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Extraction of crude polysaccharides from *Gomphidius rutilus* and their antioxidant activities in vitro

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ABSTRACT

Response surface methodology was used to optimize the extraction of crude polysaccharides from the fruiting bodies of *Gomphidius rutilus*. A central composite design was adopted to determine the combination of factors (extraction time, extraction temperature, extraction frequency, and ratio of water to raw material) that resulted in the maximum crude polysaccharide production. Results showed that the optimum extraction conditions were as follows: extraction temperature, 95 °C; ratio of water to raw material, 16; extraction time, 2.5 h; and extraction frequency, 4. Under these conditions, the experimental yield of crude polysaccharides was 8.02 ± 0.15%, which well agreed with the predicted yield. Evaluation of the antioxidant activity in vitro suggested that the crude polysaccharides had high scavenging activity for superoxide anion, hydroxyl, and 1,1-diphenyl-2-picrylhydrazyl radicals. The crude polysaccharides also showed strong reducing power. Thus, they can be used as natural antioxidants in functional foods or medicine.

1. Introduction

Mushrooms have long attracted attention as traditional food and medicine in China, Korea, and Japan. The natural products extracted from mushrooms exhibit lower toxicity and fewer side effects than chemical-based drugs; therefore, mushrooms are a potential valuable source of natural drugs (Chihara, 1992). Recently, along with the development of cytobiology, immunology, and molecular biology, mushroom polysaccharides and their composites have drawn the attention of biochemists and pharmacists because of their biological activities (Borchers, Keen, & Gershwin, 2004; Li et al., 2008; Zhang, Cui, Cheung, & Wang, 2007).

For efficient utilization, the extraction of polysaccharides should be thoroughly well studied. Generally, the extraction yields of polysaccharides are mainly affected by the extraction time, temperature, ratio of water to materials, and extraction frequency. Response surface methodology (RSM) is an effective tool for optimizing the process when many factors and interactions affect the production process (Box & Wilson, 1951). The main advantage of RSM is the reduction of the number of experimental trials needed to evaluate multiple variables and their interactions, making it less laborious and time consuming than other approaches to process optimization (Bas & Boyaci, 2007). In RSM, an experimental design such as central composite design (CCD) is generally used to fit a second-order polynomial by the least-square technique. An equation is used to describe the effect of test variables on the response and to determine the interrelationship among the variables (Wu, Cui, Tang, & Gua, 2007). RSM has already been successfully applied to the optimization of polysaccharide extraction conditions (Cui et al., 2007; Guo, Zou, & Sun, 2010; Sun, Liu, & Kennedy, 2010; Zhong & Wang, 2010; Zhu, Heo, & Row, 2010).

Gomphidius rutilus, which belongs to subphylum Basidiomycotina, is a traditional Chinese medicinal and edible fungus often found beneath pine trees. In this study, a CCD (4 factors and 5 levels) was used to optimize the production process of crude polysaccharides from the fruiting bodies of *G. rutilus*. The antioxidant activities of the crude polysaccharides were then evaluated for use in the food and pharmaceutical industries.

2. Materials and methods

2.1. Materials and chemicals

Fruiting bodies of *G. rutilus* were purchased from a local market (Fushun, Liaoning province, China). Butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), methionine (MET), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and riboflavin (RF) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Unless otherwise stated, all chemicals used were analytical grade.

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2.2. Extraction of G. rutilus crude polysaccharides (GRCPs)

The fruiting body of *G. rutilus* (1000 g) was homogenized in a blender and then defatted by ethanol at 80 °C for 6 h in a reflux apparatus. The defatted sample was treated with 80% ethanol (v/v) twice to remove some colored materials, monosaccharides, oligosaccharides, and small-molecule materials. The pretreated sample was centrifuged (2000 × *g*, 15 min) and the deposit was vacuum dried for 16 h at 60 °C to constant weight. Each dried pretreated sample (20 g) was extracted with deionized water at a designated temperature, extraction time, ratio of water to raw material, and extraction frequency. The mixture was centrifuged (2000 × *g*, 15 min) and then the supernatant was separated from the insoluble residue with a four-layer filter cloth. The extract was precipitated by the addition of ethanol to a final concentration of 75% (v/v) and incubated overnight. Polysaccharide precipitate was collected by centrifugation (2000 × *g*, 15 min) and then deproteinated by a combination of proteinase and Sevag method (Staub, 1965). The supernatant was lyophilized to obtain the crude polysaccharides. The polysaccharide content was measured by the phenol–sulfuric acid method using D-glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1958)

2.3. Experimental design and statistical analysis

After determining the preliminary range of extraction variables through a single-factor test, a CCD with four independent variables (X_1, extraction temperature; X_2, extraction time; X_3, ratio of water to raw material; and X_4, extraction frequency) at five levels was performed. For statistical calculation, the variables were coded according to Eq. (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

Table 1
Experimental domain of central composite design (CCD).

