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Optimization of polysaccharides extraction from seeds of *Pharbitis nil* and its anti-oxidant activity

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ABSTRACT

In the present study, polysaccharides were extracted from *Pharbitis nil* seeds (PNSs) by ultrasonic extraction method for the first time. Response surface methodology with Box–Behnken design was used to optimize the extraction process of PNS polysaccharides (PNSPs). Highest PNSPs yield 6.01 ± 0.15% that agreed closely with the predicted yield 5.99% were obtained under the optimal conditions as follows: ratio of water to raw material 6.5 mL/g, extraction temperature 49.0 °C, ultrasonic power 61.6 W, and extraction time 32.6 min. Preliminary characterization indicated that the sugar, uronic acid and protein contents of the product were 83.6 ± 1.61, 21.8 ± 1.25 and 16.4 ± 0.88% (w/w), respectively. FT-IR analysis revealed the general characteristic absorption peaks of polysaccharides. Besides, PNSPs showed a remarkable antioxidant activity *in vitro* in a dose-dependent manner. These may provide theoretical basis for further system research and rational development and utilization of PNS resources.

1. Introduction

Pharbitis nil Choisy (PN, morning glory), an annual climbing herb that many people use as an ornamental plant, belongs to family *Convolvulaceae* and is widely distributed throughout Southeast Asia (Bensky & Gamble, 1993). The seed of PN has been thought to be one of the most important folk medicine as a purgative drug in China, Korea, and Japan for thousands of years (Bensky & Gamble, 1993). Up to date, it has been demonstrated that the seed is rich in pharbitin (resin glycosides), gibberellins, chlorogenic acid derivatives, anthocyanins, diterpenoids and triterpene saponins that may contribute to the biological functions, such as anti-tumor, anti-fungal, cytotoxic, analgesic and gastroprokinetic activities (Jung et al., 2008; Kim, Jin, Choi, Son, & Lee, 2008; Kim, Chi, & Hong, 2009; Kim, Choi, & Lee, 2009; Ko et al., 2004; Koo et al., 1998; Lee et al., 2008; Saito, Ting, Yokoi, Shigihara, & Honda, 1993; Saito et al., 1994, 1996). However, due to pharbitin with strong purging effect and toxicity, use of PN reduces gradually in clinical application, which causes the waste of PN resources (Huang, 1998). Nowadays, many researches indicated that polysaccharides are slightly toxic and possess a wide range of biological properties, such as immunomodulatory, anti-cancer, hematopoiesis-promoting, and anti-oxidant activities (Li, Wei, You, & Lydy, 2010; Li, Li, et al., 2010; Sarker & Nahar, 2004; Shang et al., 2003; Yang, Jia, Meng, Wu, & Mei, 2006; Yang et al., 2007). To the best of our knowledge, little attention was devoted to PNSPs, especially detailed studies on extraction procedure and its preliminary characteristics.

Recently, ultrasonic extraction (UE) is the new technology that attracts much more attention in the department of extraction in recent years and it is one of the most inexpensive, simple and efficient techniques (Chen et al., 2010; Huang, Xue, Niu, Jia, & Wang, 2009; Yan et al., 2011; Zhang, Yang, Zhao, & Wang, 2009; Zhong & Wang, 2010). The mechanical effect of ultrasound is able to accelerate the extraction of active plant compounds, contained within the body of plants, due to disruption of the cell walls and enhanced mass transfer of cell contents (Hromadková, Ebringerová, & Valachovič, 1999). It offers high reproducibility at shorter times, simplified manipulation, and lowered energy input, as well as solvent consumption (Kim, Chi, et al., 2009; Kim, Choi, et al., 2009; Li, Wei, et al., 2010; Li, Li, et al., 2010; Sun, Liu, Chen, Ye, & Yu, 2011; Vilkhu, Mawson, Simons, & Bates, 2008). Nowadays, response surface methodology (RSM), as an effective tool for optimizing complex processes, is less laborious and time-consuming than

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other approaches (Box & Wilson, 1951). It can reduce the number of experimental trials needed to evaluate multiple variables and their interactions. RSM is easy to arrange and interpret experiment and has already been successfully applied to the optimization of polysaccharide extraction conditions (Chen, Zhang, Jiang, Mu, & Miao, 2012; Chen, Wang, Zhang, & Huang, 2012; Han, Jiang, & Zhang, 2011; Li, Ding, & Ding, 2007; Li et al., 2013; Rodríguez-González, Femenia, Minjares-Fuentes, & González-Laredo, 2012; XuJie & Wei, 2008; Yan et al., 2011; Yongjiang, Zhong, Jianwei, Minger, & Xueqian, 2009).

