Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.1590/fst.82221

Extraction, preparation and an assessment of the activity of carboxymethyl polysaccharide from *Panax japonicus*

Qi-Yang YU[1,2], Shuai YUAN[1,2], You-Yu YAN[2], Xi-Feng ZHANG[1]*

Abstract

A response surface method (RSM) was employed to optimize polysaccharide extraction from *Panax japonicus* (PJPS). Also, carboxymethyl substitution of PJPS (CM-PJPS) was studied using a response surface methodology. A three-variable Box–Behnken design (BBD) methodology was applied. RSM analyses revealed that conditions that maximized polysaccharide yield were as follows: a liquid-to-material ratio 22:1, an extraction temperature of 83.99 °C and an extraction duration of 2.32 h. Under optimum extraction conditions, the average absorbance value of PJPS was 0.6001, and the purity of PJPS was 98.2%. A quadratic regression model for CM-PJPS was obtained via BBD. The optimum extraction conditions for the process were determined to include a 1.38 h reaction time, 1.24 g monochloroacetic acid (MCA) and a reaction temperature of 52.85 °C. Under optimum extraction conditions, the average degree of CM-PJPS substitution was 0.9733. The structure of the PJPS was examined by means of Fourier transform infrared spectroscopy (FTIR). CM-PJPS exhibited the strongest anti-oxidant activity *in vitro*. In addition, CM-PJPS inhibited Skov3 and A2780 cancer cell proliferation. Our data revealed that CM-PJPS is a promising natural antioxidant with potential value as a food supplement and for treatment of cancer.

Keywords: *Panax japonicus*; polysaccharide; carboxymethylation; response surface; antioxidant activity.

Practical Application: The work shows a complete study about the method of polysaccharide extraction from *Panax japonicus* and the synthesized of carboxymethyl polysaccharide, and their contribution to show the potential value of carboxymethyl polysaccharide with natural antioxidant and anticancer.

1 Introduction

Panax japonicus C. A. Mey is a member of the *Araliaceae*. It is named for its rhizome-like bamboo knot. The plant is distributed mainly within Western Hubei. It is a variety included in the Chinese Pharmacopoeia and has widely been used in Chinese medicine (Tang & Eisenbrand, 1992). *P. japonicus* is of special importance to Miao medicine as a result of its capacity to nourish and strengthen, stop bleeding, dredge meridians, relax tendons, relieve pain, enhance circulation, remove blood stasis, and invigorate the spleen and stomach (CP-Chinese Pharmacopoeia, 2015). Polysaccharides from *P. japonicus* are important active ingredients of the plant and have strong immune regulatory and anti-fatigue effects (Shu et al., 2018). Domestic research on *P. japonicus* has mainly been focused on saponins, and has rarely involved the assessment of polysaccharides (Shin et al., 2015). Polysaccharides of *P. japonicus* are of global interest. These mainly consist of acidic heteropolysaccharides and dextran with complex structures consisting of galacturonic acid, galactose, rhamnose, arabinose and arabinose. After saponins, polysaccharides are believed to be the compounds that most greatly influence bioactivity of *P. japonicus* (Wang et al., 2012; Yang et al., 2014).

Polysaccharides are type of natural macromolecule in which aldose or ketose molecules are linked through a glycosidic bond. Polysaccharides are essential components of all living organisms, and are needed to maintain biological functioning (Liu et al., 2015; Xu, 2017). A large number of studies over the past decade have indicated that polysaccharides have a wide range of biological functions that include anti-cancer, anti-infection, anti-coagulation, anti-virus, and anti-radiation activities (Hetland 2003; Liu et al., 2015; Sohretoglu & Huang, 2018). The compounds also have been determined to regulate immune function, hypoglycemia, hypolipidemia, cell division and differentiation, cell growth and aging (Shu et al., 2018; Han et al., 2006; Willats et al., 1999; Ye et al., 2012). Uses of polysaccharides as drugs has very little toxicity. As a result of these findings, polysaccharide research has attracted great interest.

The pharmacological activities of polysaccharides are closely related to their structures. In order to identify polysaccharides with new or improved biological activities, researchers have altered the structures of polysaccharides. Modifications generally include adding hydroxyl, carboxyl and/or amino groups to polysaccharide residues and the formation of polysaccharide complexes (Fry et al., 2001; Charvet et al., 2011; Kadokawa et al., 1998). Each modification has a unique effect on the activity of a polysaccharide.

Received 03 Aug., 2021
Accepted 05 Sept., 2021
[1] *College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, People's Republic of China*
[2] *College of Biological and Pharmaceutical Engineering, Wuhan Polytechnic University, Wuhan, People's Republic of China*
*Corresponding author: zhangxf9465@163.com

In this study, water-soluble polysaccharides of *P. japonicus* from Enshi, Hubei Province, were extracted using water, precipitated using ethanol and carboxymethylated. Based on previous studies that assessed the feasibility of separation and purification of polysaccharides from *Panax japonicus*, carboxymethylation of the polysaccharides was optimized using a response surface methodology (RSM). Antioxidant and antineoplastic activities of carboxymethylated polysaccharides were preliminarily explored, and the effects of carboxymethylation modification of *P. japonicus* polysaccharide activity were investigated.

2. Materials and methods

2.1 Materials

P. japonicus plants were purchased from the Hubei Province of China. All chemicals and solvents were of analytical grade.

2.2 Preparation of P. japonicus polysaccharide (PJPS) extraction

The polysaccharides were extracted by water extraction and alcohol precipitation. The polysaccharide content was determined using sulfuric acid-phenol method with glucose functioning as a standard (Yao et al., 2020). Three factors affecting extraction conditions were categorized into three different levels, as specified in Supplemental Table 1. Single factor experiments were conducted to assess the effects of different extraction conditions on PJPS extraction efficiency, as follows: X_1(liquid-material ratio), X_2(extraction time) and X_3(extraction temperature). On the basis of single factor analysis, optimization of the three factors were determined, and a multi-factor response surface optimization test was carried out. Further optimization of the polysaccharide extraction process using a Box-Behnken central combination test was accomplished (Yang et al., 2016). After decompression and concentration, aqueous solutions were precipitated with 95% ethanol three times, and precipitates were re-dissolved, alcohol precipitated, and freeze dried to obtain crude PJPS.

Crude PJPS (about 80 mg) was re-dissolved in ultrapure water (4 mL), and a Cellulose DE-52 (DEAE) column (1.0 × 40 cm) was used to separate compounds. Gradient elution was carried out using 0, 0.05, 0.1, 0.2, and 0.3 mol/L NaC1 solution. Sugars were assessed using phenol sulfuric acid method and the ultraviolet absorbance was assessed at 490 nm. The major peak of polysaccharide corresponding to PJPS was concentrated and further purified using a 1.0 × 40 cm Sephadex G-100 column at a flow rate of 0.5 mL/min. PJPS fractions from the purified polysaccharide were analysed, concentrated and freeze-dried.

2.3 Carboxymethyl substitution of PJPS (CM-PJPS)

CM-PJPS was accomplished using a method described in Jiang et al. (Jiang et al., 2013). A proper amount of PJPS was dissolved in sodium hydroxide solution (2 Mol/L), stirred for 30 minutes, followed by the addition of 2g monochloroacetic acid. Then the mixture was stirred and refluxed at a certain temperature for a certain time. After the reaction was completed, it was cooled to room temperature and adjusted to pH 7 using hydrochloric acid. Further the reaction was placed in the dialysis bag and dialyzed for 48 h. After decompression and concentration, the dialysate was precipitated three times with 95% ethanol and dried to obtain CM-PJPS.

In Table 1, reaction time, MCA, and temperature were assigned to three different levels; A, B and C. The effects of A (reaction time), B (MCA) and C (reaction temperature) on the degree of polysaccharide carboxymethylation achieved were assessed using a single factor experiment. Based on single factor analysis, a response surface optimization value for each factor was determined. Carboxymethylation of polysaccharides was further optimized using the Box-Behnken central combination test.