Variables	Levels				
	−2	−1	0	1	2
Extraction temperature (X_1) (°C)	80	85	90	95	100
Extraction time (X_2) (h)	1	1.5	2	2.5	3
Ratio of water to raw material (X_3) (n)	10	12	14	16	18
Extraction frequency (X_4) (n)	2	3	4	5	6

where x_i is a coded value of the independent variable, X_i is the actual value of the independent variable, X_0 is the actual value of X_i on the center point, and ΔX_i is the value of step change. The range of independent variables and their levels are presented in Table 1. The independent variables and their ranges were chosen based on preliminary experimental results.

The design consisted of 31 experimental points (16 factorial points, 8 axial points, and 7 center points). The triplicates were performed at all design points in a randomized order. The response value in each trial was the average of duplicates.

The CCD data were analyzed by multiple regressions to fit the following quadratic polynomial model:

$$Y_k = \beta_{k_0} + \sum_{i=1}^{4} \beta_{k_i} x_i + \sum_{i=1}^{4} \beta_{k_{ii}} x_i^2 + \sum_{i<j=2}^{4} \beta_{k_{ij}} x_i x_j \quad (2)$$

where Y_k is the response function and β_{k_0} is an intercept. β_{k_i}, $\beta_{k_{ii}}$, and $\beta_{k_{ij}}$ are the coefficients of the linear, quadratic, and interactive terms, respectively. x_i and x_j represent the coded independent variables, respectively. The fitted polynomial equation was expressed as surface and contour plots to visualize the relationship between the response and experimental levels of each factor, and to deduce the optimum conditions. The regression coefficients of individual

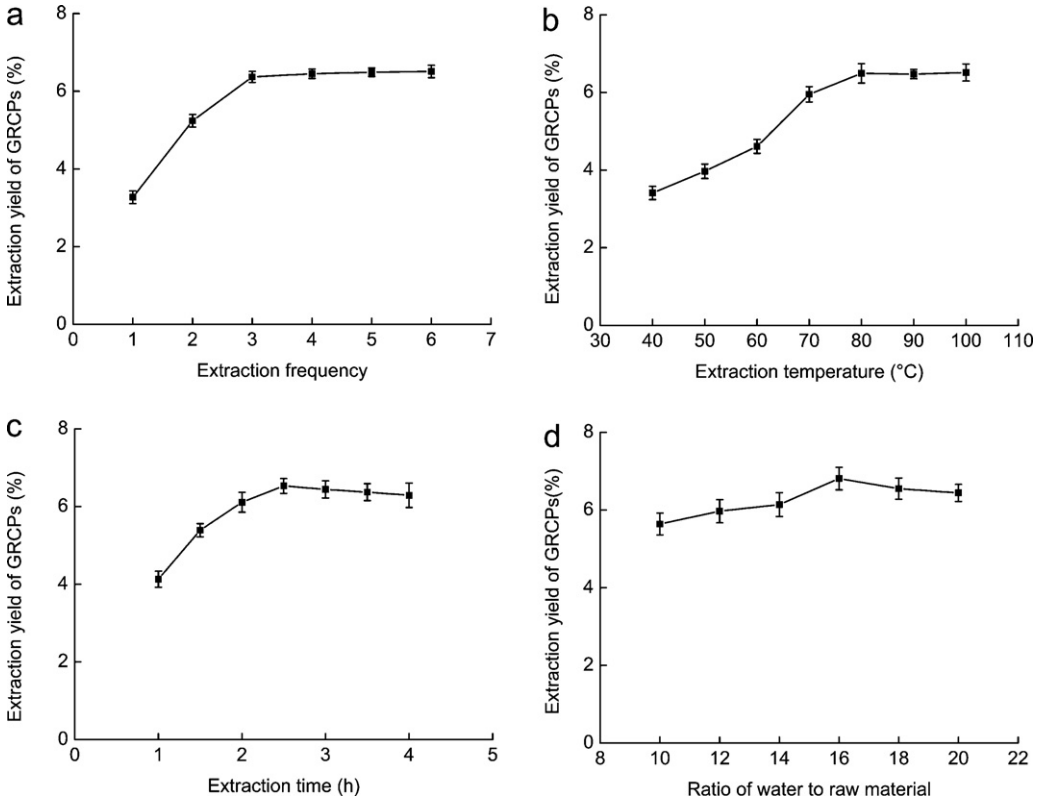

Fig. 1. Effect of different extraction parameters on the purity of GRCPs: (a) extraction frequency; (b) extraction temperature; (c) extraction time; (d) ratio of water to raw material.

linear, quadratic, and interaction terms were determined according to variable analysis. The regression coefficients were then used for statistical calculation to generate three-dimensional (3D) surface plots and contour plots from the fitted polynomial equation. Design Expert software (Version 8.0.6) was used to analyze the experimental data. *P* values less than 0.05 were considered to be statistically significant.

2.4. Antioxidant activity test in vitro

Antioxidant activity was determined according to the superoxide anion radical scavenging activity, hydroxyl radical scavenging activity, DPPH radical scavenging activity, and reducing power.