Therefore, in order to enhance the yield of PNSPs by UE, the extraction variables were optimized by employing RSM. Furthermore, its preliminary characteristics, such as sugar and protein contents, monosaccharide composition and Fourier-transform infrared (FT-IR) spectroscopy, were determined. Besides, the ABTS and DPPH radical scavenging activities were tested *in vitro*.

2. Materials and methods

2.1. Materials

The dry seeds of PN were purchased from a local market (Harbin, China) and identified by Prof. Zhenyue Wang of Heilongjiang University of Chinese Medicine. D-Glucose (Glc), D-glucuronic acid (GlcUA) and sulfuric acid (H_2SO_4) were purchased from Sigma (St. Louis, USA). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Kishida (Osaka, Japan). The common chemicals were of analytical reagent grade. All other chemicals were reagent-grade.

2.2. UE of PNSPs

The dried seeds of PN were ground (40 mesh) to obtain a fine powder. For the UE experiments, 100 g of each powder sample was mixed with an appropriate amount of distilled water in a 1000 mL beaker. UE was performed in an ultrasonic device (AS3120A, Tianjin Automatic Science Instrument Co., Ltd, China) working with designed ratio of water to raw material (2, 4, 6, 8 and 10, respectively), extraction temperature (30, 40, 50, 60 and 70 °C, respectively), ultrasonic power (40, 50, 60, 70 and 80 W, respectively), and extraction time (10, 20, 30, 40 and 50 min, respectively). Each extraction processes were performed for 3 times. When the extraction was accomplished, the resulting extract solution was mixed with four times of its volume of 95% ethanol, stirred vigorously and left overnight at 4 °C. And the precipitates were collected by centrifugation (5000 rpm, 10 min), washed with acetone and pure ethanol, and were finally dried at 40 °C to a constant weight. The crude PNSPs obtained was weighted with an analytical balance (AL204, METTLER TOLEDO, Switzerland). PNSP yield was measured using the following equation:

$$\text{PNSP yield} = \frac{\text{weight of crude polysaccharide extract (g)}}{\text{weight of each powder sample (g)}} \times 100\% \quad (1)$$

Table 1
Independent variables and their levels 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	4	6	8
Extraction temperature (°C)	X_2	40	50	60
Ultrasonic power (W)	X_3	50	60	70
Extraction time (min)	X_4	20	30	40

2.3. RSM design

Based on the preliminary test results (data not shown), the experimental range of the selected process variables is given in Table 1. Then, a Box–Behnken design (BBD) with four independent variables (X_1, ratio of water to raw material; X_2, extraction temperature; X_3, ultrasonic power; and X_4, extraction time) at three levels was performed, as shown in Table 2. For statistical calculation, the variables were coded by the following equation:

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

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

Each variable was prescribed into three levels, coded +1, 0 and −1 for high, intermediate and low value, respectively. The experimental data were fitted to the following second-order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=0}^{3}\beta_{ii}X_i^2 + \sum_{i=0}^{2}\sum_{j=i+1}^{3}\beta_{ij}X_iX_j, \quad (3)$$

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

Analysis of the experimental data and calculation of predicted responses were carried out using Design Expert software (version 8.0, Stat-Ease, Inc., Minneapolis, USA). The analysis of variance (ANOVA) tables were generated, and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significance of each term in the equation is to estimate the goodness of fit in each case. The values of R^2, adjusted-R^2 of models were evaluated to check the model adequacies.

Table 2
BBD and the response values for yields of PNSPs.