2.4 Determination carboxymethyl substitution degree

The degree of carboxymethyl substitution achieved was determined as described by Zhao et al.(Zhao & Zhang 2015). Briefly, 10 mg CM-PJPS was added to 3 mL 70% ethanol, mixed and incubated 5 min. Next 10 mL distilled water and 50 mL 0.5 mol/L sodium hydroxide solution were mixed until all the samples had dissolved. The solution was then titrated using 0.1 mol/L hydrochloric acid solution and phenolphthalein served as a chromogenic agent. Degree of substitution (DS) was calculated using the following formulae 1 and 2:

$$A = \left[V_0 M_0 - \left(V_2 - V_1\right) M\right] / m \quad (1)$$

and

$$DS = 0.162A / \left(1 - 0.058A\right) \quad (2)$$

Where V_0 is the volume sodium hydroxide (mL); V_1 is the volume of hydrochloric acid consumed by blank determination (mL); V_2 is the volume of hydrochloric acid consumed by sample determination (mL); M_0 is the concentration of sodium hydroxide; M is the concentration of hydrochloric acid; and m is the quantity of the samples (g).

Table 1. Levels and code of extraction variables used in Box-Behnken design.

Variable	Symbols	Coded levels		
	Coded	-1	0	1
Reaction time (h)	A	1	2	3
MCA (g)	B	1	1.5	2
Reaction temperature (°C)	C	45	50	55

2.5 Characterization of PJPS and CM-PJPS

Ultraviolet spectrum

To determine the absorbance of PJPS and CM-PJPS, 0.1mg/ml solutions were scanned using an ultraviolet spectrophotometer at wavelengths that ranged from 200 to 600 nm.

FTIR analysis

The composition of dried PJPS and CM-PJPS was characterized via Fourier transform infrared spectroscopy (FTIR) from 400–4,000 cm^{-1}.

Scanning electron microscopy (SEM)

Conductive film was bonded to the sample base, and evenly sprinkled with a small amount of sample, then the sample that did not stick was blown lightly with ear wash balls, further, coated with a conductive film. The morphologies of samples were observed using 500, 1,000 and 2,000× magnification using field emission SEM (Yin et al., 2019).

Monosaccharide composition analysis and Determination of molecular weight

Monosaccharide composition of purified PJPS was analyzed using a Promosil C18 column (250mm × 4.6mm × 5μm). Mobile phase A consisted of 0.1 mol/L phosphate buffer saline (PBS) with pH 6.7, and phase B consisted of acetonitrile. The flow rate was 1 mL/min. The column temperature was 30 °C, the detection wavelength was 250 nm, and the injection volume was 20 μL. Monosaccharides were identified based on comparing their mass spectra with those in the Wiley mass spectral library, and the relative abundance of each monosaccharide was calculated using the peak area normalization method (Yao et al., 2020). The molecular weights of PJPS were measured using HPSEC-MALLS-RID according to previous reported methods (Wu et al., 2016; Cheong et al., 2015).

2.6 Antioxidant activity of PJPS and CM-PJPS

Hydroxyl radical scavenging activity

To determine hydroxyl radical scavenging activity, 500 μL of 2, 4, 6, 8, and 10 mg/mL samples were mixed with 250 μL $FeSO_4$ (9 mmol/L) and 500 μL salicylic acid–ethanol solution (9 mmol/L). Then 500 μL H_2O_2 was added and the solution was incubated at room temperature for 60 min. Ascorbic acid was used as positive control, while distilled water was used as a negative control. Finally, the absorbance was measured at 510 nm. Hydroxyl radical scavenging activity was calculated using the following Equation 3:

$$Scavenging\ rate\ (\%) = [1 - (A_1 - A_2) / A_0] \times 100 \quad (3)$$

Where A_0 is the absorbance of the control (water). A_1 is the absorbance of the tested samples, and A_2 is the absorbance of the samples to which water was added, rather than H_2O_2 solution (Yang et al., 2016).

Fe^{2+} chelating activity

Fe^{2+} chelating activity was determined by mixing 400 μL of different sample concentrations (2, 4, 6, 8, and 10 mg/mL) respectively with 40 μL $FeCl_2$ (5 mmol/L), 80 μL ferrozine solution (9 mmol/L) and 1,080 μL water. Ascorbic acid was used as a positive control, while distilled water was used as a negative control. The absorbance of the mixtures was measured at 562 nm. Fe^{2+} chelating activity was calculated using the following Equation 4:

$$Scavenging\ rate\ (\%) = [1 - (A_1 - A_2) / A_0] \times 100 \quad (4)$$

Where A_0 is the absorbance of the control group (water). A_1 is the absorbance of the test group, and A_2 is the absorbance of the samples mixed with water instead of $FeCl_2$ solution.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity

To determine ABTS radical scavenging activity, 2.5 mL 7 mmol/L ABTS diammonium salt was mixed with 0.5 mL 15 mmol/L potassium persulfate. After storage in the dark for 24 h, the solution was diluted with distilled water to yield a substance with an absorbance of 0.700 at 734 nm. The diluted solution (1,600 μL) was added to 400 μL of sample solutions (2, 4, 6, 8, and 10 mg/mL) respectively. Absorbance of each solution was measured at 734 nm after they were allowed to react for 10 min at room temperature. Ascorbic acid was used as positive control, while distilled water was used as the blank. The scavenging activity of the samples was calculated according to the following Equation 5:

$$Scavenging\ rate\ (\%) = [1 - (A_1 - A_2) / A_0] \times 100 \quad (5)$$

Where A_0 is the absorbance of the control group (water was added instead of sample). A_1 is the absorbance of the test group, and A_2 is the absorbance of the samples mixed with water instead of ABTS solution.

Superoxide anion scavenging activity

To determine superoxide anion scavenging activity, 300 μL of different sample concentrations (2, 4, 6, 8, and 10 mg/mL) were mixed respectively with 900 μL Tris-HCl buffer (pH 8.0) and 90μL pyrogallol, and then to which 300 μL HCl (10mol/L) was added, further, the solutions were incubated at room temperature for 20 min. Ascorbic acid was used as positive control, and distilled water was used as a blank. The absorbance of the mixtures was measured at 420 nm. Hydroxyl radical scavenging activity was calculated using the following Equation 6:

$$Scavenging\ rate\ (\%) = [1 - (A_1 - A_2) / A_0] \times 100 \quad (6)$$

Where A_0 indicates the absorbance of the control group (water). A_1 is the absorbance of the experimental samples, and A_2 is the absorbance of the samples mixed with absolute ethanol instead of pyrogallol.

2.7 Cytotoxicity of PJPS and CM-PJPS

The cytotoxicity of PJPS was calculated according to a method reported previously (Xing et al., 2018). Human ovarian carcinoma cells (Skov3 and A2780) were cultured in DME/F12 medium (Hyclone, Logan, UT, USA), together with supplemented with fetal bovine serum (10%) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C as well as 5% CO_2 in a humidified incubator. Cells were treated respectively with different concentrations of PJPS and CM-PJPS (0, 200, 400, and 800 μg/mL), and then incubated for 24 h. An MTT assay was used to evaluate cell proliferation as well as the cytotoxicity of PJPS and CM-PJPS. The experiment was repeated three times.

3 Results and discussion

3.1 Optimization of polysaccharide extraction conditions

Using the absorbance as an index for detection, the influence of each factor on extraction of PJPS was investigated. Specific experimental results determined using the (Box-Behnken design) BBD were shown in Supplemental Table 2. Using Design-Expert 8.0.6 software, results of Supplemental Table 2 were analyzed and the following quadratic regression Equation 7 was obtained:

$$Y = -2.14745 + 0.010448X_1 + 0.036760X_2 + 0.062164X_3 + 1.95000E-004X_1X_2 + 6.25000E - 005X_1X_3 + 1.01000E - 003X_2X_3 - 3.67150E-004X_1^2 - 0.027165X_2^2 - 3.92150E - 004X_3^2 \quad (7)$$

Design-Expert 8.0.6 software was used to further analyze the experimental results determined via BBD shown in Supplemental Table 3. From Supplemental Table 3, X_1, X_2, X_3, X_2X_3, X_1^2, X_2^2, X_3^2 significantly impacted the response value ($P < 0.01$), and X_1X_3 significantly impacted the response value ($P < 0.05$). The judgment coefficient value ($R^2 = 0.9932$) indicated that data correlated with the model and identified experimental factors that most greatly influenced the response value. The calibration coefficient of $R^2_{Adj} = 0.9845$ indicates that 98.45% of observed experimental data variability can be explained by this regression model. Coefficient of variation (CV) = 0.89%, which shows that the reliability and accuracy of the experiment was good, and precision was determined to be 28.098. The *F* value of the model was 113.64, which indicates that the model reached a significance level of P < 0.0001. In addition, lack of fit was 0.36 (P = 0.7878 > 0.05), which indicates that there is no significant relationship between the missing values and pure error. The regression model shown was used to guide further experiments, including determining the two-factor effect of the model. Results of these analyses were shown in Figure 1.