2.4.1. Superoxide radical (O_2^-) scavenging assay

Superoxide anion radical scavenging activity was determined according to the method of Stewar and Beewley (1980). The reaction mixture in a final volume of 3 mL contained the following reagents (final concentrations): 13 mM MET, 10 mM RF, 75 μM NBT, 100 mM EDTA, 50 mM phosphate buffer (pH 7.8), and GRCPs (5–250 mg/L). After illuminating the reaction mixture with a fluorescent lamp at 25 °C for 30 min, the absorbance was measured at 560 nm using BHT as a positive control. The superoxide radical scavenging capability was calculated by the following formula:

$$\text{scavenging rate}\,(\%) = \frac{A_0 - A_1}{A_0} \times 100\% \quad (3)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of GRCPs/BHT.

2.4.2. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured according to the method of Winterbourn and Sutton (1984). The reaction mixture contained 1 mL of 0.15 M phosphate buffer (pH 7.4), 1 mL of 40 μg/mL safranin, 1 mL of 0.945 mM EDTA-Fe(II), 1 mL of 3% (v/v) H_2O_2, and 0.5 mL of GRCPs (5–250 mg/L). After incubating at 37 °C for 30 min, the absorbance was measured at 560 nm using BHT as a positive control. The EC_{50} value (mg/L) of GRCPs or BHT was the effective concentration at which 50% of the hydroxyl radicals were scavenged. The hydroxyl radical scavenging activity was expressed as follows:

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

where A_0 is the absorbance of the blank and A_1 is the absorbance of GRCPs/BHT.

2.4.3. DPPH scavenging assay

DPPH radical scavenging activity was carried out according to the method of Shimada, Fujikawa, and Yahara (1992) with slight modifications. The reaction mixture contained 2 mL of DPPH (0.1 μM in 95% ethanol) and 2 mL of GRCPs (5–250 mg/L). The mixture was incubated at 25 °C for 15 min, and the absorbance of the mixture was determined at 517 nm using BHT as a positive control. The EC_{50} value (mg/L) of GRCPs was the effective concentration at which 50% of the DPPH radicals were scavenged. The antioxidant activity of GRCPs was evaluated according to the following formula:

$$\text{scavenging rate}\,(\%) = \left(1 - \frac{A}{A_0}\right) \times 100\% \quad (5)$$

where A is the absorbance of GRCPs/BHT and A_0 is the absorbance of the DPPH solution.

2.4.4. Determination of the reducing power of GRCPs

The reducing power of GRCPs was evaluated according to the method of Deng et al. (2011). The reaction mixtures contained 2.5 mL of phosphate buffer (pH 6.6, 0.2 M), 2.5 mL of potassium ferricyanide (1%, w/v), and GRCPs (5–250 mg/L). After incubating at 50 °C for 20 min, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture to end the reaction and then the mixture was centrifuged (1200 × *g*, 10 min). About 2.5 mL of the supernatant was collected and mixed with 2.5 mL of deionized water and 0.5 mL of $FeCl_3$ (0.1%, w/v). After incubating at room temperature for 15 min, the absorbance was measured at 700 nm using BHT as a positive control.

3. Results and discussion

3.1. Effect of the extraction frequency on the GRCPs yield

The effect of the extraction frequency on the GRCPs yield is shown in Fig. 1a. Different extraction frequencies were carried out while fixing the other extraction conditions as follows: extraction temperature, 80 °C; extraction time, 3 h; and ratio of water to raw material, 20. The polysaccharide yield rapidly increased with increased extraction frequency from 1 to 3, reaching the critical value (6.37 ± 0.14%) when the extraction frequency was 3. The yields did not change with further increased extraction frequency.

3.2. Effect of the extraction temperature on the GRCPs yield

The extraction coefficient increases with increased temperature because of increased polysaccharide solubility (Braga, Moreschi, & Meireles, 2006). To examine the effect of different temperatures on the GRCPs yield, extraction was carried out at different extraction temperatures. The extraction frequency, extraction time, and ratio of water to raw material were fixed at 3, 3 h, and 20, respectively. As shown in Fig. 1b, with increased extraction temperature from 40 °C to 80 °C, the yield continued to increase and reached the maximum value (6.49 ± 0.24%) at 80 °C. Although the extraction yield was also high from 80 °C to 100 °C, this high temperature range can increase the cost of the industrialization extraction process.

3.3. Effect of the extraction time on the GRCPs yield

Fig. 1c shows the effect of the extraction time on the GRCPs yield while fixing the other extraction conditions as follows: extraction frequency, 3; extraction temperature, 80 °C; and ratio of water to raw material, 20. With increased extraction time from 1 h to 2.5 h, the extraction yield remarkably increased and reached the peak value (6.53 ± 0.19%) at 2.5 h. Afterwards, the GRCPs yield insignificantly changed with prolonged extraction time. This result indicated that an extraction time of 2.5 h was sufficient to obtain the product.

3.4. Effect of the ratio of water to raw material on the GRCPs yield

The GRCPs yield was affected by different ratios of water to raw material, as shown in Fig. 1d. Extraction was carried out at different ratios of water to raw material while fixing the other extraction parameters as follows: extraction temperature, 80 °C; extraction time, 2.5 h; and extraction frequency, 3. The yield continued to increase with increased ratio and reached the peak value (6.81 ± 0.29%) at 16. When the ratio exceeded 16, the GRCPs yield insignificantly changed. Based on the above single-factor experiments, extraction frequency the following conditions were adopted in the RSM experiments: extraction time, 2–4 h; extraction temperature, 80–100 °C; extraction frequency, 2–6; ratio of water to raw material, 12–20.