Run	Coded variable levels				Yield of PNSPs (%)	
	X_1	X_2	X_3	X_4	Experimental	Predicted
1	−1	−1	0	0	3.74	3.99
2	1	−1	0	0	4.06	4.18
3	−1	1	0	0	3.57	3.66
4	1	1	0	0	4.35	4.31
5	0	0	−1	−1	2.86	2.98
6	0	0	1	−1	3.88	3.75
7	0	0	−1	1	4.54	4.88
8	0	0	1	1	4.86	4.95
9	−1	0	0	−1	3.04	2.99
10	1	0	0	−1	3.36	3.40
11	−1	0	0	1	4.62	4.54
12	1	0	0	1	4.95	4.96
13	0	−1	−1	0	3.88	3.59
14	0	1	−1	0	4.36	4.40
15	0	−1	1	0	5.01	4.92
16	0	1	1	0	3.65	3.90
17	−1	0	−1	0	4.09	3.96
18	1	0	−1	0	4.16	4.08
19	−1	0	1	0	4.16	4.08
20	1	0	1	0	4.83	4.79
21	0	−1	0	−1	3.17	3.27
22	0	1	0	−1	3.15	3.07
23	0	−1	0	1	4.82	4.73
24	0	1	0	1	4.98	4.72
25	0	0	0	0	5.86	5.85
26	0	0	0	0	5.69	5.85
27	0	0	0	0	5.83	5.85
28	0	0	0	0	5.98	5.85
29	0	0	0	0	5.89	5.85

Additional confirmation experiments were subsequently conducted to verify the validity of the statistical experimental design.

2.4. Chemical analysis of PNSP

The sugar content was measured by the phenol–sulfuric acid method using D-glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and the protein content was determined by Bradford method using bovine serum albumin as the standard (Bradford, 1976). The content of uronic acid was determined by carbazole–sulfuric acid method using D-glucuronic acid as the standard (Bitter & Muir, 1962). Three replicate data were obtained.

2.5. FT-IR spectroscopy analysis of PNSP

The crude PNSPs were identified using a FT-IR spectrophotometer (FTIR-8400S, Shimadzu Co., Japan) in the frequency range of 4500–400 cm^{-1} by KBr pressed-disk method (Yamamoto & Masui, 1995). 2 mg of PNSP sample was mixed with 300 mg of KBr powder, grinded and pressed into a 1 mm pellets for FT-IR measurement. Three replicate spectra were obtained.

2.6. Antioxidant activity assays of PNSP

2.6.1. ABTS radicals scavenging assay

The ABTS assay was performed following Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993), with some modifications. ABTS was dissolved in 0.01 M PBS (pH 7.4) to a 7 mM concentration. The ABTS radical cation ($ABTS^+$) was generated by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 16 h. 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. Each sample (0.2 mL) with various concentrations (0.08, 0.16, 0.31, 0.63, 1.25, 2.5 and 5.0 mg/mL) was mixed with 2.0 mL of $ABTS^+$ solution. After reacting for 20 min at room temperature, the absorbance at 734 nm was immediately measured and recorded. Vitamin C was used as standard. Each sample was measured in triplicate and averaged. ABTS radicals scavenging effect was calculated according to the following equation:

$$\text{ABTS scavenging effect}\,(\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100, \quad (4)$$

where A_0, A_{734} of ABTS without sample; A_1, A_{734} of sample and ABTS; and A_2, A_{734} of sample without ABTS.

2.6.2. DPPH radicals scavenging assay

The DPPH assay was carried out according to the method of Ge, Duan, Fang, Zhang, and Wang (2009) with some modifications. Briefly, 2.0 mL DPPH solution (0.2 mM in 95% ethanol) was mixed with 2.0 mL of related tested samples with different concentrations (0.08, 0.16, 0.31, 0.63, 1.25, 2.5 and 5.0 mg/mL) in the tubes. After 30 min at room temperature, the absorbance was measured at 517 nm. Vitamin C was used as standard. The DPPH radical scavenging effect was calculated according to the following equation:

$$\text{DPPH scavenging effect}\,(\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100, \quad (5)$$

where A_0, A_{517} of DPPH without sample, A_1, A_{517} of sample and DPPH, and A_2, A_{517} of sample without DPPH.

2.7. Statistical analysis

The Design-Expert V8.0 was used to determine the analysis of variance for RSM experiments. Comparison of means was performed by one-way analysis of variance (ANOVA) followed by Duncan's test by SPSS statistical software (Version 17.0).