Analysis using Design Expert 8.0.6 showed that the optimum conditions for PSPF extraction included a ratio of liquid to material, 22:1, an extraction temperature of 83.99°C, and an extraction time of 2.32 h. Under these conditions, the predicted absorbance of the experimental sample was 0.6208. In order to verify the feasibility of the response surface results, optimal conditions were further optimized and validated. The optimal extraction conditions were applied to confirmative the predictive capacity of the model. An average value 0.6001was produced as a result of five independent laboratory experiments, and the standard deviation was determined to be 0.017. The relative error between experimental and predicted values was determined to be 3.4%, which indicated that the response surface results were reliable. Additionally, polysaccharide purity was 90%.

3.2 Optimization experiment of CM-PJPS

The optimum conditions for single factor test were determined to require a reaction time of 2 h, 1.5 g monochloroacetic acid and a reaction temperature of 50 °C. Using substitution degree as a detection index, the influence of the three factors on levels of *P. japonicus* polysaccharide carboxymethyl substitution was investigated. BBD factor level results were shown in Table 2.

Design-Expert 8.0.6 software was used to analyze results of Table 2. The following quadratic regression Equation 8 for three variables was obtained:

$$Y = -24.92896 + 2.10369A + 4.00334B + 0.83105C + 0.20370AB - 0.036045AC - 0.045020BC - 0.16391A2 - 0.76553B2 - 6.86230E - 003C2 \quad (8)$$

Experimental results were further analyzed using Design-Expert 8.0.6 software. The results were shown in Table 3.

Results of Table 3 indicated that A, B, C, AB, AC, BC, A^2, B^2, C^2 significantly impacted the response value ($P < 0.01$). The judgment coefficient ($R^2 = 0.9992$) showed that the model was reliable and experimental factors influenced the response value. The calibration coefficient of $R^2_{Adj} = 0.9982$ indicated that 99.82% of experimental data variability could be explained by this regression model. CV = 1.33% indicated that the reliability and accuracy of the model was good, and precision was determined to be 77.180.

The *F* value of the model was 968.53, which indicated that the model reached a significant level ($P < 0.0001$). In addition, the Lack of fit was determined to be 6.13 ($P = 0.0562 > 0.05$), which showed that there was no significant relationship between the missing value and pure error. Since the regression model was determined to be able to guide the experiment, further analysis of the two-factor effect of the model was carried out, and results were shown in Figure 2.

Design Expert 8.0.6 analysis revealed that the optimal conditions of polysaccharide extraction included a reaction duration of 1.38 h, 1.24 g monochloroacetic acid and a reaction temperature of 52.85 °C. Under these conditions, the predicted degree of substitution was determined to be 0.9723. In order to verify the feasibility of response surface results, the optimal conditions were optimized and validated. The average value determined after six repeated experiments was 0.9733 and the standard deviation was 0.0073. These results revealed that the experimental results were stable under conditions, and the relative error with the predicted value was 0.10%, indicating that the results of the response surface were reliable.

The activity of polysaccharides is directly or indirectly affected by their molecular structure. The structure of polysaccharides includes primary structure, secondary structure, tertiary structure

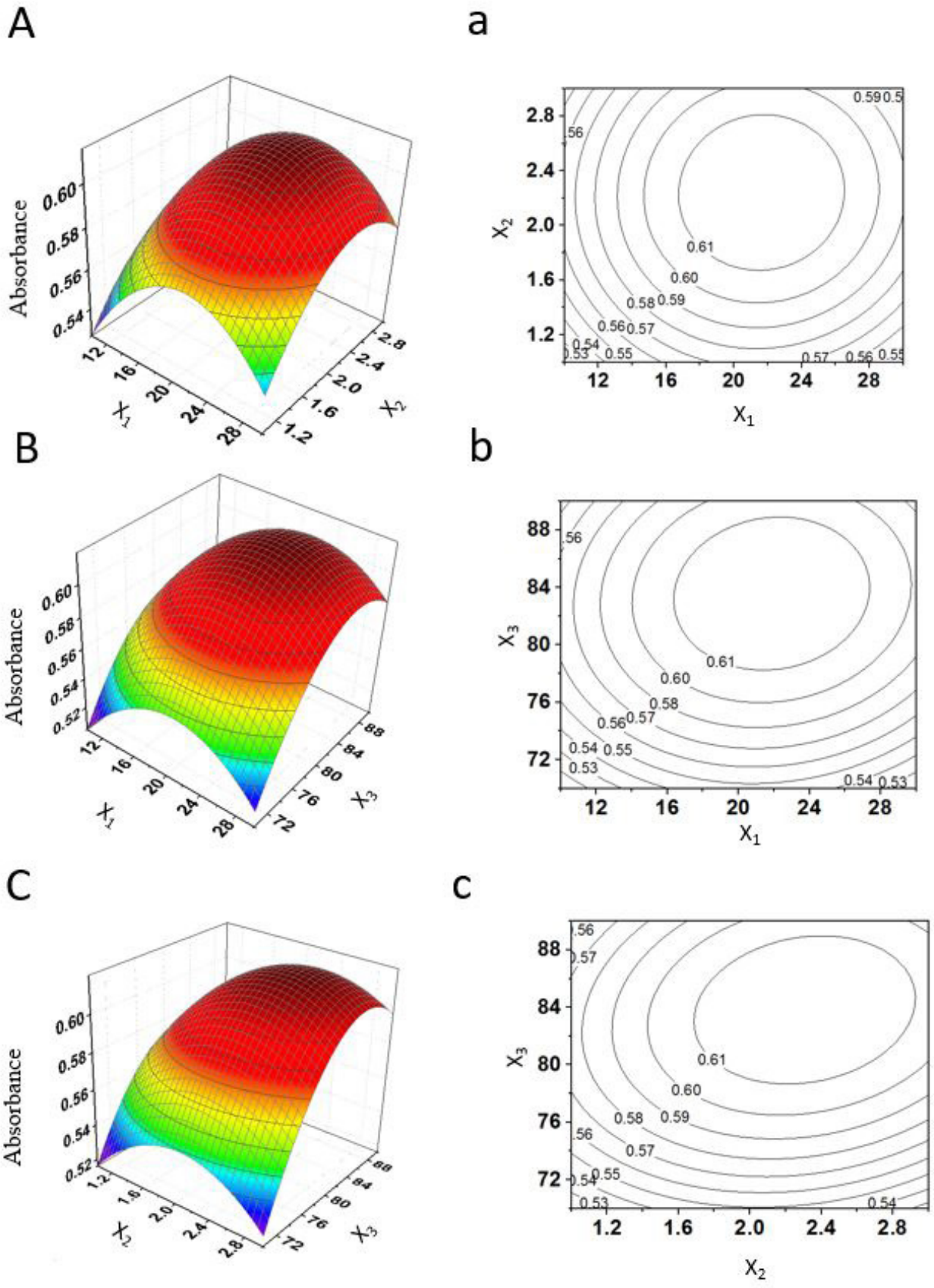

Figure 1. Response surface plots showing effect of variables on PJPS yield. Effects of (X_1) liquid-material ratio, (X_2) extraction time, h, and (X_3) extraction temperature (°C) on PJPS extraction yield are shown.

Table 2. Experimental Design and Results of CM-PJPS Box-Behnken.

Run	A	B	C	Degree of substitution (DS)
1	3	1.5	55	0.3887
2	2	1	45	0.4900
3	2	1.5	50	0.9374
4	3	1	50	0.4952
5	2	1.5	50	0.9240
6	1	1.5	55	0.8427
7	2	1.5	50	0.9346
8	2	2	45	0.5824
9	2	1.5	50	0.9340
10	1	1.5	45	0.4446
11	3	2	50	0.5579
12	2	2	55	0.4237
13	2	1	55	0.7815
14	2	1.5	50	0.9317
15	1	2	50	0.4552
16	1	1	50	0.7999
17	3	1.5	45	0.7115

Table 3. Analysis of variance of the experimental results of the CM-PJPS.

