up to its maximum amount of 32.70 ± 1.7% at 40 mL/g, and then decreased when ratio of water to raw material continued to rise. This phenomenon can be explained that the higher the ratio of water to raw material was, the lower the concentration and viscosity of the extraction solvent would be, according to Xu et al. (2014). Hence more polysaccharides molecules could dissolve in water and the extraction yield increased. But the intensity of cavitation falls down with an increase in the viscosity of the medium, since the formation of cavitation requires the negative pressure in the rarefaction region of wave function overcome the natural cohesive forces (Gogate & Pandit, 2004; Majumdar, Kumar, & Pandit, 1998; Xu et al., 2014). Therefore, after 40 mL/g, further increase of ratio of water to material would not increase the extraction yield. Thus, ratio of water to raw material range of 30–50 mL/g was favorable for extracting HPs, being selected for further optimization in BBD design.

### 3.1.2. *Effect of extraction temperature on extraction yield of HPs*

Extraction temperature is also a very important parameter that would influence the extraction efficiency for UAE. To investigate the effect of temperature on extraction yield of HPs, extraction was conducted at different temperatures (40, 50, 60, 70 and 80 °C, respectively), when the other extraction variables were set as follows: ratio of water to raw material 40 mL/g, ultrasonic power 60 W, and extraction time 30 min. As shown in Fig. 1B, the extraction yield increased when temperature increased from 40 to 60 °C, and then it declined, reaching a maximum (32.53 ± 1.4%) at approximately 60 °C. The positive effect of extraction temperature can be explained that as the temperature increases, the polysaccharides diffusion coefficient increases, resulting in an enhanced solubility of the polysaccharides in the extracting solvent. However, when temperature became higher the extraction yield of HPs decreased drastically probably due to that high temperature could destroy the structure of polysaccharides and lead to degradation. It was also due to that the vapor pressure of the heated liquid was elevated, allowing cavitation to be achieved at lower ultrasound intensity, thus the strength of shear forces was reduced in the vicinity of the bubble (Mason & Lorimer, 2002). Therefore, extraction temperature range of 50–70 °C was selected as optimal in the BBD experiment.

### 3.1.3. *Effect of ultrasonic power on extraction yield of HPs*

Ultrasonic power is believed to be the driving force for the complete dispersion of liquid into solid (Chen, Luo, Gao, & Zhu, 2011). In this experiment, the effects of different ultrasonic power on the yield of HPs were investigated (40, 50, 60, 70 or 80 W, respectively), with the other extraction variables as follows: ratio of water to raw material 40 mL/g, extraction temperature 60 °C, and extraction time 30 min. As can be seen in Fig. 1C, the extraction yield of HPs continued to increase with the increasing of ultrasonic power and reached the maximum value (32.72 ± 1.5%) with the ultrasonic power 60 W. But when the ultrasonic power was greater than 60 W, the HP yield decreased slightly. It can be explained that the higher ultrasonic power produced more cavitation bubbles leading to stronger pressure capable of tearing the fungus cell wall and faster mass transfer, which was considered as the "saturation effect" (Contamine, Wilhelm, Berlan, & Delmas, 1995). At the same time, too high power also caused hydrolyzation and aggregation of polysaccharides, leading to viscosity of the polysaccharides decrease and the extraction yield decrease (Zhang et al., 2013). Therefore, ultrasonic power range of 50–70 W was considered to be optimal in the present experiment.

### 3.1.4. *Effect of extraction time on extraction yield of HPs*

Extraction time is another important factor that would influence the extraction efficiency for UAE. To investigate the effect of extraction time on extraction yield of HPs, the extraction process was carried out using extraction time from 10 to 50 min, while other parameters were as follows: ratio of water to raw material 40 mL/g, extraction temperature 60 °C, and ultrasonic power 60 W. The effect of extraction time on the yield is presented in