3. Results and discussion

3.1. Optimization of extraction of PNSPs by RSM

3.1.1. Statistical analysis and the model fitting

The experimental conditions with four variables including X_1 (ratio of water to raw material), X_2 (extraction temperature), X_3 (ultrasonic power) and X_4 (extraction time) were shown in Table 1. And Table 2 shows the complete design matrix together with the response values obtained. The multiple regression analysis was applied to the experiment data, and the response variable and the test variables are related by the following second-order polynomial equation according to coded values:

$$\begin{aligned} Y = {} & 5.85 + 0.21X_1 - 0.052X_2 + 0.21X_3 + 0.78X_4 + 0.12X_1X_2 \\ & + 0.15X_1X_3 + 2.500E - 003X_1X_4 - 0.46X_2X_3 + 0.045X_2X_4 \\ & - 0.18X_3X_4 - 0.90X_1^2 - 0.92X_2^2 - 0.73X_3^2 - 0.98X_4^2, \end{aligned} \quad (6)$$

where Y is the yield of PNSPs (%) calculated using the regression model; X_1, X_2, X_3 and X_4 are the coded variables for ratio of water to raw material, extraction temperature, ultrasonic power and extraction time, respectively.

Analysis of variance (ANOVA) was used to analyze the experimental data. The higher it is the better degree of correlation between the actual and predicted values (Li et al., 2013). As shown in Table 3, the significant response surface model with high R^2 and adj-R^2 values were 0.9748 and 0.9495, respectively, which showed a good agreement between the experimental and the predicted values of the PNSPs yield with goodness-of-fit of the regression equation. The value of the coefficient of variation (CV) was 4.65, which clearly stated that, the deviations between experimental and predicted values are low, high degree of precision and also showed a good reliability of the conducted experiments.

The P-value is used as a tool to check the significance of each coefficient, and it also indicates the interaction strength between each independent variable (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). A small P-value less than 0.05 indicate a significant coefficient. From the analysis, the low model P-value was found to be <0.0001, which indicated that the response surface quadratic model was highly statistically significant. ANOVA (Table 3) indicated that the linear coefficients (X_1, 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_2X_3) had a significant effect, with small P-values ($P < 0.01$). The other coefficients (X_2, X_1X_2, X_1X_3, X_1X_4 and X_2X_4) were found insignificant ($P > 0.05$). The parameter estimates and the corresponding P-values suggest that, among the independent variables, ratio of water to raw material X_1, ultrasonic power X_3 and extraction time X_4 were significantly correlated with the yield of PNSPs.

3.1.2. Analysis of response surface and verification of predictive model

Through 3D response surface plots and their respective 2D contour plots using the Design-Expert software, the suitability of the model equation for predicting the optimum response values can be tested under the selected optimal conditions. In addition, the shapes of the contour plots indicate whether the mutual interactions between the variables are significant or not (Li et al., 2013). As presented in Figs. 1 and 2, two variables within the experimental range are depicted in 3D surface plots when the other two variables are kept constant at zero level and different shapes of the contour plots indicated different interactions between the variables.

Table 3
ANOVA for response surface quadratic model of PNSP extraction.

	Source	Sum of squares	*DF*	Mean square	*F* value	*p*-Value Prob. > *F*
	Model	22.56	14	1.61	38.63	<0.0001[a]
Linear effects	X_1	0.52	1	0.52	12.38	0.0034[a]
	X_2	0.032	1	0.032	0.77	0.3957
	X_3	0.52	1	0.52	12.48	0.0033[a]
	X_4	7.22	1	7.22	173.14	<0.0001[a]
Interaction effects	$X_1 \times X_2$	0.053	1	0.053	1.27	0.2791
	$X_1 \times X_3$	0.090	1	0.090	2.16	0.1640
	$X_1 \times X_4$	2.500×10^{-5}	1	2.500×10^{-5}	5.993×10^{-4}	0.9808
	$X_2 \times X_3$	0.85	1	0.85	20.29	0.0005[a]
	$X_2 \times X_4$	8.100×10^{-3}	1	8.100×10^{-3}	0.19	0.6662
	$X_3 \times X_4$	0.12	1	0.12	2.94	0.1087
Quadratic effects	X_1^2	5.21	1	5.21	124.78	<0.0001[a]
	X_2^2	5.49	1	5.49	131.48	<0.0001[a]
	X_3^2	3.43	1	3.43	82.20	<0.0001[a]
	X_4^2	6.27	1	6.27	150.34	<0.0001[a]
	Residual	0.58	14	0.042		
	Lack of fit	0.54	10	0.054	4.84	0.0711[b]
	Pure error	0.045	4	0.011		
	Cor. total	23.14	28			
	R^2	0.9748				
	Adj. R^2	0.9495				
	Pred. R^2	0.8627				
	Adeq. precision	19.535				
	C.V. %	4.65				