Variables	Sum of squares	df	Mean square	F-value	p -Value
model	0.72	9	0.080	968.53	< 0.0001
A	0.019	1	0.019	228.83	< 0.0001
B	0.037	1	0.037	452.90	< 0.0001
C	5.413E-03	1	5.413E-003	65.45	< 0.0001
AB	0.041	1	0.041	501.73	< 0.0001
AC	0.13	1	0.13	1571.00	< 0.0001
BC	0.051	1	0.051	612.69	< 0.0001
A^2	0.11	1	0.11	1367.80	< 0.0001
B^2	0.15	1	0.15	1864.78	< 0.0001
C^2	0.12	1	0.12	1498.45	< 0.0001
Residual	5.789E-04	7	3.494E-05		
Lack of fit	4.755E-04	3	4.705E-05	6.13	0.0562
Pure error	1.034E-04	4	2.586E-05		
Correlation total	0.72	16			
$R^2 = 0.9992$	$R^2_{Adj} = 0.9982$	$R^2_{Pred} = 0.9892$	CV = 1.33%		

p:Significance test; CV: Coefficient of variation; F: F test; df: Degree of Freedom.

and quaternary structure. The activity of polysaccharides can be changed by modifying its molecular structure. Molecular modification can affect the activity of polysaccharides by changing their spatial structure, molecular weight, substituent type, number and position (Getachew & Chun, 2017; Xie et al., 2020). There are many methods for the modification of polysaccharides, including physical method, chemical method and biological method (Liu et al., 2018; Xie et al., 2020; Zhu et al., 2019). In addition to the modification of the main chain, the branched chain of polysaccharides can also be modified (Lee et al., 2017; Xiao et al., 2020). The common methods of modification of polysaccharides or oligosaccharides include acidification, phosphorylation, acetylation, alkylation, sulfonyl formation, carboxymethylation, etc (Cai et al., 2018; Xu et al., 2019). After molecular modification, the bioactivity of polysaccharides has been improved, and even new functions have been produced (Huang & Huang 2017).

3.3 Analyze the characterization of PJPS and CM-PJPS

Separation and purification of PJPS

As shown in Figure 3A and B, crude PJPS was purified with DE-52, revealing five peaks. The major peak (neutral polysaccharide) was corresponded to PJPS (Figure 3A). Then, PJPS was further purified using SephadexG-100 column chromatography and eluted using deionised water. PJPS was

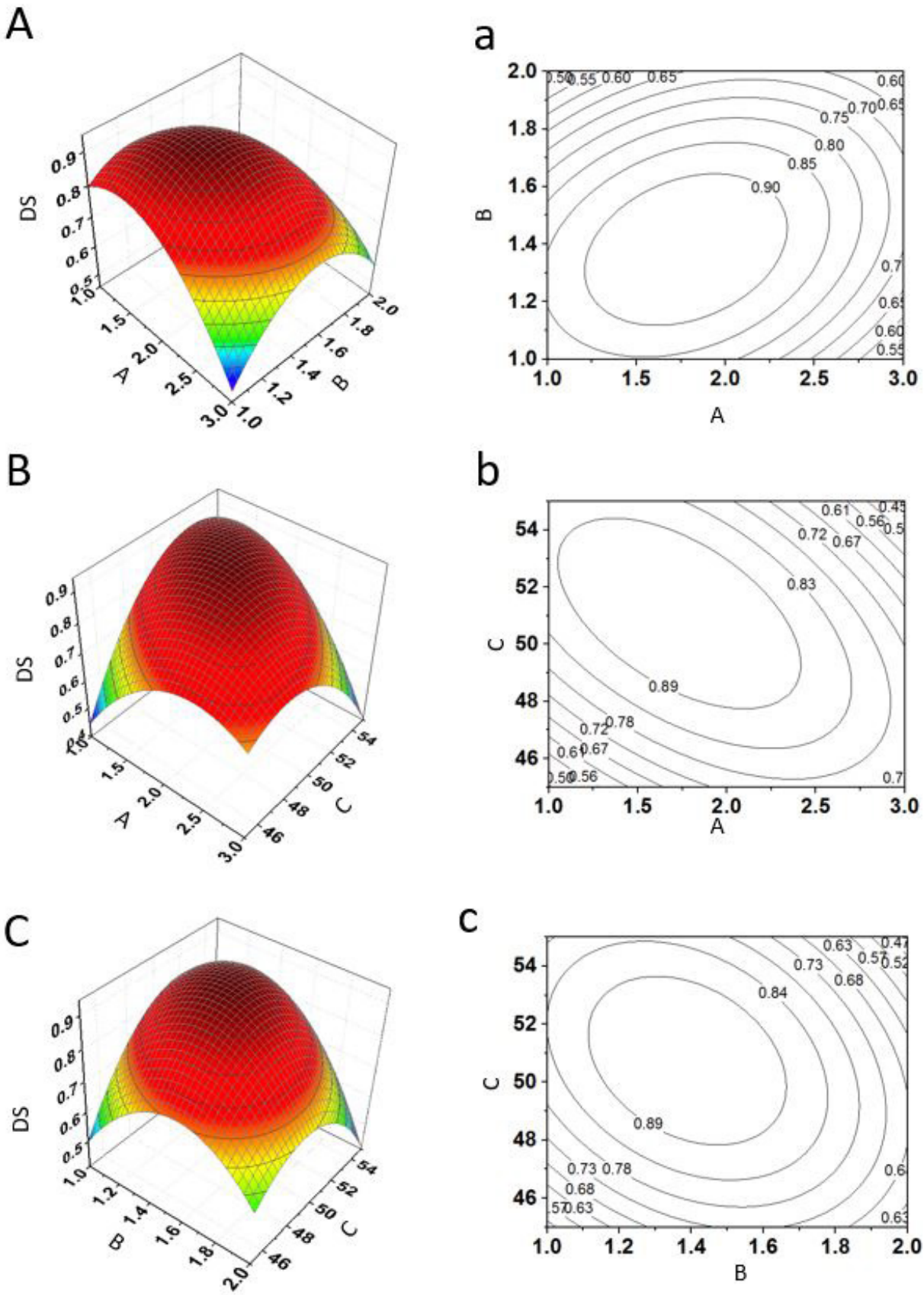

Figure 2. Response surface plots showing the effect on CM-PJPS. Effects of (A) reaction time; (B) MCA (g) and (C) reaction temperature (°C) on the degree of substitution of CM-PJPS.