**Table 3**
ANOVA for response surface quadratic model.

| Variables | Sum of squares | *DF* | Mean square | *F* value | *p*-value |
|---|---|---|---|---|---|
| | | | | | Prob. > *F* |
| Model | 342.06 | 14 | 24.43 | 46.91 | <0.0001[a] |
| $X_1$ | 0.65 | 1 | 0.65 | 1.25 | 0.2832 |
| $X_2$ | 28.68 | 1 | 28.68 | 55.05 | <0.0001[a] |
| $X_3$ | 35.26 | 1 | 35.26 | 67.70 | <0.0001[a] |
| $X_4$ | 24.91 | 1 | 24.91 | 47.83 | <0.0001[a] |
| $X_1 \times X_2$ | 31.87 | 1 | 31.87 | 61.18 | <0.0001[a] |
| $X_1 \times X_3$ | 1.33 | 1 | 1.33 | 2.56 | 0.1318 |
| $X_1 \times X_4$ | 4.35 | 1 | 4.35 | 8.35 | 0.0119[a] |
| $X_2 \times X_3$ | 65.04 | 1 | 65.04 | 124.88 | <0.0001[a] |
| $X_2 \times X_4$ | 1.27 | 1 | 1.27 | 2.43 | 0.1414 |
| $X_3 \times X_4$ | 41.41 | 1 | 41.41 | 79.50 | <0.0001[a] |
| $X_1^2$ | 50.30 | 1 | 50.30 | 96.58 | <0.0001[a] |
| $X_2^2$ | 51.57 | 1 | 51.57 | 99.02 | <0.0001[a] |
| $X_3^2$ | 33.86 | 1 | 33.86 | 65.01 | <0.0001[a] |
| $X_4^2$ | 30.40 | 1 | 30.40 | 58.36 | <0.0001[a] |
| Residual | 7.29 | 14 | 0.52 | | |
| Lack of fit | 3.52 | 10 | 0.35 | 0.37 | 0.9063[b] |
| Pure error | 3.77 | 4 | 0.94 | | |
| Cor total | 349.35 | 28 | | | |
| $R^2$ | 0.9791 | | | | |
| $R^2_{adj}$ | 0.9583 | | | | |
| $R^2_{pred}$ | 0.9251 | | | | |
| Adeq precision | 23.877 | | | | |
| C.V. % | 2.54 | | | | |

[a] Significant ($p < 0.05$).
[b] Not significant ($p > 0.05$).

Fig. 1D. When extraction time varied from 10 to 30 min, the variance of extraction yield was relatively rapid and reached the maximum extraction yield (32.72 ± 1.6%) at 8 min, and then decreased as the extraction proceeded. These results can be explained that ultrasonic could enhance the mass transfer and makes the release and diffusion of the polysaccharides easier into water. However, extended extraction time (>30 min) may result in the degradation of the polysaccharides, which is induced by their thermal instability (Ying, Han, & Li, 2011). Therefore, after the maximum extraction yield was achieved, longer time of ultrasonic extraction was not necessary. The result indicated that time range of 20–40 min was considered to be optimal in the present experiment.

### 3.2. Optimization of HP extraction by BBD

#### 3.2.1. Predicted model and statistical analysis

The range of four independent variables was based on the results of the preliminary single-factor experiments, as shown in Table 1. The design matrix and corresponding results of BBD experiments to evaluate the four independent variables including $X_1$ (ratio of water to raw material), $X_2$ (extraction temperature), $X_3$ (ultrasonic power) and $X_4$ (extraction time) are presented in Table 2. By applying multiple regression analysis on the experimental data, the response variable and the test variables are related by the following quadratic equation:

$$Y = 32.57 - 0.23X_1 + 1.55X_2 - 1.71X_3 + 1.44X_4 + 2.82X_1X_2 + 0.58X_1X_3 + 1.04X_1X_4 - 4.03X_2X_3 - 0.56X_2X_4 - 3.22X_3X_4 - 2.78X_1^2 - 2.82X_2^2 - 2.28X_3^2 - 2.16X_4 \quad (7)$$

