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Original Research Article

Extraction optimization and characterization of polysaccharide antioxidants from Pinellia ternata (Thunb) Breit rhizome

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Abstract

Purpose: *To investigate the optimum extraction conditions of polysaccharides from Pinellia Rhizoma (PRP) and their antioxidant activities.*
Methods: Response surface methodology (RSM) was applied to optimize the water extraction conditions of PRP by Box-Benhnken design (BBD). A high performance liquid chromatography (HPLC) method was performed for determining the monosaccharide composition, while a high performance gel permeation chromatography (HPGPC) method was established for determining the molecular weight distribution of PRP. In addition, scavenging DPPH and superoxide anion radical scavenging assays were used to evaluate the antioxidant activities of PRP.
Results: *As a result, the optimum extraction conditions of PRP were as follows: extraction time, 103 min; solid-liquid ratio, 24 g/mL; and extraction no., 3. Under these conditions, the maximum extraction yield of PRP was 2.47 %, which matched the optimum value (2.55 %) predicted by RSM. The results of HPLC analysis suggest that the monosaccharide composition of PRP was mannose (Man), galactose acid (Gal acid), galactose (Gal), glucose (Glc), and arabinose (Ara) in molar ratios of 5.76:2.20:2.64:4.57:1, respectively, and the molecular weight ranged from 13592 to 445065. Furthermore, PRP showed marked antioxidant activities with the highest DPPH free radical scavenging rate of 71.5 % and O^{2-} free radical scavenging rate of 87.5 % at a concentration of 10 mg/mL.*
Conclusion: *RSM is a rapid and effective statistical technique for optimizing conditions for PRP extraction. Furthermore, PRP is a potential natural source of antioxidants.*

Keywords: *Pinellia rhizoma, Polysaccharides Optimization extraction, Monosaccharide composition, Antioxidant activity*

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INTRODUCTION

Pinellia ternata (Thunb.) Breit. is an important medicinal plant from the Araceae family, and is widely distributed in Anhui, Sichuan and Hubei provinces of China. Its Rhizoma (PR) has been used in Traditional Chinese Medicines (TCMs) for its antiemetic, antitussive, anti-tumor, antimicrobial, anti-obesity, anticonvulsant activities and other therapeutic efficacies [1-6]. Studies have shown that the main constituents of PR are proteins, alkaloids, polysaccharides and organic acids [7-10]. Recently, it has been reported that an increasing number of polysaccharides from natural sources have excellent antioxidant activities [11].

Polysaccharides are important constituents of PR, which have been reported to possess significant anti-tumor effects [12-14]. However, little information is available in the literatures regarding the antioxidant effect of PRP.

The aim of the present study was to apply RSM to optimize the extraction conditions of PRP, furthermore, monosaccharide composition and molecular weight of PRP were investigated in order to characterize the structure of PRP.

EXPERIMENTAL

Chemicals and reagents

Standard monosaccharides (Mannose, Glucose, Galactose, Galactose acid, and Arabinose), and dextrans of different molecular weights were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); Phenol, concentrated sulfuric acid (H_2SO_4) and 3-Methyl-1-phenyl-2-pyrazolin-5-one (PMP), trifluoroacetic acids (TFA) were purchased from the Sino Pharm chemical reagents (Shanghai, China); 1,1-diphenyl-2-picrylhydrazyl (DPPH), Vitamin C (Vc) and 1,2,3-trihydroxy-benzene were purchased from Sigma Co (St. Louis, MO, USA). All other solvents and chemical reagents used in this study were of analytical reagent grade.

Extraction procedure

Pinellia Rhizoma was purchased from Sichuan New Lotus Chinese Herbal Pieces Co., Ltd. (Chengdu, China) in April, 2016 (batch no. D1601137), and was authenticated by Professor Chun-Jie Wu (College of Pharmacy, Chengdu University of Traditional Chinese Medicine). A voucher specimen (no. sPR-20160715) was deposited in the herbarium of College of Pharmacy, Chengdu University of Traditional Chinese Medicine (Chengdu, China).