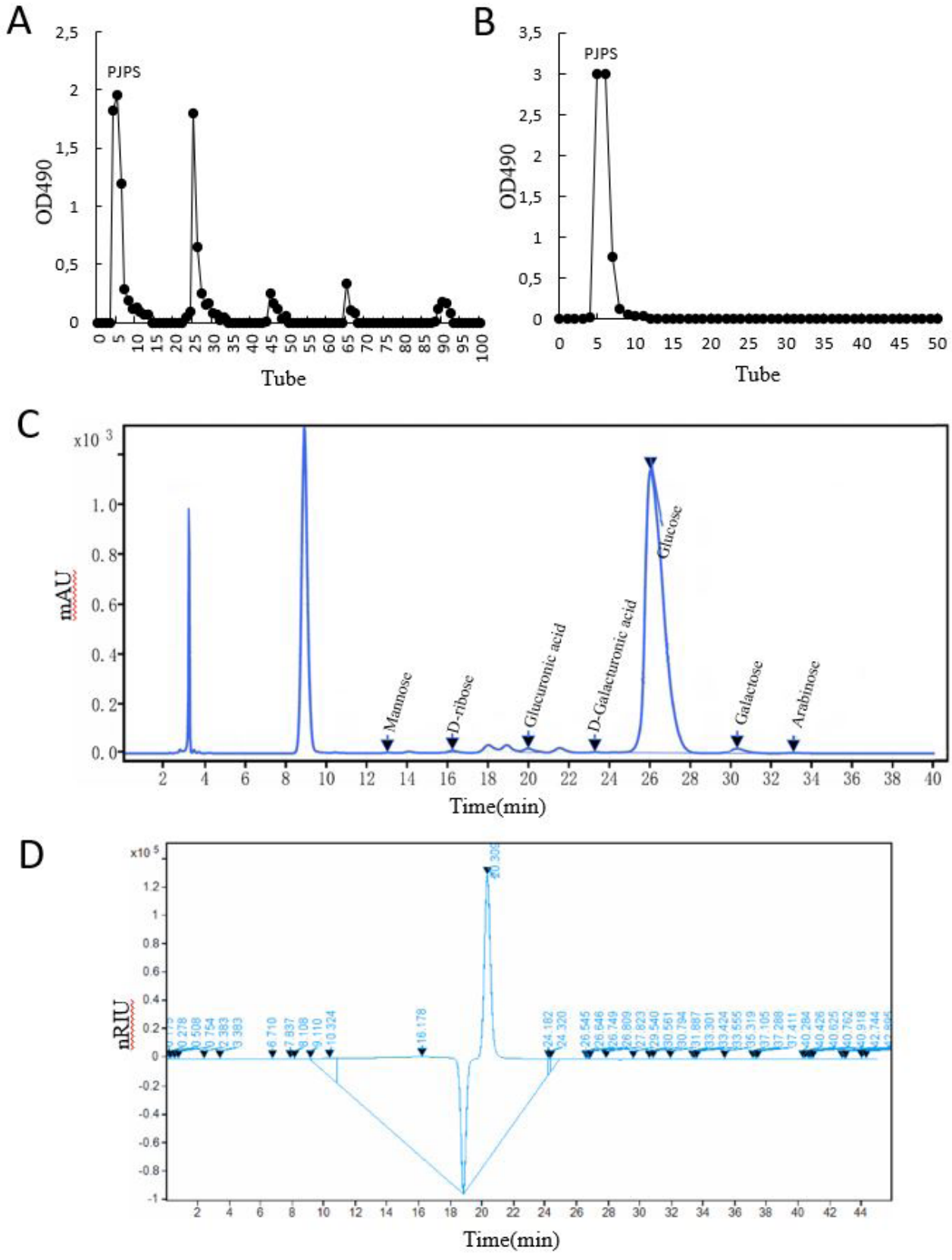

Figure 3. Isolation and analysis of PJPS. (A) Elution curve of PJPS using the DEAE-52 ion-exchange column. The eluent was H_2O and NaCl (0.1, 0.2, 0.3 and 0.5 mol/L) with a flow rate of 0.5 mL/min (10 min/tube); (B) Elution curves of PJPS using the Sephadex column. The eluent was H_2O with a flow rate of 0.4 mL/min (20 min/tube); (C) Monosaccharide composition of PJPS; (D) GPC chromatogram of PJPS for molecular weight determination with HPSEC-MALLS-RID system.

unimodal, and peaks were symmetrical, which indicated that the sample was relatively uniform (Figure 3B).

Monosaccharide composition and Molecular weight of PJPS

As shown in Figure 3C, the monosaccharide composition of PJPS included Mannose (0.08%), D-ribose (0.53%), Glucuronic acid (1.03%), D-Galacturonic acid (0.14%), Glucose (96.99%) and Galactose (1.23%). Differences observed may have been caused by extraction method differences (ultrafiltration or hot water extraction) that affected peak area. The molecular weight of PJPS was determined by HPSEC-MALLS-RID. The weight average molecular weight (Mw) was 251890 g/mol and the number average molecular weight (Mn) was 10240 g/mol (Figure 3D).

UV absorption

As shown in the Figure 4A, UV absorption of PJPS and CM-PJPS (DS of H-CM-PJPS and M-CM-PJPS were 0.97 and 0.78, respectively) were observed from 200–600 nm, which indicated that modification did not significantly affect polysaccharide absorption.

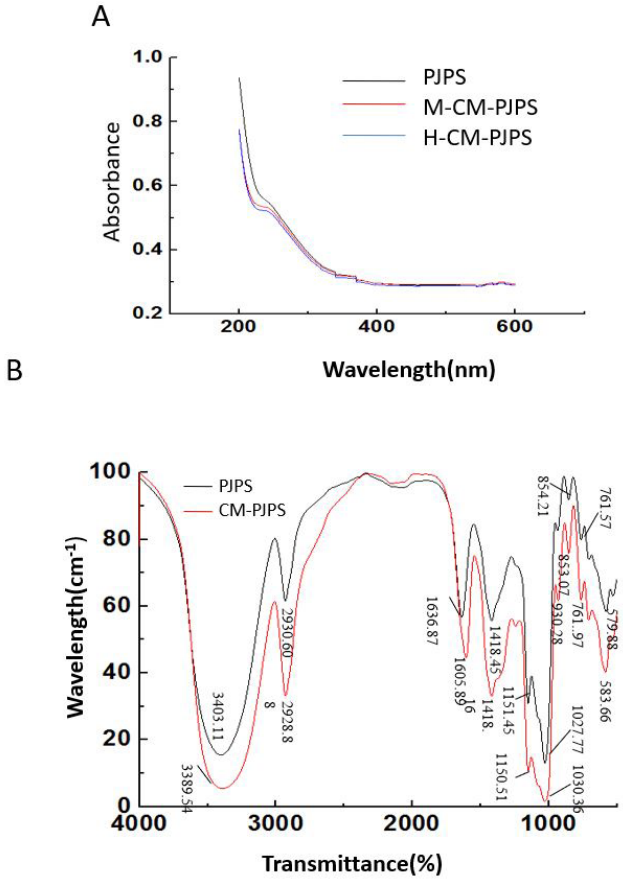

Figure 4. Ultraviolet spectroscopy and the FT-IR spectra of PJPS and CM-PJPS. (A) Ultraviolet spectra of PJPS and CM-PJPS; (B) FT-IR spectra of PJPS and CM-PJPS.

FTIR spectrophotometric analysis

As shown in Figure 4B, polysaccharide peaks occurred at 3407 cm^{-1} (O-H stretching vibration) and 2930 cm^{-1} (C-H stretching vibration). Peaks at 1605 cm^{-1} and 1418 cm^{-1}, which were characteristic of asymmetric and symmetric stretching vibrations of -COO-, respectively, indicated that the carboxymethyl had been successfully attached to the polysaccharide chain.

Scanning electron microscopy

Scanning electron microscopy (SEM) is an effective method for assessing polysaccharide morphology. The method involves assessing interactions between an electron beam and sample, producing a high-resolution, three-dimensional image of a sample. Generally, it is used to identify the surface structure of samples. Figure 5 shows that PJPS is porous, middle degree of substitution of CM-PJPS(M-CM-PJPS) is loosely porous, and high degree of substitution of CM-PJPS(H-CM-PJPS) forms flaky aggregation, which appears even more loosely packed than M-CM-PJPS.

3.4 Antioxidant analysis

As shown in Figure 6, CM-PJPS has hydroxyl radical, metal ion radical, ABTS radical and superoxide radical ion scavenging activity. After carboxymethyl modification, the antioxidant capacity of PJPS significantly improved. However, the hydroxyl radical scavenging activity of unmodified polysaccharide was also strong. The removal rate of metal ions by polysaccharides of H-CM-PJPS was significantly higher than those of M-CM-PJPS and PJPS, and the maximum removal rate was determined to be 56.90%, while the positive ascorbic acid control possessed no significant removal rate. The free radical scavenging rate of modified PJPS was significantly higher than that of unmodified PJPS, and the maximum scavenging rate was 73.26%, which was lower than that of the ascorbic acid positive control. Modified polysaccharide superoxide radical ion scavenging rates were significantly higher than those of unmodified polysaccharides. The maximum scavenging rate was determined to be 72.73%, which was less than that of ascorbic acid (positive control).