where $Y$ is the predicted yield of HPs, and $X_1$, $X_2$, $X_3$ and $X_4$ are the coded parameters for ratio of water to raw material, extraction temperature, ultrasonic power, and extraction time, respectively. In the BBD analysis, the $p$-value is used as a tool to check the significance of each coefficient, and the smaller the $p$-value is, the more significant the corresponding coefficient is (Zhong & Wang, 2010). The analysis of variance (ANOVA) was performed to evaluate the predictive model and the variables. As shown in Table 3, the "Model *F*-value" is 46.91 and $p$-value is less than 0.0001, which indicated that the response surface quadratic model was significant and can be used to optimize the extraction variables. Additionally, the linear coefficients ($X_2$, $X_3$ and $X_4$), quadratic coefficients ($X_1^2$, $X_2^2$, $X_3^2$ and $X_4^2$) and cross product coefficients ($X_1X_2$, $X_1X_4$, $X_2X_3$ and $X_3X_4$) had a significant effect, with small $p$-values ($p < 0.05$). The other coefficients ($X_1$, $X_1X_3$, $X_2X_4$) were found insignificant ($p > 0.05$). At the same time, the "Lack of Fit *F*-Value" of 0.37 ($p > 0.05$) implied the Lack of Fit was not significant relative to the pure error. The determination coefficient ($R^2 = 0.9791$) indicated that only 2.09% of the total variations were not explained by the model. The value of adjusted determination coefficient ($R^2_{adj}$) was 0.9583, which also confirmed that the model was highly significant. Moreover, the value of the coefficient of variation (C.V. %) was 2.54, which clearly indicated a better precision and reliability of the experimental values.

#### 3.2.2. Analysis of response surface and verification of predictive model

As graphical representations of the regression equation, three-dimensional response surface and two-dimensional contour plots are very useful to visualize the relationship between independent and dependent variables and the interactions between two variables. Different shapes of the contour plots indicated different interactions between the variables. Circular contour plot means the interactions between the corresponding variables are negligible, while elliptical contour suggests the interactions between the corresponding variables are significant (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). Among these four variables (ratio of water to raw material, extraction temperature, ultrasonic power, and extraction time) as shown in Figs. 2 and 3, two variables were kept constant at zero level, when the other two variables within the experimental range were depicted in the plots.

The extraction yield ($Y$) of HPs affected by ratio of water to raw material ($X_1$) and extraction temperature ($X_2$) is shown in Figs. 2A and 3A with ultrasonic power ($X_3$) and extraction time ($X_4$) fixed at a zero level. The extraction yield ($Y$) increases rapidly when ratio of water to raw material ($X_1$) and extraction temperature ($X_2$) increase in the range of 30.0–41.3 mL/g and 50.0–63.4 °C, respectively; but beyond 41.3 mL/g and 63.4 °C, extraction yield ($Y$) decreases slightly. The elliptical contour plot shown in Fig. 3A indicated the mutual interactions between ratio of water to raw material ($X_1$) and extraction temperature ($X_2$) were significant, which was in good agreement with the results in Table 3. The same trends were depicted in Figs. 2C and 3C, of which Fig. 3C showed a similar elliptical contour plot.

As shown in Figs. 2B and 3B, when extraction temperature ($X_2$) and extraction time ($X_4$) were fixed at 0 levels, ratio of water to raw material ($X_1$) and ultrasonic power ($X_3$) demonstrated quadratic effects on the extraction yield ($Y$). However, results in Table 3 showed that interactions between the variables are insignificant ($p > 0.05$). Also, the presented circular contour plot means interactions between ratio of water to raw material ($X_1$) and ultrasonic power ($X_3$) were not significant. The same trends were depicted in Figs. 2E and 3E, of which Fig. 3E showed a similar circular contour plot.

Meanwhile, a great increase in extraction yield ($Y$) resulted when the extraction temperature ($X_2$) was increased in the range from 50 to 70 °C (Figs. 2D and 3D). At lower ultrasonic power ($X_3$) (about 50 W) and higher extraction temperature ($X_2$) (about 70 °C), high yield of HPs was obtained. It indicated that the greater yield could be obtained when the moderate extraction temperature and a lower level of the ultrasonic power were selected. The same trends were depicted in Figs. 2F and 3F.