Crude polysaccharide extraction was carried out using a method described previously [15] with some modifications. The *Pinellia Rhizoma (PR)* powder was extracted with 95 % ethanol (1 : 3, w/v) for 2 h by reflux. After filtration, the residue was air-dried and re-extracted twice with distilled water by reflux. The obtained solutions were filtered by filter paper, and the filtrate was concentrated at 50 °C under vacuum. Amylase (20 mL) was added to remove the starch, and the excess amylase was removed by boiling for 5 min. After centrifuging at 5000 rpm for 10 min, 95 % ethanol was added to the supernatant to make the ethanol content of 80 % (v/v), and then stored overnight at 4 °C. After centrifugation (5000 rpm, 10 min), the precipitates were collected and washed thrice with anhydrous ethanol, acetone and aether, and finally dried at 50 °C to obtain the PRP. The phenol-sulfuric acid method [16] was used to determine the polysaccharide content of PRP. The percentage extraction yield (%) of PRP was calculated by using the following equation (Eq 1).

$$\text{Yield (\%)} = (C_1/C_0)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where C_0 is the weight of raw material and C_1 is the weight of crude polysaccharide.

Optimization of PRP extraction

Three major influence factors affecting the extraction yield of PRP were selected: extraction time (A, min); number of extraction (B) and solid-liquid ratio (C, g/mL). These three factors were set at three different levels coded -1, 0, +1 to represent the low, medium and high grade, respectively. The yield of polysaccharides was used as the response value, and the code of the levels and factors are shown in Table 1.

Monosaccharide composition analysis

The monosaccharide composition of PRP sample was determined by HPLC with pre-column derivatization. The hydrolysis of polysaccharides was performed using previously described methods [17]. PRP (20 mg) was hydrolyzed in TFA (2 M, 5 mL) for 5 h at 110 °C in a sealed test tube. The hydrolysate was cooled to room temperature, and then the supernatant was obtained after centrifugation (5000 rpm, 15 min) and dried at 50 °C using a rotary evaporator. The residue was washed with methanol three times to remove the TFA, and then dissolved in 2 mL distilled water.

Table 1: Factors and levels of RSM experiments

Symbol	Independent variable	Code level		
		-1	0	1
A	Extraction time (min)	90	120	150
B	Number of extraction	1	2	3
C	Solid-liquid ratio (g/mL)	10	20	30

The pre-column monosaccharide derivatives were prepared using PMP reagents according to a previous method [18]. Man, Gal acid, Glu, Gal, and Ara were used as monosaccharide standards, and dissolved in distilled water (2 mM). PMP solution (0.5 M, 0.2 mL) and NaOH solution (0.3 M, 0.2 mL) were added into the hydrolyzed polysaccharide sample (0.2 mL) and monosaccharide standard solutions (2 mM), and then maintained in a water bath at 70 °C for 60 min. After incubation, the mixture was then completely neutralized by adding 0.2 mL HCl (0.3 M). Subsequently, 1 mL trichloromethane was added, and mixed thoroughly by vortexing. The organic phase was carefully discarded and the aqueous phase was diluted to 5 mL with distilled water.

The monosaccharide analysis was performed on a CAPCELL PAK MG II S5 C18 column (4.6 mm × 250 mm, 5 μm) by Agilent 1260 HPLC system (Agilent, USA). The mobile phase was composed of 0.05 M phosphate buffer (pH = 6.8) and acetonitrile (82 : 18, v/v), the flow rate was 0.8 mL/min, and the injection volume was 5 uL. The column temperature was maintained at 35 °C, and the detection wavelength at 245 nm.

Molecular weight distribution test

HPGPC was used to determine the molecular weight (M_W) distribution of polysaccharides sample according to a previous report [20]. A series of dextran with different molecular weights, viz, 1.26×10^4, 7.38×10^4, 1.10×10^5, 2.89×10^5, and 4.96×10^5 g/mol were used to construct a calibration curve. PRP (20 mg) was dissolved in distilled water (2 mL), and centrifuged (10,000 rpm, 5 min). The supernatant was filtered through a 0.22 mm filter before injection. The TSK-Gel G4000 SW_{XL} (7.8mm × 30 cm, 8 μm) column was used to separate the sample at 40 °C with distilled water as mobile phase at a flow rate of 0.8 mL/min. The sample was detected by Alltech 2000 ES evaporative light-scattering detector; the injection volume was 20 uL. The data were analyzed by EZChrom Elite software (Agilent Technology, CA).