[a] Means significance ($p < 0.01$).
[b] Not significant ($p > 0.05$).

As shown in Figs. 1a and 2a, when ultrasonic power (X_3) and extraction time (X_4) were fixed at 0 levels, ratio of water to raw material (X_1) and extraction time (X_2) demonstrated quadratic effects on the extraction yields. However, results showed that interactions between the variables are insignificant ($P > 0.05$) (seen in Table 3). A circular contour plot means interactions between ratio of water to raw material (X_1) and extraction time (X_2) were not significant (Yan et al., 2011). Additionally, increases in ratio of water to raw material (X_1) and extraction time (X_2) produce a maximum in the extraction yield value. The same trends were depicted in other figures of Figs. 1 and 2, except Figs 1d and 2d that showed an obvious elliptical contour plot. An elliptical contour plot indicated the interactions between extraction time (X_2) and ultrasonic power (X_3) were significant (Yan et al., 2011). It is consistent with the significant *F* value ($P < 0.01$) in Table 3.

Based on response surface plots, contour plots and variance analysis, optimum operational conditions for maximizing

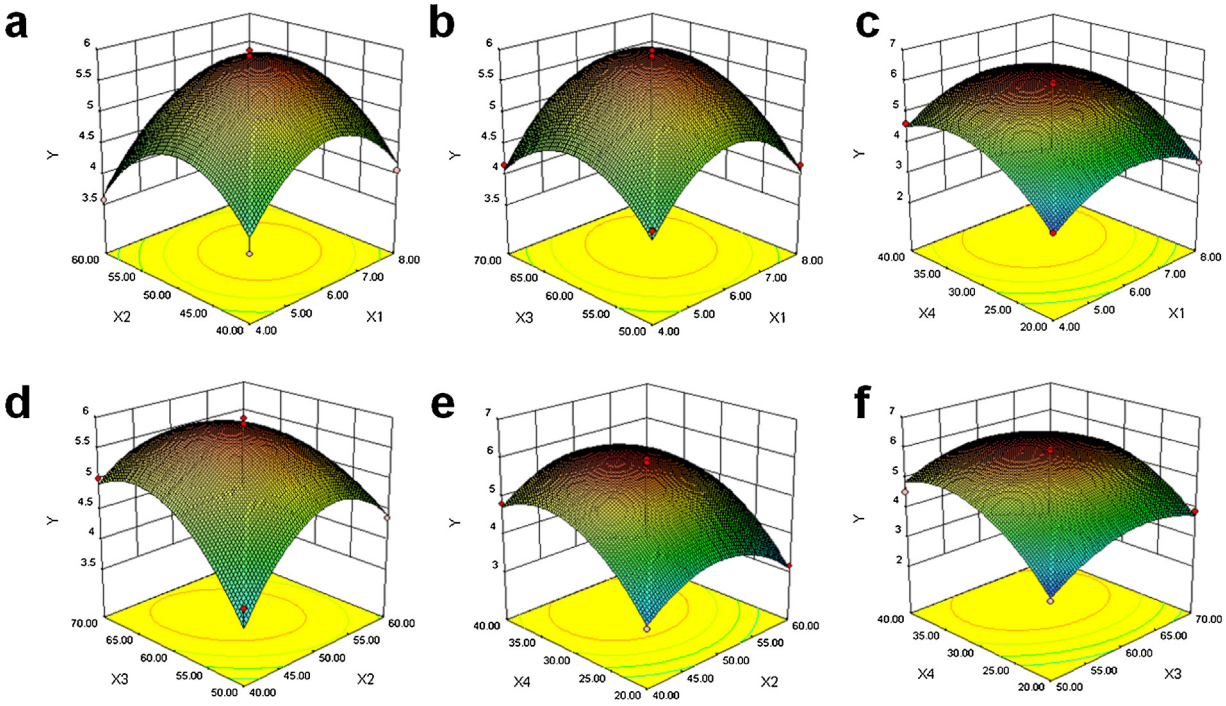

Fig. 1. Response surface plots showing effects of the variables on the yield of PNSPs.