By analyzing the plots, the optimal conditions for HP extraction obtained were as follows: ratio of water to raw material,

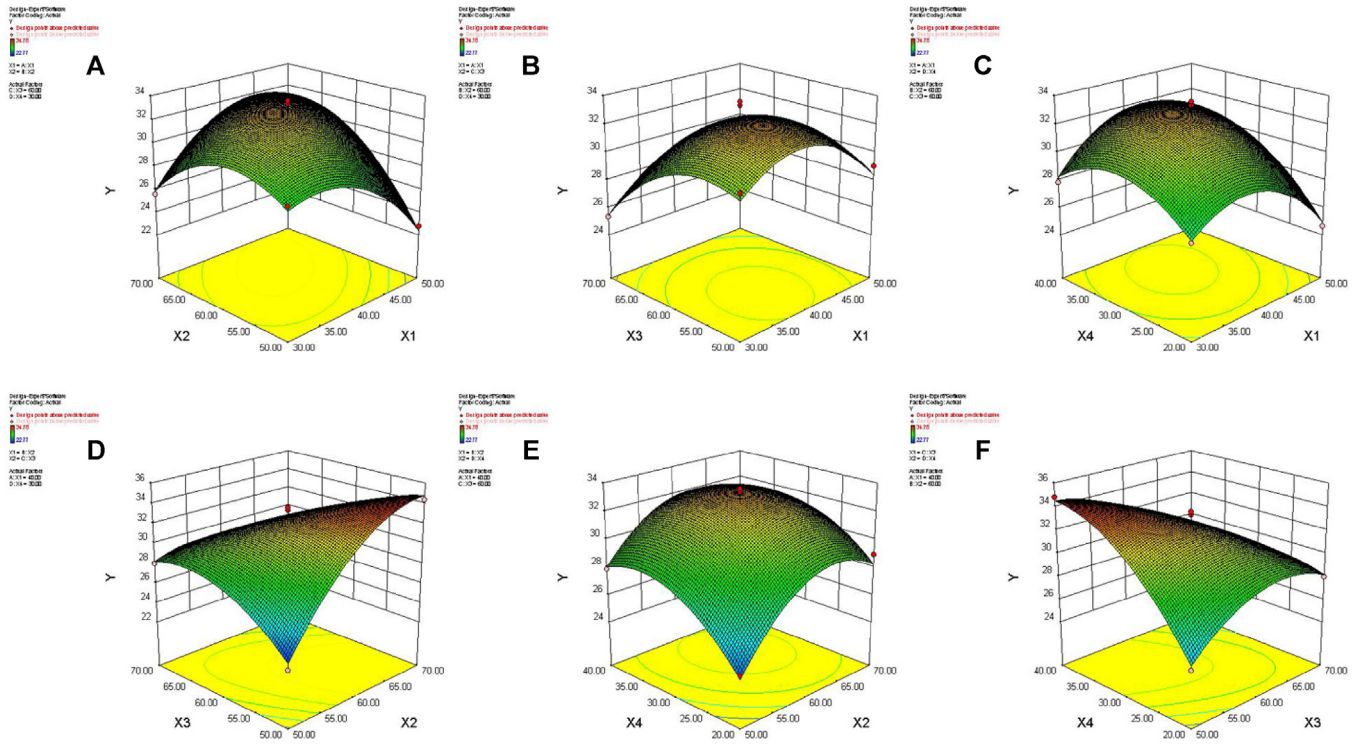


**Fig. 2.** Response surface plots (A–F) showing the effect of variables ($X_1$: ratio of water to raw material, mL/g; $X_2$: extraction temperature, °C; $X_3$: ultrasonic power, W; and $X_4$: extraction time, min) on the extraction yield ($Y$) of HPs.

46.0 mL/g; extraction temperature, 68.9 °C; ultrasonic power, 51.3 W; and extraction time, 36.8 min. To validate the adequacy of the model equations, a verification experiment was carried out under optimum conditions as mentioned above. Under these optimal conditions, highest HP yield 36.8 ± 0.12% ($n$ = 3) that agreed closely with the predicted yield 36.6% were obtained. Therefore, the results indicated suitability of the model employed and the success of BBD in optimizing the extraction conditions for HPs.