Table 2
Response surface central composite design and results for extraction yield of GRCPs.

No.	X_1, extraction temperature (°C)	X_2, extraction time (h)	X_3, ratio of water to raw material	X_4, extraction frequency	Extraction yield of GRCPs (%)
1	−1 (85)	−1 (1.5)	−1 (12)	−1 (3)	5.89
2	−1 (85)	−1 (1.5)	−1 (12)	1 (5)	5.78
3	−1 (85)	−1 (1.5)	1 (16)	−1 (3)	5.85
4	−1 (85)	−1 (1.5)	1 (16)	1 (5)	6.25
5	−1 (85)	1 (2.5)	−1 (12)	−1 (3)	6.55
6	−1 (85)	1 (2.5)	−1 (12)	1 (5)	6.45
7	−1 (85)	1 (2.5)	1 (16)	−1 (3)	6.23
8	−1 (85)	1 (2.5)	1 (16)	1 (5)	6.44
9	1 (95)	−1 (1.5)	−1 (12)	−1 (3)	6.21
10	1 (95)	−1 (1.5)	−1 (12)	1 (5)	6.21
11	1 (95)	−1 (1.5)	1 (16)	−1 (3)	6.17
12	1 (95)	−1 (1.5)	1 (16)	1 (5)	6.49
13	1 (95)	1 (2.5)	−1 (12)	−1 (3)	6.72
14	1 (95)	1 (2.5)	−1 (12)	1 (5)	7.78
15	1 (95)	1 (2.5)	1 (16)	−1 (3)	7.78
16	1 (95)	1 (2.5)	1 (16)	1 (5)	7.49
17	−2 (80)	0 (2)	0 (14)	0 (4)	5.78
18	2 (100)	0 (2)	0 (14)	0 (4)	6.88
19	0 (90)	−2 (1)	0 (14)	0 (4)	5.49
20	0 (90)	2 (3)	0 (14)	0 (4)	7.15
21	0 (90)	0 (2)	−2 (12)	0 (4)	5.98
22	0 (90)	0 (2)	2 (18)	0 (4)	7.42
23	0 (90)	0 (2)	0 (14)	−2 (2)	6.11
24	0 (90)	0 (2)	0 (14)	2 (6)	6.34
25	0 (90)	0 (2)	0 (14)	0 (4)	7.22
26	0 (90)	0 (2)	0 (14)	0 (4)	7.22
27	0 (90)	0 (2)	0 (14)	0 (4)	7.22
28	0 (90)	0 (2)	0 (14)	0 (4)	7.22
29	0 (90)	0 (2)	0 (14)	0 (4)	7.22
30	0 (90)	0 (2)	0 (14)	0 (4)	7.22
31	0 (90)	0 (2)	0 (14)	0 (4)	7.22

Table 3
Analysis of variance for the fitted quadratic polynomial model.

Source	SS	DF	MS	*F*-value	Prob > *F*
Model	11.1634	14	0.7974	10.15	<0.0001
Residual	1.257	16	0.0786		
Lack of fit	1.257	10	0.1257		
Pure error	0.000	6	0.000		
Cor. total	12.4208	30			
	$R^2 = 0.9598$; $R_{adj}^2 = 0.9072$; $CV = 4.22$				

3.5. Statistical analysis and model fitting

The design matrix and corresponding results of RSM experiments for determining the effects of the four independent variables, including X_1, X_2, X_3, and X_4, are listed in Table 2. The GRCPs yield ranged from 5.71% to 7.66%. The maximum GRCPs yield was recorded under the following experimental conditions: extraction temperature, 95 °C; extraction time, 2.5 h; ratio of water to raw material, 16; and extraction frequency, 5. Multiple regression analysis performed on the experimental data revealed that the response and test variables were related by the following second-order polynomial equation (6):

$$Y = 7.22 + 0.3171X_1 + 0.4129X_2 + 0.1663X_3 + 0.0813X_4 + 0.1748X_1X_2 + 0.0569X_1X_3 + 0.0431X_1X_4 - 0.0144X_2X_3 + 0.0169X_2X_4 - 0.0131X_3X_4 - 0.2018{X_1}^2 - 0.2043{X_2}^2 - 0.1093{X_3}^2 - 0.2280{X_4}^2 \quad (6)$$

The fitting statistics of the extraction yield Y for the selected quadratic predictive model is shown in Table 3. The determination coefficient ($R^2 = 0.9598$) was determined by the ANOVA of the quadratic regression model, indicating that only 4.02% of the total variations were not explained by the model. The adjusted determination coefficient ($R_{adj}^2 = 0.9072$) also confirmed that the model was highly significant. At the same time, the very low coefficient of variation of 4.22 clearly indicated a very high degree of precision and reliability of the experimental values. The model was found to be adequate for prediction within the range of experimental variables. The regression coefficient values of Eq. (6) are listed in Table 4. The P values were used as a tool to check the significance of each coefficient, which in turn indicated the pattern of interactions between variables. A smaller P indicated a more significant corresponding coefficient (Guo et al., 2010). Table 4 shows that the linear coefficients (X_1, X_2, and X_3), a quadratic term coefficient (${X_1}^2$, ${X_2}^2$, and ${X_4}^2$), and interaction product coefficients (X_1X_2) were significant, with very small P values ($P < 0.05$). The other coefficients were insignificant ($P > 0.05$). The full model was made 3D with contour plots to predict the relationships between the independent and dependent variables.