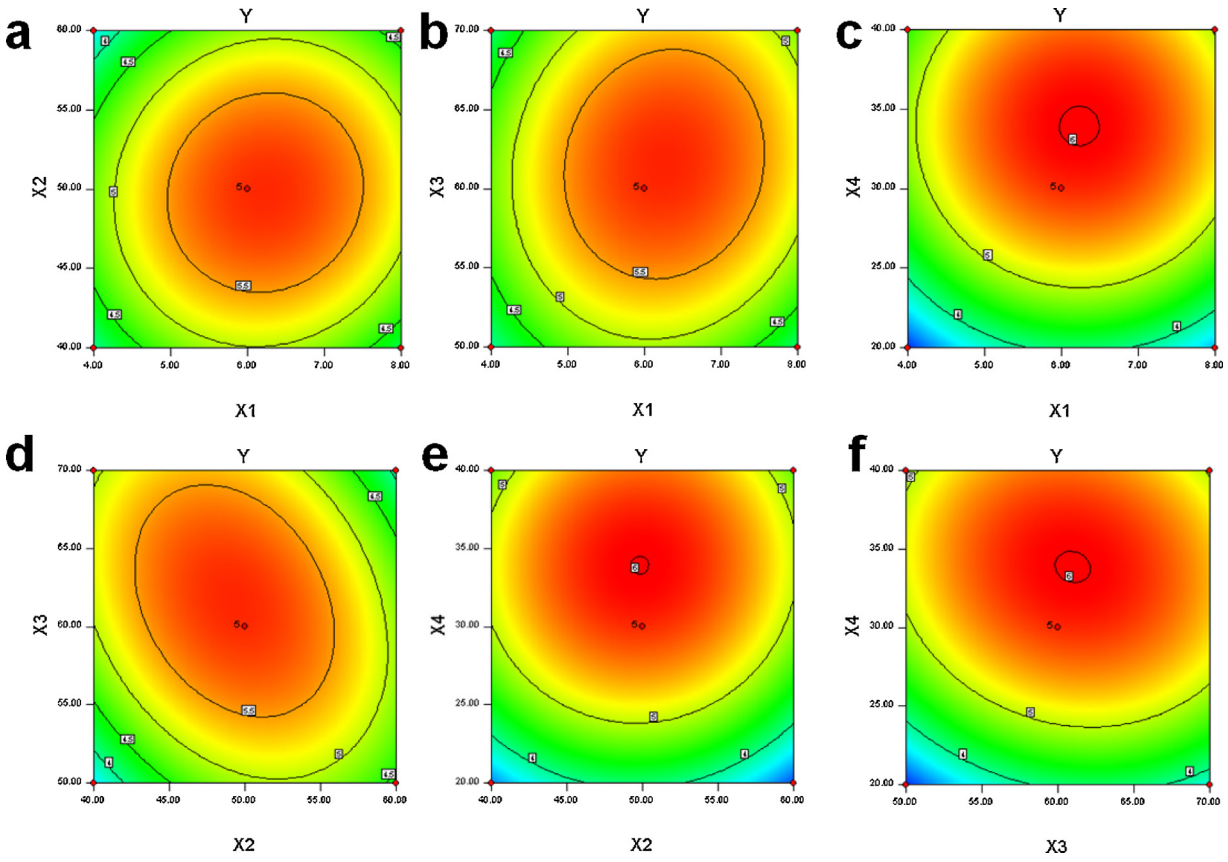

Fig. 2. Contour plots showing effects of the variables on the yield of PNSPs.

extraction yield can be predicted as follows: ratio of water to raw material 6.5 mL/g, extraction temperature 49.0 °C, ultrasonic power 61.6 W, and extraction time 32.6 min. To validate the adequacy of the model equations, a verification experiment is carried out under optimum conditions as mentioned above. Under the optimum operating conditions determined, the yield of PNSPs is $6.01 \pm 0.15\%$ ($n = 3$), which was well-matched with the predicted value 5.99% obtained from the model.