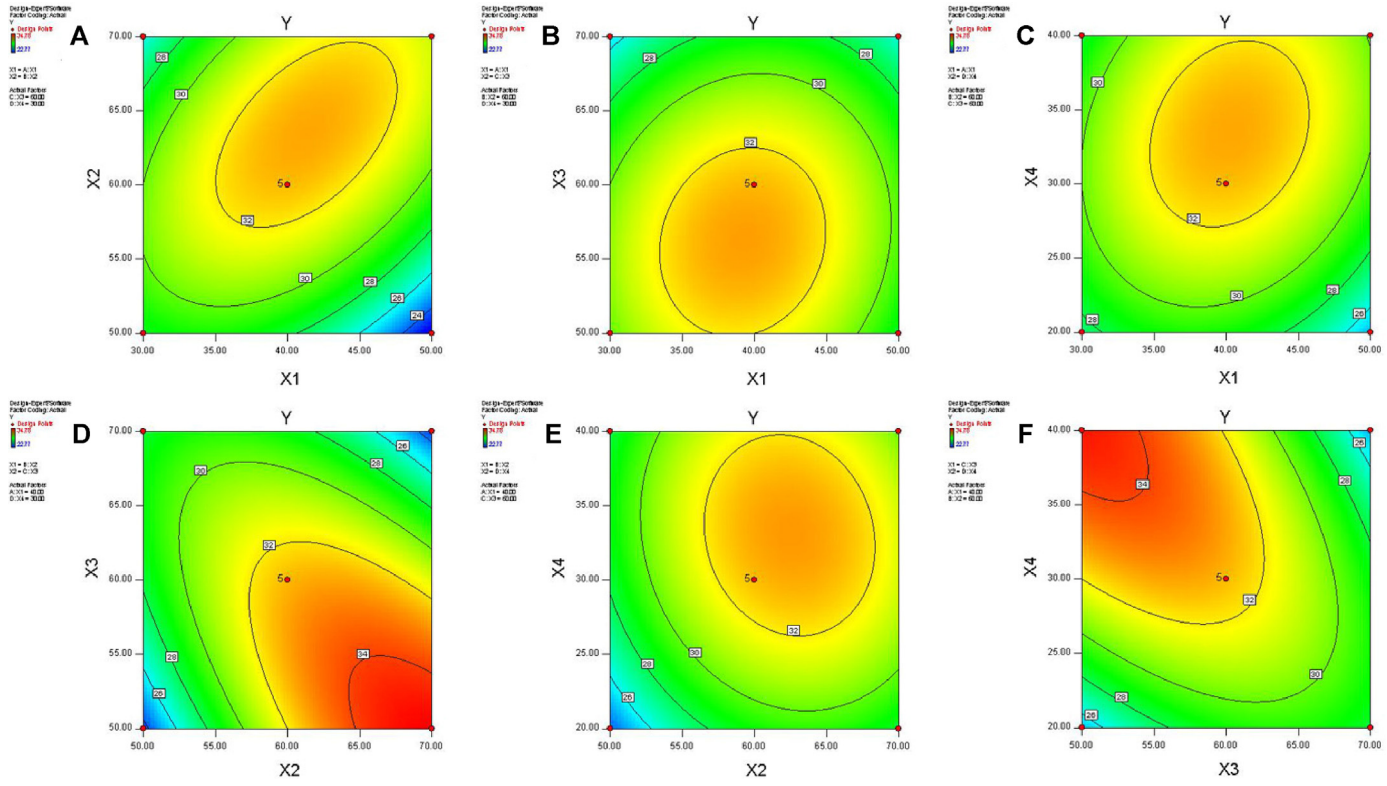


**Fig. 3.** Contour plots (A–F) showing the effect of variables ($X_1$: ratio of water to raw material, mL/g; $X_2$: extraction temperature, °C; $X_3$: ultrasonic power, W; and $X_4$: extraction time, min) on the extraction yield ($Y$) of HPs.