Polysaccharides are a kind of important natural product resources. Extensive studies have shown that active polysaccharides have a variety of biological activities, including antioxidant, anti-inflammatory, hypoglycemic, hypolipidemic and so on. Researchers at home and abroad are very concerned about the antioxidant capacity of active polysaccharides, especially in vitro antioxidant activity, and have published a large number of studied results (Chen et al., 2009; Zhao et al., 2014; Pereira et al., 2012). The antioxidant mechanism of antioxidants can be summarized as: elimination of active free radicals, elimination of inactive oxidation factors, combination of metal ions and so on. There are many factors affecting the antioxidant activity of polysaccharides, which may include the source, chemical structure, molecular weight, purity and spatial structure of polysaccharides (Huang et al., 2017; Mei et al., 2017; Mirzadeh et al., 2020).

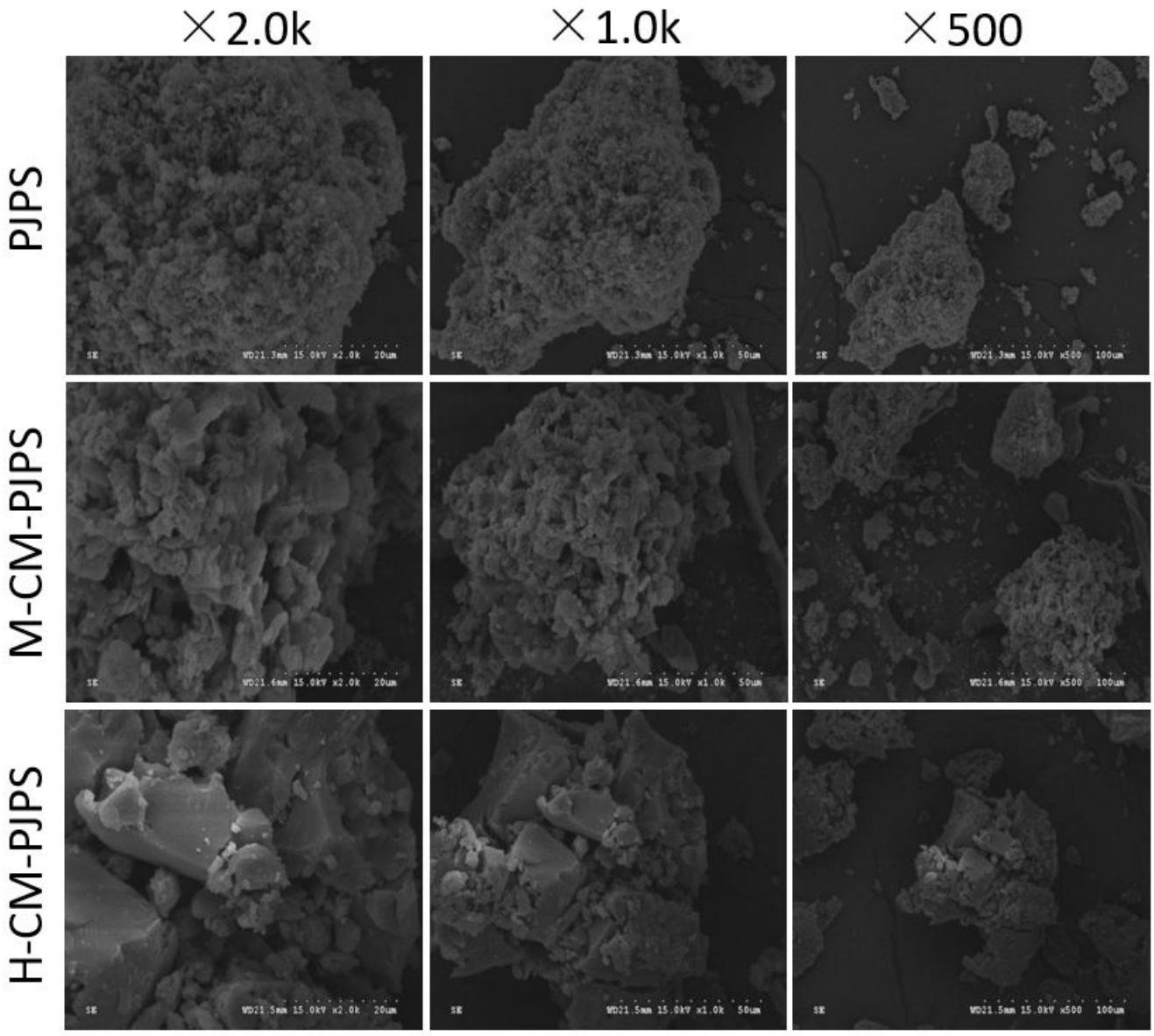

Figure 5. Morphology of PJPS and CM-PJPS observed by SEM at different magnifications.

3.5 Analysis of anticancer results

Data from Figure 7 indicated that PJPS did not inhibit A2780 and Skov3 tumor cell survival after modification, the inhibitory effect of polysaccharides on tumor cell survival was significantly enhanced. As concentration increased the inhibitory effect of H-CM-PJPS was enhanced relative to that of M-CM-PJPS. The A2780 tumor cell survival was 66.15%. The lowest survival rate of Skov 3 cancer cells was 76.08%.

Polysaccharide has a wide range of biological functions. It can not only be used as energy supply material and basic components of some substances in the body, but also participates in the process of intercellular recognition, regulation of immune energy supply, transportation of intercellular substances, cell transformation, apoptosis of tumor cells and so on. Among which the research on the antitumor activity of polysaccharides has attracted the most attention, and polysaccharides has been applied in clinical practice, such as lentinan, Polyporus umbellatus polysaccharides, Ganoderma lucidum polysaccharides, Coriolus versicolor polysaccharides, astragalus polysaccharides, etc (Li et al., 2020; Sohretoglu & Huang 2018; Zhang & Zhao 2019). Polysaccharides can directly kill tumor cells by inducing differentiation or apoptosis of tumor cells and affecting oncogene expression, or improve host anti-tumor immune function by activating macrophages, activating lymphocytes, promoting cytokine secretion and activating complement, so as to inhibit tumor and play a better anti-tumor effect (Fu et al., 2014; Liu et al., 2019).

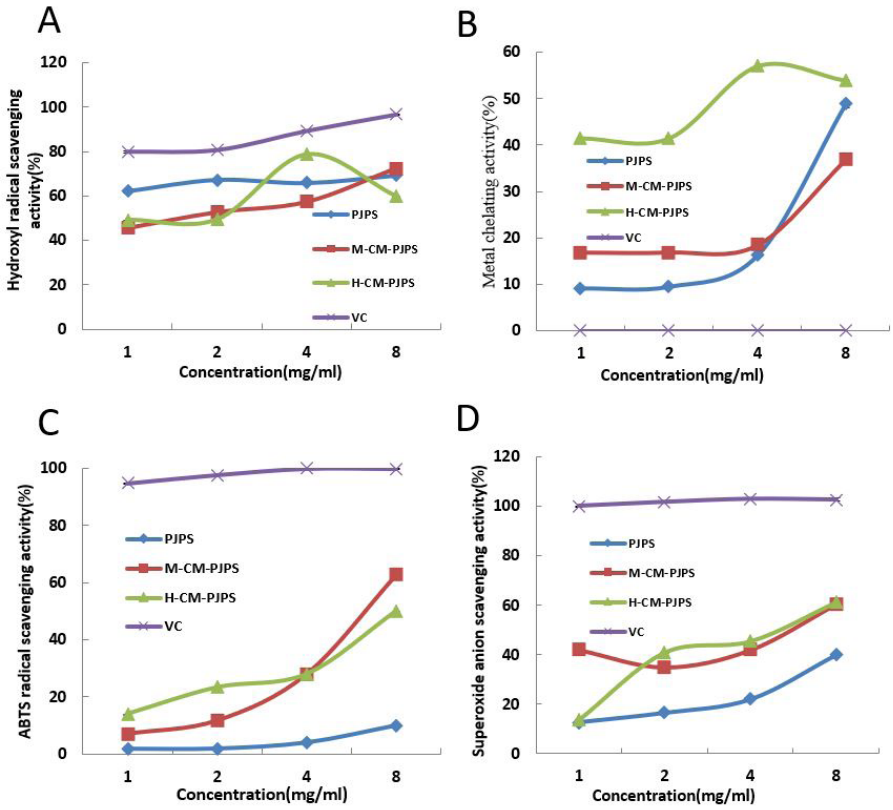

Figure 6. Antioxidant capacity of PJPS and CM-PJPS. (A) Scavenging of hydroxyl radicals by PJPS and CM-PJPS; (B) Metal chelating activity of PJPS and CM-PJPS; (C) Scavenging of ABTS radicals by PJPS and CM-PJPS; (D) Scavenging of superoxide anions by PJPS and CM-PJPS.