DPPH radical scavenging assay

The DPPH radical scavenging test was performed by the method reported by Bartolomeuws *et al* [21]. Different concentrations of PRP (1, 2, 4, 6, 8, 10 mg/mL) were prepared, and 2 mL of DPPH solution (0.04 mM) were added and mixed. The solutions kept in the dark at room temperature for 30 min, and then measured the absorption at 517 nm. Vitamin C (Vc) was used as positive control. DPPH free radical scavenging rate (D) of the sample was computed as in Eq 2.

$$D\ (\%) = \{1 - (A_1 - A_2)/A_0\}100 \quad \ldots\ldots\ldots\ldots \quad (2)$$

where A_0 is the absorbance of the control (distilled water), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (ethanol instead of DPPH solution).

Superoxide anion (O^{2-}) radical scavenging assay

The O^{2-} radical scavenging test was carried out according to a previous report by Wang *et al* [22]. Sample solutions (1 mL) of different concentrations of PRP (1, 2, 4, 6, 8, 10 mg/mL) were prepared and 4.5 mL Tris-HCl buffer (50 mM, pH=8.2) were added. After incubation at 25 °C for 10 min, 0.1mL pyrogallol (25 mM) were added and mixed. Concentrated hydrochloric acid (0.5 mL) was added to stop the reaction. Finally, the absorption was measured at 320 nm. Vc was used as positive control. O^{2-} scavenging rate (S) of the sample was calculated using Eq 3.

$$S\ (\%) = \{1 - (B_1 - B_2)/B_0\}100 \quad \ldots\ldots\ldots\ldots \quad (3)$$

where B_0 is the absorbance of the control (distilled water), B_1 is the absorbance of the sample, and B_2 is the absorbance of the sample only (distilled water instead of pyrogallol solution).

Statistical analysis

The results were analyzed with SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). The experimental data from RSM was analyzed using version 8.0.5 Design-Expert software (Stat-Ease Inc, Minneapolis, MN, USA). Analysis of variance (ANOVA) was used to analyze the results, and $p < 0.05$ was considered statistically significant.

RESULTS

Optimized extraction conditions

RSM data based on BBD of PRP extraction are shown in Table 2. The entire design consisted of 17 experiments based on BBD, and all the experiments were performed in random order.

The regression equation (Eq 4) was obtained by quadratic polynomial using Design-expert 8.05 b software.

$$Y = 2.47 + 0.078A + 0.12B + 0.056C + 0.067AB + 0.013AC - 0.005BC - 0.15A^2 - 0.10B^2 - 0.068C^2 \quad \ldots\ldots\ldots \quad (4)$$

Table 2: Experimental points of BBD and the experimental data

Run	A	B	C	Yield (%,Y)
1	0	0	0	2.52
2	0	-1	1	2.22
3	1	-1	0	2.11
4	0	1	-1	2.39
5	-1	0	-1	2.12
6	-1	-1	0	2.09
7	0	-1	-1	2.12
8	0	0	0	2.45
9	1	1	0	2.48
10	0	1	1	2.47
11	0	0	0	2.43
12	1	0	1	2.41
13	-1	0	1	2.23
14	0	0	0	2.47
15	1	0	-1	2.25
16	0	0	0	2.49
17	-1	1	0	2.19

The results of significant test and the variance analysis of the model are shown in Table 3. The three factors (A, B and C) had significant effects on the extraction yield of PRP. Further analysis of the equation variance showed that the model had a higher F value (F = 49.16) and a lower P value ($p < 0.0001$), indicating that this model was highly significant. The lack-of-fit (0.7849) was not significant demonstrating that the quadratic polynomial model selected by the study had a high degree of significance. The total determination coefficient of the regression model R^2 = 0.9844 and the adjusted determination coefficient R^2adj = 0.9644, which indicated the model had good fitting degree with small experimental error. Thus, the regression equation model established was valid and could be applied to analyze and predict the extraction conditions of PRP.

The response surface and contour plots of the effects of three factors on the extraction yield of the PRP were obtained according to the multiple regression equation. The response surface and the contour plot are shown in Figure 1. It could be seen from the figure that the interaction between extraction time and number of extraction had a significant effect on the extraction yield of PRP. The yield of PRP was not affected by the interactions between other factors.

The optimal extraction conditions of PRP were as follows: the extraction time was 103.13 min, number of extraction was 2.73, and the solid-liquid ratio of 24.23 g/mL. The predicted value of extraction yield of PRP was 2.55 %. To validate the predicted conditions, the optimal extraction conditions were modified as: extraction time of 103 min, number of extraction of 3 and the solid-liquid ratio of 24 g/mL. The above conditions were used to extract the PRP three times and the mean yield of PRP was 2.47 %. Therefore, the optimal extraction conditions of PRP based on RSM were accurate and reliable.