Table 4
Regression coefficients of the predicted quadratic polynomial model.

Parameter	Estimate	Standard error	*t* ratio	*P*-value
X_1	0.3171	0.0572	5.541	0.000
X_2	0.4129	0.0572	7.216	0.000
X_3	0.1663	0.0572	2.905	0.010
X_4	0.0813	0.0572	1.420	0.175
X_1X_1	−0.2018	0.0524	−3.849	0.001
X_1X_2	0.1744	0.0701	2.488	0.024
X_1X_3	0.0569	0.0701	0.812	0.429
X_1X_4	0.0431	0.0701	0.615	0.547
X_2X_2	−0.2043	0.0524	−3.897	0.001
X_2X_3	−0.0144	0.0701	−0.205	0.840
X_2X_4	0.0169	0.0701	0.241	0.813
X_3X_3	−0.1098	0.0524	−2.084	0.054
X_3X_4	−0.0131	0.0701	−0.187	0.854
X_4X_4	−0.2280	0.0524	−4.350	0.000

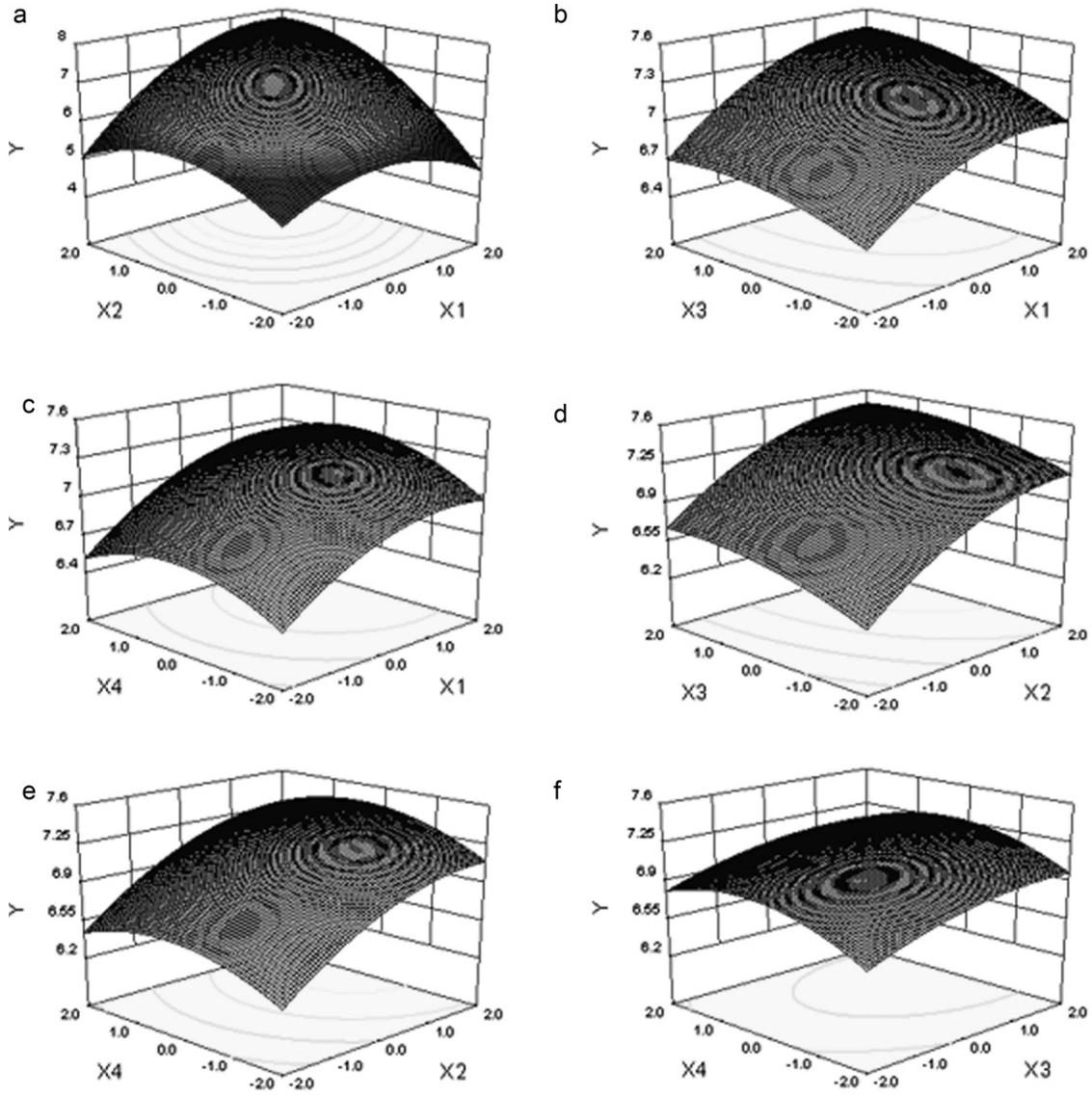

Fig. 2. Response surface plots (3D) showing the effects of variables (X_1: extraction temperature; X_2: ratio of water to raw material; X_3: extraction time; X_4: extraction frequency) on the response Y (yield of GRCPs).