**Table 4**
Total carbohydrate and protein contents and monosaccharide compositions for HPs.

| Sample | Carbohydrate (%) | Protein (%) | Monosaccharide compositions (%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Fucose | Arabinose | Xylose | Mannose | Galactose | Glucose |
| HPs | 85.3 ± 1.3[a] | 10.4 ± 0.8[a] | 5.82 | 13.11 | 16.88 | 15.85 | 11.40 | 36.94 |

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

### 3.3. Chemical analysis

The chemical analysis results of crude HPs, prepared under the optimal extraction condition, are presented in Table 4. Carbohydrate and protein contents of the HPs were determined as 85.3 ± 1.3 and 10.4 ± 0.8% (w/w), respectively, which showed a high carbohydrate content. Besides, the monosaccharide composition of HPs was analyzed by GC–MS. As shown in Table 4, HPs were mainly composed of five monosaccharides, including fucose (5.82%), arabinose (13.11%), xylose (16.88%), mannose (15.85%), galactose (11.40%) and glucose (36.94%), which showed a high glucose content and a low fucose content.

### 3.4. Antioxidant activity

Reactive oxygen species (ROS) or free radicals produced by some carcinogens, such as aromatic hydrocarbons, preservatives and betel nut extract, play an important role in initiation, promotion and growth of cancer in human body (Debbasch et al., 2001; Lai, Lin, Yang, Liu, & Hung, 2007; Myhre & Fonnum, 2001). Furthermore, ROS was thought to be able to damage tumor suppressor genes, leading to acceleration of cancer cell growth, involving a series of free radicals such as superoxide anion radicals ($O_2^{\bullet -}$) and hydroxyl radical species (•OH), generated as by-products in the process of cell metabolism (Kang, 2002; Ramana, Boldogh, Izumi, & Mitra, 1998). Therefore, antioxidants mainly inhibit cancer by effectively removing excess free radicals to reduce the gene mutation, improving the immune function, and protecting tumor suppressor genes.

#### 3.4.1. ABTS radical scavenging activity

The ABTS radical scavenging assay has been widely applied to evaluating antioxidant activity because of its good separation from the visible-light range with a maximum absorption at 734 nm (Wu et al., 2006). The scavenging effects of HPs on ABTS radicals were measured as shown in Fig. 4A. The scavenging ability of HPs on ABTS radical was 58.3 ± 1.1% at 0.2 mg/mL, and increased to 83.6 ± 1.2% at 1.0 mg/mL in a concentration-dependent manner. This result indicated that the HP was a good scavenger for ABTS radicals.

#### 3.4.2. Superoxide radical scavenging activity

Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals, being important for the antioxidative defense (Marklund & Marklund, 1974). The results of superoxide radical scavenging assay of HPs are described in Fig. 4B, with an increasing scavenging rate. Although the scavenging activity of HPs was a little weaker than that of BHT, it can work well in higher concentrations. The scavenging rate (83.1 ± 1.5%) of HPs was achieved with the concentration of 1 mg/mL, and at the same time the rate of BHA was 94.5 ± 2.4%. Therefore, HPs had an appreciable scavenging capacity on superoxide radicals.

#### 3.4.3. Hydroxyl radical scavenging activity

Hydroxyl radicals are one of representative reactive oxygen species generated in the body (Yuan, Zhang, Fan, & Yang, 2008). The mechanism of hydroxyl radical scavenging is related to the transition metal ions. Hydroxyl radicals can easily inflict tissue damage or cell death, thus removing hydroxyl radicals is important for the protection of living systems. Fig. 4C shows that the hydroxyl radical scavenging activity of HPs were concentration dependent (0–1.0 mg/mL), and the maximum scavenging rate was approximately 62.0 ± 2.3% at 1.0 mg/mL. But when the concentrations