Monosaccharide composition analysis

HPLC chromatograms of the monosaccharide composition of PRP are shown in Figure 2. The results indicated that PRP was composed of Man, Gal acid, Glc, Gal, and Ara with a molar ratio of 5.76 : 2.20 : 4.57 : 2.64 : 1.

Molecular weight distribution

The GPC chromatogram of PRP is shown in Figure 3. A standard calibration curve [Log (M) = - 0.00163 RT + 6.712 (r = 0.9965)] was obtained by a series of dextran standards. The calibration

Table 3: Analysis of variance (ANOVA) for the fitted quadratic polynomial model

Source	Sum of squares	df	Mean square	F -Value	*P*-value
Model	0.39	9	0.044	49.16	< 0.0001
A	0.048	1	0.048	54.21	0.0002
B	0.12	1	0.12	138.21	< 0.0001
C	0.025	1	0.025	28.56	0.0011
AB	0.018	1	0.018	20.56	0.0027
AC	6.250E-004	1	6.250E-004	0.71	0.4288
BC	1.000E-004	1	1.000E-004	0.11	0.7468
A^2	0.096	1	0.096	108.30	< 0.0001
B^2	0.045	1	0.045	50.88	0.0002
C^2	0.020	1	0.020	22.29	0.0022
Residual	6.205E-003	7	8.864E-004	-	-
Lack of fit	1.325E-003	3	4.417E-004	0.36	0.7849
Pure error	4.880E-003	4	1.220E-003	-	-
Cor total	0.40	16	-	-	-
Std.dev.	0.9844	-	-	-	-
Mean	0.9644	-	-	-	-
CV (%)	0.9276	-	-	-	-

curves showed good linearity in the range of 1.26×10^4 to 4.96×10^5 g/moL. RT, Mw, Number-average Molecular Weight (Mn) and relative content of PRP were obtained by using GPC software (Agilent, USA) (Table 4). The results of HPGPC analysis showed that the PRP had a broad M_W range. There were six constituents with different molecular weights level in PRP and the average Mw values were approximately 445065, 329918, 176010, 119024, 99238 and 13592.