3.6. Optimization of GRCPs extraction conditions

The 3D response surface and contour plots were obtained by Design Expert software (Version 8.0.6). The results of GRCPs yield as affected by the extraction temperature (X_1), extraction time (X_2), ratio of water to raw material (X_3), and extraction frequency (X_4) are shown in Figs. 2 and 3.

The main goal of RSM was to identify the optimum values of the independent variables efficiently so that the responses are maximized (Wu et al., 2007). In the response surface and contour plots, the GRCPs yield was obtained along with two continuous variables while fixing the other two variables at their respective zero level (center value of the testing ranges). In the two figures, the maximum predicted value indicated by the surface was confined in the smallest ellipse in the contour diagram. Elliptical contours were obtained when perfect interaction existed between the independent variables (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). The independent variables and maximum predicted values from the figures corresponded with the optimum values of the dependent variables (responses) obtained by the equations.

The 3D response surface and contour plots based on the independent variables (extraction temperature and extraction time) are shown in Figs. 2a and 3a. The other two independent variables (ratio of water to raw material and extraction frequency) were kept at a zero level. The maximum GRCPs yield was achieved when the extraction temperature and extraction time were 98 °C and 2.86 h, respectively. Figs. 2b and 3b show the GRCPs yield with varied extraction temperature and ratio of water to raw material at fixed extraction time (zero level) and extraction frequency (zero level). The yield was found to increase with increased extraction temperature from 80 °C to 98 °C, and then slightly decreased. The yield also increased with increased ratio of water to raw material from 10 to 16, and then decreased when the ratio ranged from 16 to 18. The GRCPs yield as affected by different extraction temperatures and numbers of extraction are shown in Figs. 2c and 3c, wherein two variables (extraction time and ratio of water to raw material) were fixed at zero levels. This finding indicated that the maximum GRCPs yield was achieved when the extraction temperature and ratio of water to raw material was 16 °C and 4, respectively. Figs. 2d and 3d show the 3D response surface and contour plots at varied