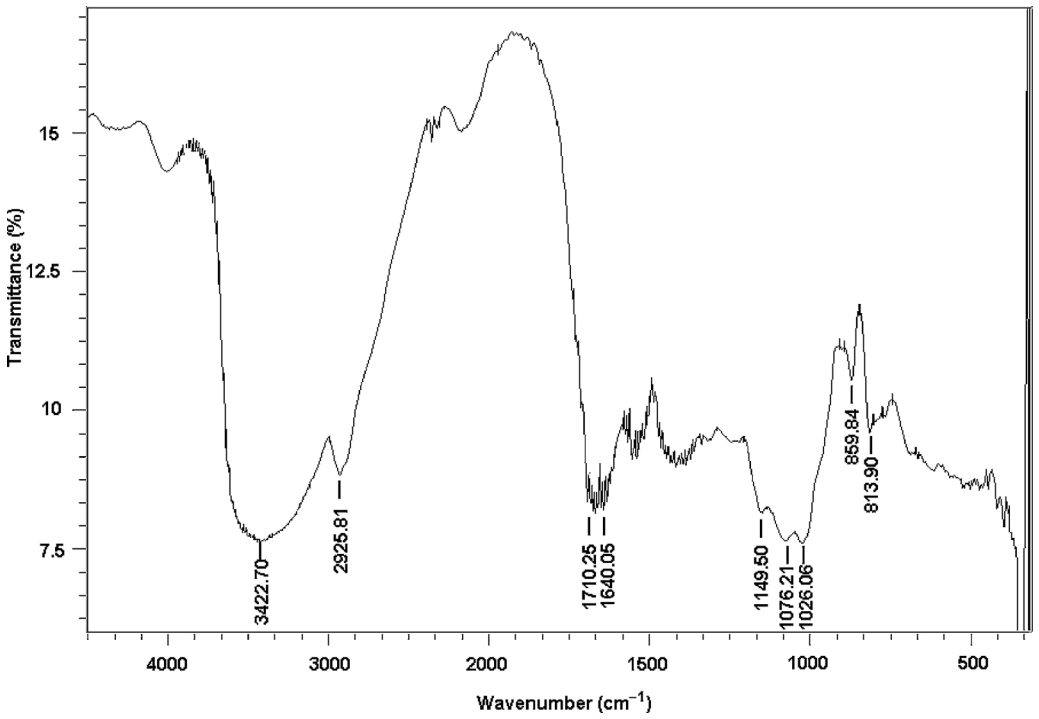

Fig. 3. FT-IR spectroscopy of PNSPs between 400 and 4500 cm^{-1}.

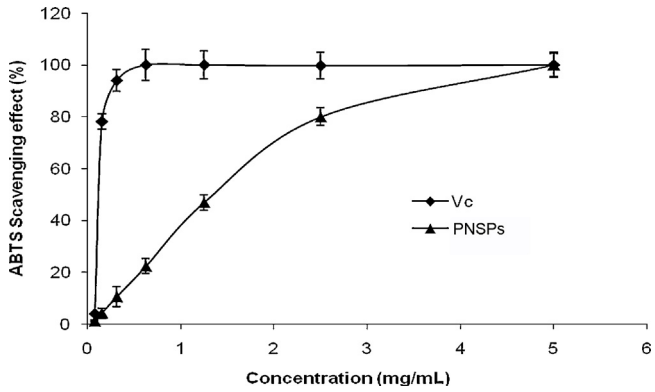

Fig. 4. ABTS radical scavenging activity of PNSPs.