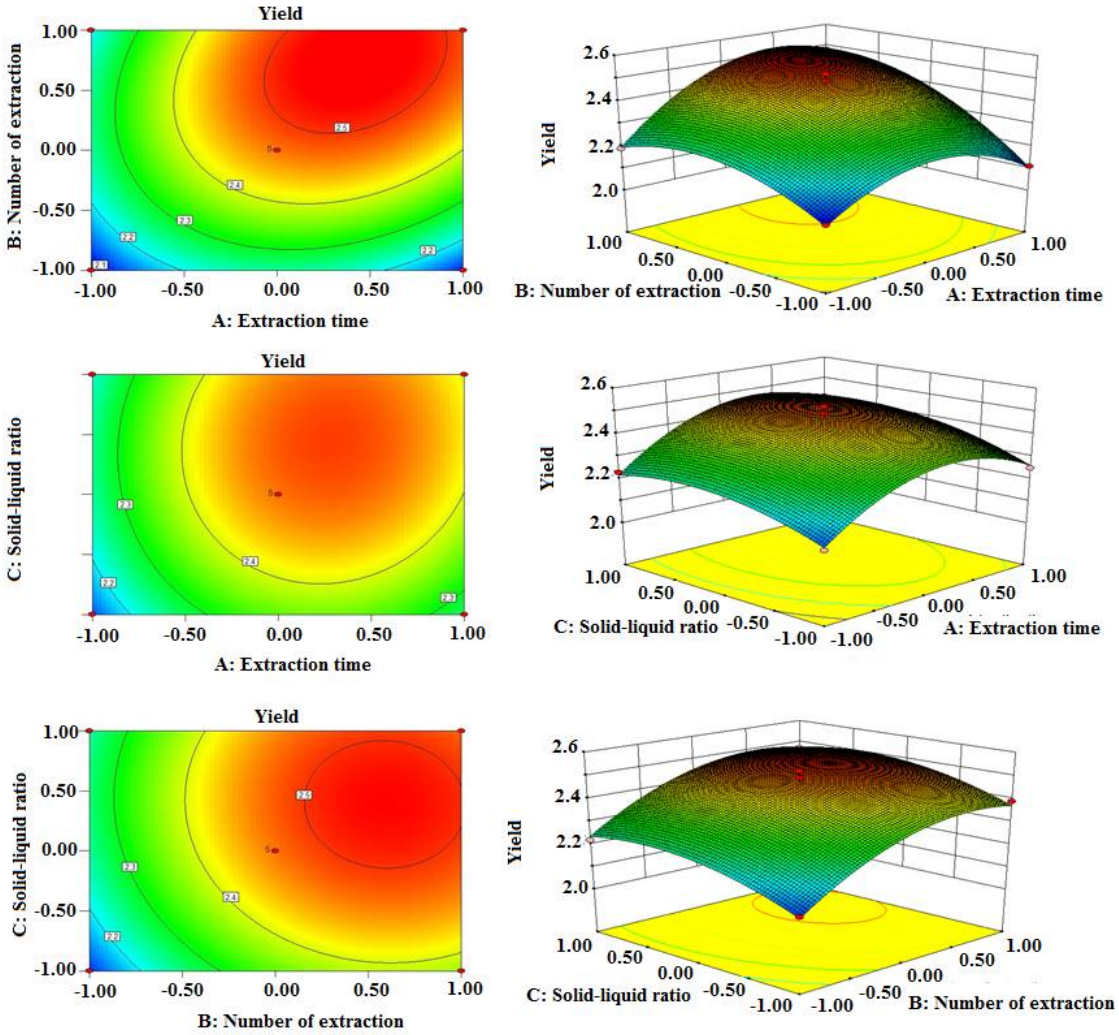

Figure 1: Contour plots and response surface for the yield of PRP

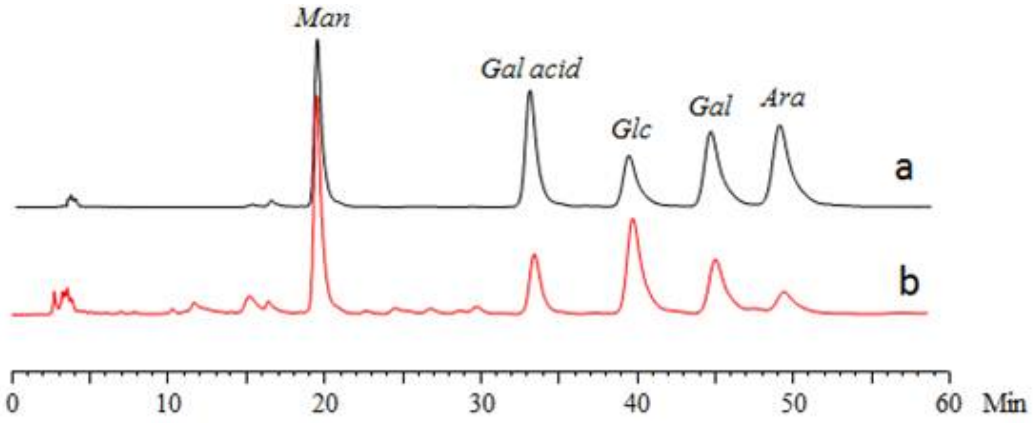

Figure 2: HPLC chromatograms of PMP derivatives of monosaccharide standards (a) and hydrolysate of PRP (b)

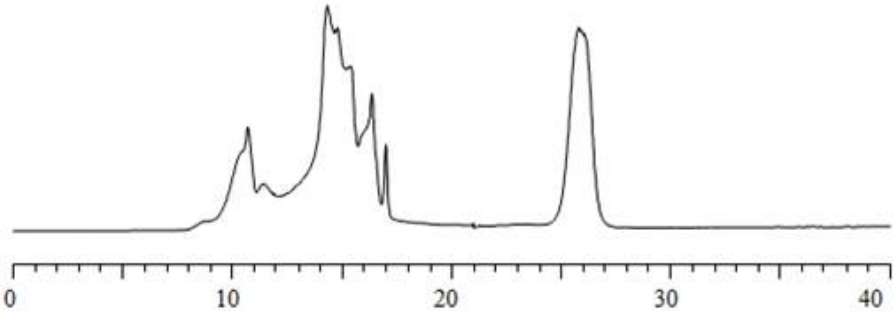

Figure 3: HPGPC-ELSD chromatogram of molecular weight distribution of PRP.