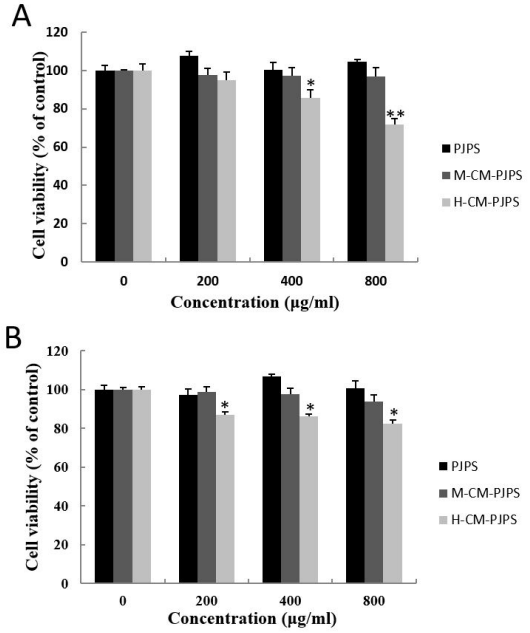

Figure 7. Effect of PJPS and CM-PJPS on cellular viability and morphology. Skov3 and A2780 cells were incubated with different PJPS and CM-PJPS concentrations for 24 h, and cell viability was determined using a CCK-8 assay. (A) Viability and morphological features of A2780 cells are shown; (B) Viability and morphological features of Skov3 cells are shown. Data are expressed as the mean ± SD of three independent experiments (*P < 0.05, **P < 0.01). Abbreviations: CCK, cell counting kit-8.

4 Conclusion

A single factor experiment revealed that optimal PJPS extraction conditions established using RSM contained a liquid to material ratio, 22:1, an extraction temperature of 83.99 °C, and an extraction duration of 2.32 h. Under these conditions, the purity of polysaccharide reached 90%. Single factor experiments with RSM also were used to determine optimal conditions for CM-PJPS. To produce optimal levels of CM-PJPS, a reaction duration of 1.38 h, 1.24 g MCA and a temperature of 52.85°C was determined. Under these conditions, the degree of substitution of polysaccharides reached 97%. After CM-PJPS, antioxidant capacity and anticancer activity of polysaccharides were significantly improved.

Conflict of interest

The authors have declared that no competing interest exists.

Availability of data and material

The data used to support the findings of this study are included within the article.

Author contributions

Zhang XF designed the study. Yu QY, Yuan S, Yan YY and Zhang XF collected data. All authors agreed the final version.

Acknowledgements

This work was supported by High level talents research fund project of Qingdao Agricultural University in China (1120043) and Research start up project of Wuhan Polytechnic University (2017257).

References

Cai, Y., Chen, P., Wu, C., Duan, J., & Bai, H. (2018). Sulfated modification and biological activities of polysaccharides derived from *Zizyphus jujuba cv*. Jinchangzao. *International Journal of Biological Macromolecules*, 120(Pt A):1149-1155. http://dx.doi.org/10.1016/j.ijbiomac.2018.08.141.

Charvet, R., Soula, R., & Soula, O., inventors; ADOCIA, LYON (FR), assignee. (2011). Polysaccharides comprising carboxyl functional groups substituted via esterification by a hydrophobic alcohol. US 20110112039 A1.

Chen, X., Liu, Y., Bai, X., Wen, L., Fang, J., Ye, M., & Chen, J. (2009). Hypoglycemic Polysaccharides from the Tuberous Root of Liriope spicata. *Journal of Natural Products*, 72(11), 1988-1992. http://dx.doi.org/10.1021/np900346d. PMid:19874043.

Cheong, K. L., Wu, D. T., Zhao, J., & Li, S. P. (2015). A rapid and accurate method for thequantitative estimation of natural polysaccharides and their fractions usinghigh performance size exclusion chromatography coupled with multi-anglelaser light scattering and refractive index detector. *Journal of Chromatography. A*, 1400, 98-106. http://dx.doi.org/10.1016/j.chroma.2015.04.054. PMid:25990349.

CP-Chinese Pharmacopoeia. (2015). *National Pharmacopoeia Committee of China* (pp. 138-139). Beijing*:* China Medical Science and Technology Press.

Fry, S. C., Dumville, J. C., & Miller, J. G. (2001). Fingerprinting of polysaccharides attacked by hydroxyl radicals in vitro and in the cell walls of ripening pear fruit. *The Biochemical Journal*, 357(Pt 3), 729-737. http://dx.doi.org/10.1042/bj3570729. PMid:11463343.

Fu, J., Wang, Z., Huang, L., Zheng, S., Wang, D., Chen, S., Zhang, H., & Yang, S. (2014). Review of the botanical characteristics, phytochemistry, and pharmacology of Astragalus membranaceus (Huangqi). *Phytotherapy Research*, 28(9), 1275-1283. http://dx.doi.org/10.1002/ptr.5188. PMid:25087616.

Getachew, A. T., & Chun, B. S. (2017). Molecular modification of native coffee polysaccharide using subcritical water treatment: structural characterization, antioxidant, and DNA protecting activities. *International Journal of Biological Macromolecules*, 99, 555-562. http://dx.doi.org/10.1016/j.ijbiomac.2017.03.034. PMid:28283450.

Han, C. R., Ma, Y. Q., & Tang, J. (2006). Extraction of polysaccharide from Auricularia auricular and its hypoglycemia activity(Chinese). *Journal of Food Science and Biotechnology*, 25, 111-114.

Hetland, G. (2003). Anti-Infective Action of Immuno-modulating Polysaccharides (β-Glucan and Plantago Major L. Pectin) against Intracellular (Mycobacteria sp.) and Extracellular (Streptococcus Pneumoniae sp.) Respiratory Pathogens. *Current Medicinal Chemistry - Anti-Infective Agents*, 2(2), 135-146. http://dx.doi.org/10.2174/1568012033483123.

Huang, G., & Huang, H. (2017). The derivatization and antitumor mechanisms of polysaccharides. *Future Medicinal Chemistry*, 9(16), 1931-1938. http://dx.doi.org/10.4155/fmc-2017-0132. PMid:29076350.

Huang, G., Mei, X., & Hu, L. (2017). The antioxidant activities of natural polysaccharides. *Current Drug Targets*, 18(11), 1296-1300. http://dx.doi.org/10.2174/1389450118666170123145357. PMid:28117001.

Jiang, L. M., Fan, C. M., Nie, S. P., Zhou, H. L., Li, J. Y., & Xie, M. Y. (2013). Preparation and Immunoregulatory Activity of Carboxymethyl Polysaccharide from the Seeds of Plantago asiatica. *Shipin Kexue*, 34, 10-14. http://dx.doi.org/10.7506/spkx1002-6630-201322003.

Kadokawa, J. I., Sato, M., Karasu, M., Tagaya, H., & Chiba, K. (1998). Synthesis of Hyperbranched Aminopolysaccharides. *Angewandte Chemie*, 37(17), 2373-2376. http://dx.doi.org/10.1002/(SICI)1521-3773(19980918)37:17<2373::AID-ANIE2373>3.0.CO;2-B.

Lee, J. H., Lee, Y. K., & Chang, Y. H. (2017). Effects of selenylation modification on structural and antioxidant properties of pectic polysaccharides extracted from *Ulmus pumila* L. *International Journal of Biological Macromolecules*, 104(Pt A):1124-1132. http://dx.doi.org/10.1016/j.ijbiomac.2017.06.121.

Li, W., Hu, X., Wang, S., Jiao, Z., Sun, T., Liu, T., & Song, K. (2020). Characterization and anti-tumor bioactivity of astragalus polysaccharides by immunomodulation. *International Journal of Biological Macromolecules*, 145, 985-997. http://dx.doi.org/10.1016/j.ijbiomac.2019.09.189. PMid:31669273.