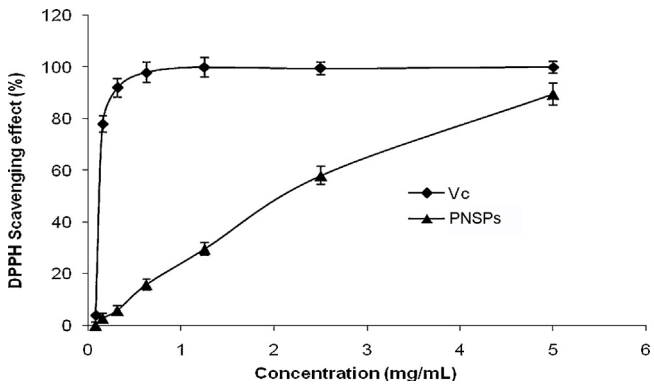

Fig. 5. DPPH radical scavenging activity of PNSPs.

3.2. Chemical analysis

In the present study, crude PNSPs were prepared by UE under the optimal extraction conditions. Then their chemical analysis was carried out, and sugar, uronic acid and protein contents of the resulting PNSPs were 83.6 ± 1.61, 21.8 ± 1.25 and 16.4 ± 0.88% (w/w), respectively.

3.3. FT-IR spectroscopy

In order to further characterize PNSPs and identify the fundamental groups present in its structure, FT-IR analysis was carried out. As shown in Fig. 3, it displayed a broad and intense peak around 3422.70 cm^{-1}, which were due to the hydroxyl groups stretching vibration. The weak peak toward 2925.81 cm^{-1} was attributed to the C—H anti-symmetrical stretching vibration. The peaks 1710.25 and 1640.05 cm^{-1} suggested the presence of the ester carbonyl groups (C=O) and carboxylate (COO—) stretching band, respectively, indicating that there were esterified and free carboxyl groups present in PNSPs. In addition, the absorptions at 1026.06, 1076.21 and 1149.50 cm^{-1} indicated the α-pyranose ring (He et al., 2007; Zhao, Kan, Li, & Chen, 2005). The band between 950 and 700 cm^{-1} in the spectrum exhibited the characteristic absorption at 813.90 cm^{-1} due to the presence of mannose (Chen, Zhang, et al., 2012; Chen, Wang, et al., 2012b).

3.4. Antioxidant activity

3.4.1. ABTS radical scavenging activity of PNSPs

The ABTS radical scavenging assay has been widely applied to evaluating antioxidant activity due to the good separation from the visible-light range with a maximum absorption at 734 nm within a short reaction time (Wu et al., 2006). The total antioxidant ability of PNSPs was measured by means of scavenging a protonated radical ABTS and compared with Vitamin C (Vc) as a control standard, as shown in Fig. 4. The ABTS radical was scavenged by PNSPs in a concentration dependant manner with the maximum percentage of inhibition 100%, equal to that of vitamin C, observed at 5 mg/mL. It claims that PNSP is an efficient antioxidant and a free radical scavenger.

3.4.2. DPPH radical scavenging activity of PNSPs

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activity of antioxidants, with a maximum absorption at 517 nm (Yu et al., 2007). The DPPH radical scavenging effects of PNSPs were measured, and the results are shown in Fig. 5, which depicts DPPH scavenging abilities of different concentrations of PNSPs and is compared with Vc as a control standard. When the concentration of PNSPs was 5 mg/mL, the scavenging effects of PNSPs on DPPH increased at 89.6%, which was closed to Vc. The results indicated that PNSPs had a noticeable effect on scavenging DPPH free radicals, especially at high concentrations.

4. Conclusions

An efficient UE technique was employed to extract crude PNSPs and the corresponding extraction parameters were optimized by BBD. By analyzing the second-order polynomial model, a maximum extraction yield 6.01 ± 0.15% was obtained under the following conditions: ratio of water to raw material 6.5 mL/g, extraction temperature 49.0 °C, ultrasonic power 61.6 W, and extraction time 32.6 min. The experimental yield agreed closely with the predicted yield 5.99% obtained from the model. In addition, chemical analysis indicated that total sugar, uronic acid and protein contents of the resulting PNSPs were 83.6 ± 1.61, 21.8 ± 1.25 and 16.4 ± 0.88% (w/w), respectively, which showed a high carbohydrate content. Moreover, FT-IR results revealed the general characteristic absorption peaks of polysaccharides and exhibited the characteristic absorption of mannose. Besides, the evaluation of anti-oxidant activity suggested that PNSPs exhibited significant protection against ABTS and DPPH radicals and could be explored as a functional food ingredients.

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