Table 4: Molecular weight distribution of PRP

Peak no.	Retention time (min)	Mw (g/mol)	Mn (g/mol)	Mw/Mn	Relative content (%)
1	11.221	445065	440830	1.010	15.83
2	12.118	329918	328425	1.004	0.66
3	18.514	176010	174895	1.006	24.43
4	19.757	119024	118561	1.004	0.13
5	20.140	99238	99156	1.001	1.56
6	26.627	13592	13454	1.010	57.38

DPPH radical scavenging effect of PRP

The results in Figure 4 showed that the antioxidant activity by DPPH radical scavenging assay. The highest scavenging rate of PRP was 71.5 % at a concentration of 10 mg/mL. The 50 % inhibitory concentration (IC_{50}) was calculated by modified Karber's method [23]. As a result, the IC_{50} of DPPH radical scavenging activity of PRP was 2.07 mg/mL, while the IC_{50} of Vc was 0.024 mg/mL.

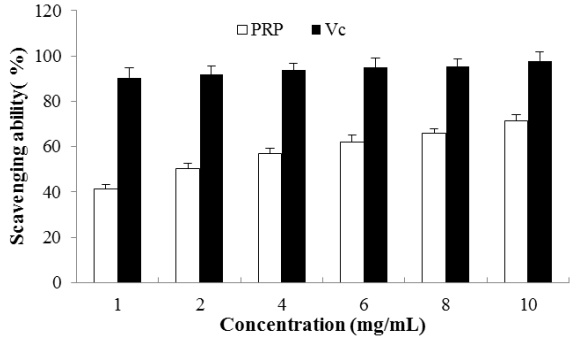

Figure 4: Scavenging effects of PRP on DPPH radicals with V_c as a positive control. Results are as mean value (n = 3)

Superoxide anion radical scavenging effect of PRP

The O^{2-} free radical scavenging rate of PRP was 87.5 % at the concentration of 10 mg/mL, while that of Vc was 98.7 %. The IC_{50} of O^{2-} free radical of PRP was 1.37 mg/mL, and that of Vc was 0.044 mg/mL, as shown in Figure 5. The results indicated that the O^{2-} scavenging ability of Vc was stronger than PRP.

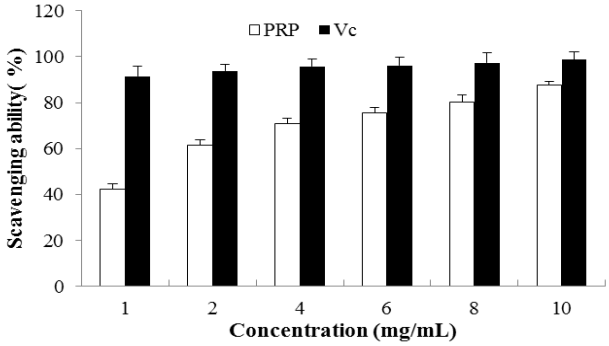

Figure 5: Scavenging effects of PRP on superoxide radicals with V_c as a positive control. Data are mean ± SD (n = 3)

DISCUSSION

Water extraction method is the most commonly used method for the extraction of plant polysaccharides [24]. Extraction variables have great effects on the extraction yield of polysaccharides. Therefore, it is necessary to optimize the extraction method in order to improve the yield of polysaccharides.

Studies [25,26] have indicated that RSM is an outstanding and effective statistical technique for complex optimization processes by reducing the number of experimental trials compared with traditional methods. Thus, it is widely used to optimize the extraction process of polysaccharides [27]. By employing RSM, the effects of factors on the extraction yield of PRP were achieved by quadratic regression design, and the reliability of the model was validated.

The results demonstrate that RSM is a rapid and effective statistical technique for PRP extraction.

Monosaccharide composition and molecular weight distribution are two important factors closely related to the activities of natural polysaccharides [28]. In the present study, chemical analysis indicated that the main monosaccharide components of PRP were Man, Gal acid, Glu, Gal, and Ara sugar with a molar ratio of 5.76:2.20:4.57:2.64:1, and the molecular weight ranged from 13592 to 445065.

It is reported that oxidative stress can result in cell death and tissue damage and cause a large number of diseases, such as cancer, inflammation and ageing, etc. [15]. Polysaccharides extracted from natural materials have been reported to have significant antioxidant activities based on large amounts of antioxidant assays *in vitro* and *in vivo* [29]. The present study indicates_that PRP has good antioxidant activities as shown by scavenging DPPH and scavenging superoxide anion radicals data.

CONCLUSION

RSM is a rapid and effective statistical technique for PRP extraction. Furthermore, PRP has good antioxidant activity, and thus, can potentially be developed as a source of natural antioxidants for the food and pharmaceutical industries.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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