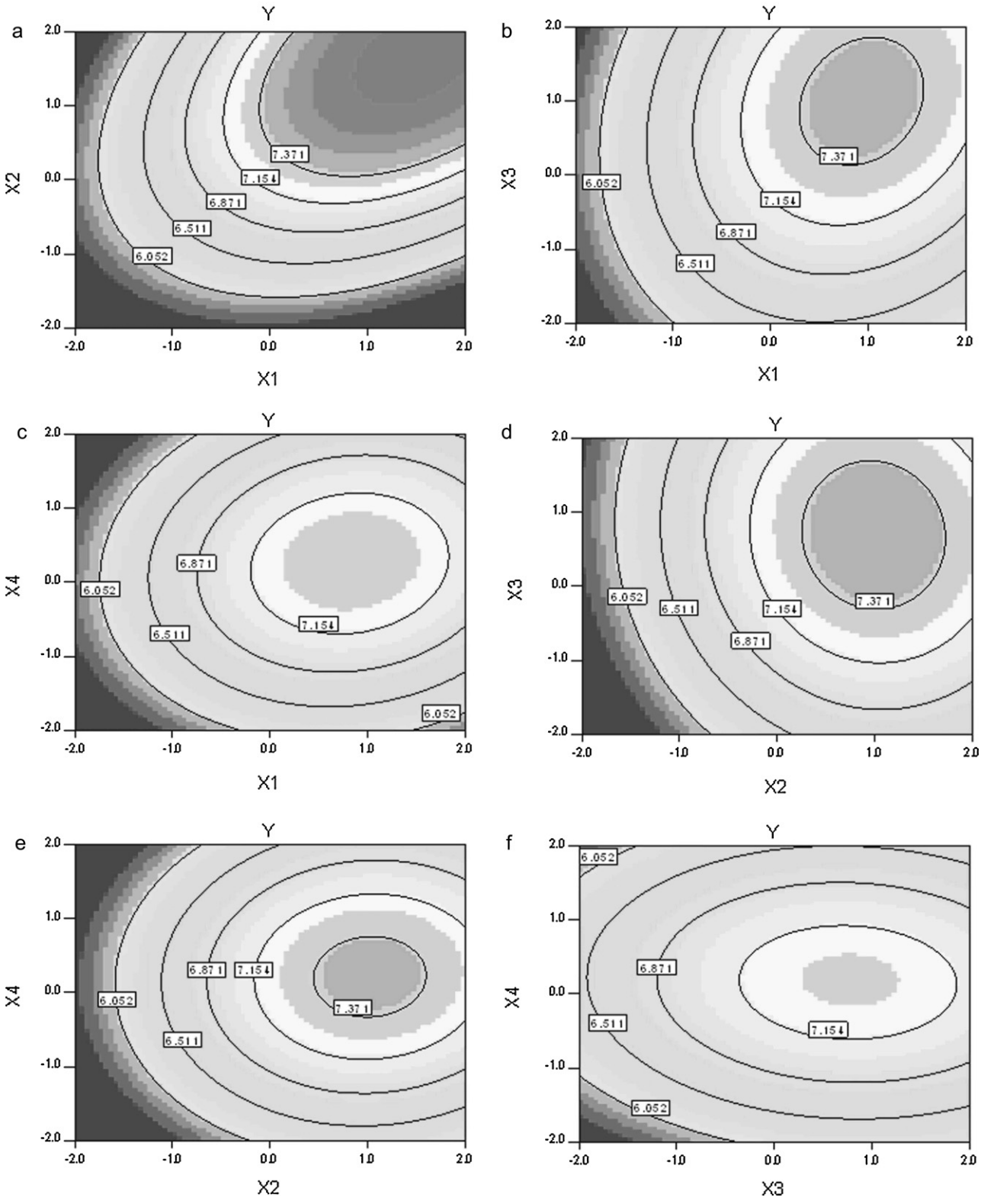

Fig. 3. Contour plots (2D) showing the effects of variables (X_1: extraction temperature; X_2: ratio of water to raw material; X_3: extraction time; X_4: extraction frequency) on the response Y (yield of GRCPs).

extraction time and ratio of water to raw material while fixing the extraction temperature (zero level) and extraction frequency (zero level). The GRCPs yield increased with increased extraction time from 1 h to 2.86 h, but did not increase with further increased extraction time. The GRCPs yield reached the maximum value when the ratio of water to raw material was 16; beyond this level, the GRCPs yield did not further increase. Figs. 2e and 3e show that the 3D response surface and contour plots presented the GRCPs yield with varied extraction times and extraction frequency while fixing the extraction temperature (zero level) and ratio of

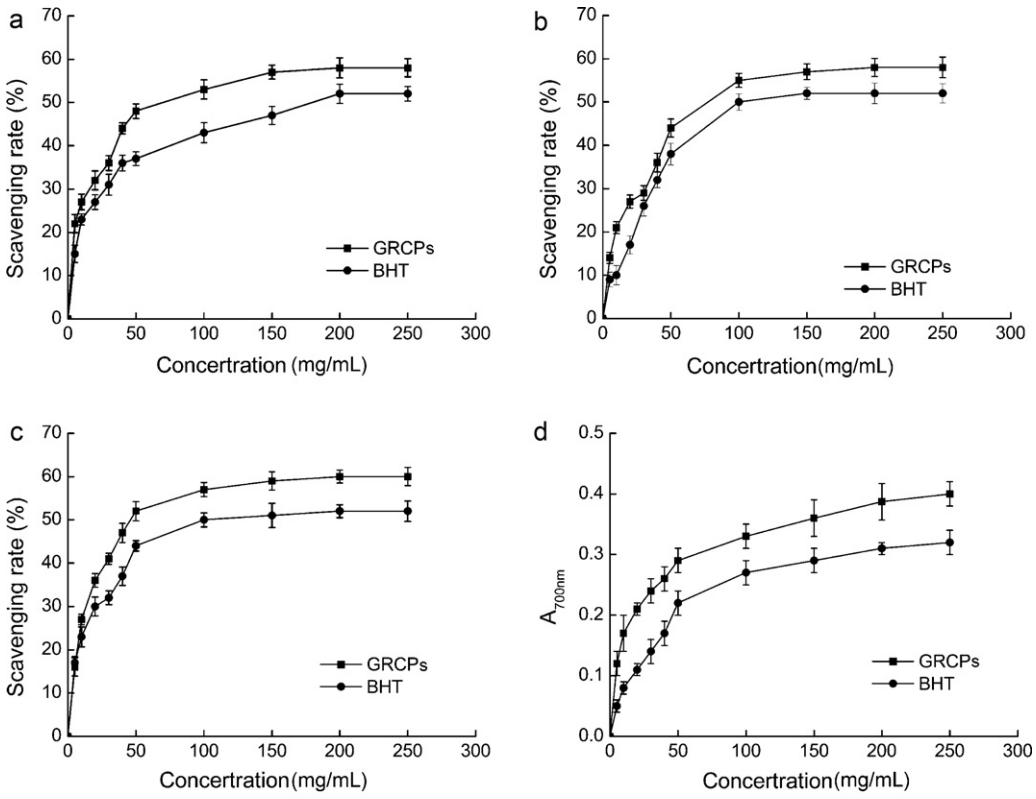

Fig. 4. Antioxidant activity assay of GRCPs and BHT. (a) Scavenging effects on superoxide anion radicals. (b) Scavenging effects on hydroxyl radicals. (c) Scavenging effects on DPPH. (d) Reducing power of GRCPs and BHT.

water to raw material (zero level). This finding indicated that the maximum GRCPs yield was achieved when the extraction time and extraction frequency was at the threshold levels of 2.86 h and 4, respectively. Figs. 2f and 3f show that the 3D response surface and contour plots at varied ratios of water to raw material and extraction frequency while fixing the extraction temperature (zero level) and extraction time (zero level). The yield was found to increase with increased extraction frequency from 2 to 4, but decreased beyond 4. The yield was found to increase rapidly with increased ratio of water to raw material from 10 to 16, but reached the maximum plateau region and did not further increase beyond a ratio of 16.