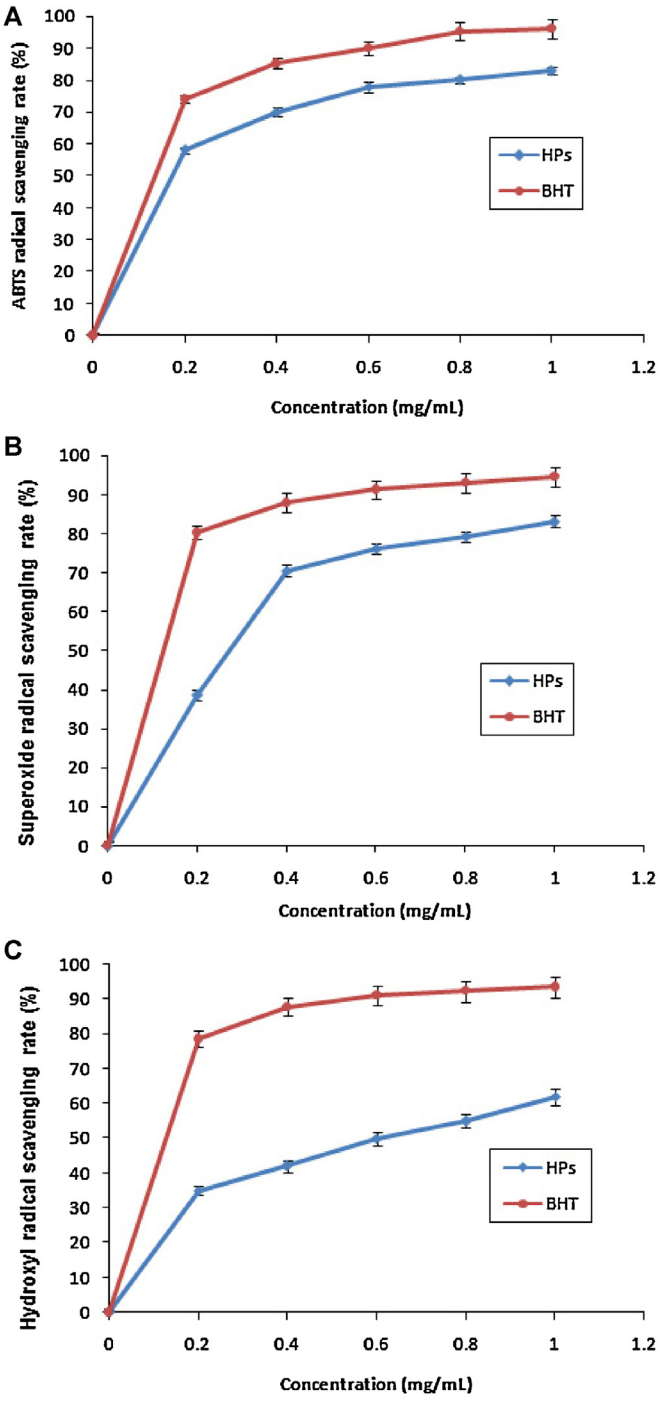


**Fig. 4.** Anti-oxidant effects of HPs. Scavenging effects of HPs on ABTS radicals (A) superoxide radicals (B), and hydroxyl radicals (C). Values are means ± SD ($n$ = 3).

increased from 0.2 to 1.0 mg/mL, BHT did not show any obvious increasing scavenging effects. The result proved that HPs were a good scavenger for hydroxyl radicals.

## 4. Conclusions

UAE technology was performed for HP extraction in order to increase the extraction yield. The optimal extraction conditions were optimized by single-factor design and BBD. The optimum variables given by BBD were as follows: ratio of water to raw material, 46.0 mL/g; extraction temperature, 68.9 °C; ultrasonic power, 51.3 W; and extraction time, 36.8 min. Under these conditions, the experimental yield was obtained as 36.8 ± 0.12% ($n$ = 3), which corresponded with the predicted value well. In addition, chemical analysis indicated that HPs showed a high carbohydrate content, composed of fucose, arabinose, xylose, mannose, galactose and glucose. Besides, HPs exhibited positive radical scavenging activities against ABTS, superoxide anion and hydroxyl radicals *in vitro*. Thus, HPs could be explored as a natural antioxidant food ingredient. These may further provide theoretical basis for the widely application of HPs in medicine and health care products.

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