Figs. 2 and 3 show that the optimal extraction conditions of GRCPs from the fruiting bodies of *G. rutilus* were as follows: extraction temperature, 98 °C; ratio of water to raw material, 16; extraction time, 2.86 h; and extraction frequency, 4. Among the four extraction parameters studied, the extraction time and extraction temperature were both the most significant factors that affected the GRCPs yield followed by the ratio of water to raw material and extraction frequency.

3.7. Verification of the predictive model

The suitability of the model equations for predicting the optimum response values was determined under the above optimized conditions. This set of conditions was determined to be optimum by RSM and used to validate experimentally and predict the values of the responses using the model equation. Compared with the predicted value (7.95), the mean value of 8.02 ± 0.15 ($n = 3$) obtained from real experiments demonstrated the validation of the RSM model and indicated that the model was adequate for the extraction process.

3.8. Antioxidant activity

Antioxidant activities have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, binding of transition metal ion catalysts, etc. (Frankel & Meyer, 2000). In this experiment, the antioxidative activities of GRCPs in vitro were evaluated using different biochemical methods of superoxide anion, hydroxyl, and DPPH radical scavenging assay, as well as reducing power analysis.

Superoxide anion is one of the precursors of singlet oxygen and hydroxyl radicals; therefore, it indirectly initiates lipid peroxidation. Moreover, the presence of superoxide anion can aggravate cellular damage because it produces other kinds of free radicals and oxidizing agents (Athukorala, Kim, & Jeon, 2006). The results of superoxide radical scavenging assay are shown in Fig. 4a. The scavenging rates of GRCPs and BHT for superoxide radical were directly proportional to their concentrations. The inhibition percentage of GRCPs was remarkably higher than that of BHT within the test dosage range. The EC_{50} value of GRCPs was 66.8 ± 2.3 mg/L, which was about 36% that of BHT (187.4 ± 2.6 mg/L), indicating that the GRCPs significantly affected the scavenging of superoxide anion radical.

Hydroxyl radicals can easily cross cell membranes, readily react with most biomolecules (including carbohydrates, proteins, lipids, and DNA in cells), and inflict tissue damage or cell death. Thus, removing hydroxyl radicals is important for the protection of living systems (Cheng, Ren, Li, Chang, & Chen, 2002). As shown in Fig. 4b, the hydroxyl radical scavenging activity of GRCPs was concentration dependent and slightly higher than that of BHT within the test dosage range. The EC_{50} value of GRCPs for hydroxyl radical scavenging activity was 78.4 ± 3.5 mg/L, which did not

significantly differ from the scavenging effect of BHT (101.2 ± 3.2 mg/L).

Meanwhile, DPPH free radical is stable, with a maximum absorption at 517 nm and the ability to undergo scavenging by an antioxidant readily. Thus, DPPH is widely accepted as a tool for evaluating the free radical scavenging activities of natural compounds (Leong & Shui, 2002). The scavenging effects of GRCPs on the DPPH radical were measured, and the results are shown in Fig. 4c.

GRCPs and BHT showed evident scavenging activity on DPPH radical in a concentration-dependent manner within a relatively low concentration range of 5–100 mg/L. However, increased scavenging activities of GRCPs and BHT did not significantly increase within a relatively high concentration range of 100–250 mg/L. Furthermore, the DPPH scavenging activities of GRCPs were higher than that of BHT at all concentration points. The EC_{50} value of GRCPs was 43.6 ± 1.6 mg/L, which was higher than 102.3 ± 4.2 mg/L.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Kallithraka, Bakker, & Clifford, 2001). The reducing power of GRCPs and BHT were investigated, and the results are shown in Fig. 4d. The reducing capacity of GRCPs was remarkably higher than that of BHT. The results showed that the GRCPs had potential antioxidant properties.

4. Conclusion

Using contour and surface plots in RSM was effective for estimating the effect of four independent variables (extraction temperature, extraction time, ratio of water to raw material, and extraction frequency). The optimum set of independent variables was graphically obtained to determine the desired levels of crude polysaccharide extraction. The optimal experimental extraction yield of $8.02 \pm 0.15\%$ was obtained when the extraction temperature was 98 °C, the ratio of water to raw material was 16, the extraction time was 2.86 h, and the extraction frequency was 4. Under these optimized conditions, the experimental GRCPs yield well agreed with the predicted yield of 7.95%. GRCPs had enormous potential for use as a novel antioxidant because it displayed positive radical scavenging activity for superoxide anion, hydroxyl, and DPPH radicals, as well as strong reducing power in vivo. This study provided essential information for the large-scale production of polysaccharides from *G. rutilus*, which can be used as functional food or antioxidant.

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