Liu, J., Willför, S., & Xu, C. L. (2015). A review of bioactive plant polysaccharides: Biological activities, functionalization, and biomedical applications. *Bioactive Carbohydrates and Dietary Fibre*, 5(1), 31-61. http://dx.doi.org/10.1016/j.bcdf.2014.12.001.

Liu, S., Zhuang, X., Zhang, X., Han, W., Liu, Y., Sun, D., & Guo, W. (2018). Enzymatic modification of rice bran polysaccharides by enzymes from grifola frondosa: natural killer cell cytotoxicity and antioxidant activity. *Journal of Food Science*, 83(7), 1948-1955. http://dx.doi.org/10.1111/1750-3841.14148. PMid:29939404.

Liu, Z., Gao, T., Yang, Y., Meng, F., Zhan, F., Jiang, Q., & Sun, X. (2019). Anti-cancer activity of porphyran and carrageenan from red seaweeds. *Molecules (Basel, Switzerland)*, 24(23), 4286. http://dx.doi.org/10.3390/molecules24234286. PMid:31775255.

Mei, X., Yi, C. K., & Huang, G. L. (2017). Preparation methods and antioxidant activities of polysaccharides and their derivatives. *Mini-Reviews in Medicinal Chemistry*, 17(10), 863-868. http://dx.doi.org/10.2174/1389557517666170116114657. PMid:28093971.

Mirzadeh, M., Arianejad, M. R., & Khedmat, L. (2020). Antioxidant, antiradical, and antimicrobial activities of polysaccharides obtained by microwave-assisted extraction method: a review. *Carbohydrate Polymers*, 229, 115421. http://dx.doi.org/10.1016/j.carbpol.2019.115421. PMid:31826454.

Pereira, L. P., da Silva, K. E. S., da Silva, R. O., Assreuy, A. M. S., & Pereira, M. G. (2012). Anti-inflammatory polysaccharides of Azadirachta indica seed tegument. *Revista Brasileira de Farmacognosia*, 22(3), 617-622. http://dx.doi.org/10.1590/S0102-695X2012005000031.

Shin, B. K., Kwon, S. W., & Park, J. H. (2015). Chemical diversity of ginseng saponins from Panax ginseng. *Journal of Ginseng Research*, 39(4), 287-298. http://dx.doi.org/10.1016/j.jgr.2014.12.005. PMid:26869820.

Shu, G., Jiang, S., Mu, J., Yu, H., Duan, H., & Deng, X. (2018). Antitumor immunostimulatory activity of polysaccharides from Panax japonicus C. A. Mey: roles of their effects on CD4+ T cells and tumor associated macrophages. *International Journal of Biological Macromolecules*, 111, 111430-111439. http://dx.doi.org/10.1016/j.ijbiomac.2018.01.011. PMid:29317237.

Sohretoglu, D., & Huang, S. L. (2018). Ganoderma lucidum Polysaccharides as an Anti-cancer Agent. *Anti-Cancer Agents in Medicinal Chemistry*, 18(5), 667-674. http://dx.doi.org/10.2174/1871520617666171113121246.

Tang, W. C., & Eisenbrand T. G. (1992). Panax japonicus C. A. Mey. In W. C. Tang & T. G. Eisenbrand. *Chinese drugs of plant origin* (pp. 739-743). Berlin: Springer-Verlag Berlin Heidelberg.

Wang, R. F., Chen, P., Jia, F., Tang, J., Ma, F., & Xu, B. (2012). Characterization and antioxidant activities of polysaccharides from Panax japonicus

C. A. Meyer. *Carbohydrate Polymers*, 88(4), 1402-1406. http://dx.doi.org/10.1016/j.carbpol.2012.02.026.

Willats, W. G., Steele-King, C. G., Marcus, S. E., & Knox, J. P. (1999). Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. *The Plant Journal*, 20(6), 619-628. http://dx.doi.org/10.1046/j.1365-313X.1999.00629.x. PMid:10652134.

Wu, D. T., Lam, S. C., Cheong, K. L., Wei, F., Lin, P. C., Long, Z. R., Lv, X. J., Zhao, J., Ma, S. C., & Li, S. P. (2016). Simultaneous determination of molecular weights and contents of water-soluble polysaccharides and their fractions from *Lycium barbarum* collected in China. *Journal of Pharmaceutical and Biomedical Analysis*, 129, 210-218. http://dx.doi.org/10.1016/j.jpba.2016.07.005. PMid:27429371.

Xiao, Z., Zhou, W., & Zhang, Y. (2020). Fungal polysaccharides. *Advances in Pharmacology*, 87, 277-299. http://dx.doi.org/10.1016/bs.apha.2019.08.003. PMid:32089236.

Xie, L., Shen, M., Hong, Y., Ye, H., Huang, L., & Xie, J. (2020). Chemical modifications of polysaccharides and their anti-tumor activities. *Carbohydrate Polymers*, 229, 115436. http://dx.doi.org/10.1016/j.carbpol.2019.115436. PMid:31826393.

Xing, S., Zhang, X., Ke, H., Lin, J., Huang, Y., & Wei, G. (2018). Correction to: physicochemical properties of polysaccharides from Dendrobium officinale by fractional precipitation and their preliminary antioxidant and anti-HepG2 cells activities in vitro. *Chemistry Central Journal*, 12(1), 103. http://dx.doi.org/10.1186/s13065-018-0471-9. PMid:30328010.

Xu, Y. (2017). Hierarchical materials. In R. Xu & Y. Xu. (Eds.), *Modern inorganic synthetic chemistry* (2nd ed., pp. 545-574). USA: Elsevier. http://dx.doi.org/10.1016/B978-0-444-63591-4.00019-7.

Xu, Y., Wu, Y. J., Sun, P. L., Zhang, F. M., Linhardt, R. J., & Zhang, A. Q. (2019). Chemically modified polysaccharides: synthesis, characterization, structure activity relationships of action. *International Journal of Biological Macromolecules*, 132, 970-977. http://dx.doi.org/10.1016/j.ijbiomac.2019.03.213. PMid:30965077.

Yang, T., Zhang, S., Wang, R., Li, D., Hu, Y., Nie, J., Zhao, X., Wang, Q., Chen, Y., Zheng, Y., & Chen, P. (2016). Polysaccharides from Rhizoma Panacis Majoris and its anti-oxidant activity. *International Journal of Biological Macromolecules*, 86, 756-763. http://dx.doi.org/10.1016/j.ijbiomac.2016.01.091. PMid:26826292.

Yang, X., Wang, R., Zhang, S., Zhu, W., Tang, J., Liu, J., Chen, P., Zhang, D., Ye, W., & Zheng, Y. (2014). Polysaccharides from Panax japonicus C. A. Meyer and their antioxidant activities. *Carbohydrate Polymers*, 101, 386-391. http://dx.doi.org/10.1016/j.carbpol.2013.09.038. PMid:24299787.

Yao, Y. L., Shu, C., Feng, G., Wang, Q., Yan, Y. Y., Yi, Y., Wang, H. X., Zhang, X. F., & Wang, L. M. (2020). Polysaccharides from Pyracantha fortuneana and its biological activity. *International Journal of Biological Macromolecules*, 150, 1162-1174. http://dx.doi.org/10.1016/j.ijbiomac.2019.10.125. PMid:31794823.

Ye, M., Chen, W. X., Qiu, T., Yuan, R. Y., Ye, Y. W., & Cai, J. M. (2012). Structural characterisation and anti-ageing activity of extracellular polysaccharide from a strain of Lachnum sp. *Food Chemistry*, 132(1), 338-343. http://dx.doi.org/10.1016/j.foodchem.2011.10.087. PMid:26434299.

Yin, Z., Zhang, W., Zhang, J., Liu, H., Guo, Q., Chen, L., Wang, J., & Kang, W. (2019). Two novel polysaccharides in Psoralea corylifolia L and anti-A549 lung cancer cells activity in vitro. *Molecules (Basel, Switzerland)*, 24(20), 3733. http://dx.doi.org/10.3390/molecules24203733. PMid:31623207.

Zhang, S., & Zhao, Y. (2019). Lentinan protects cardiomyocytes against hypoxia-induced injury by regulation of microRNA-22/Sirt1. *Artificial Cells, Nanomedicine, and Biotechnology*, 47(1), 3938-3946. http://dx.doi.org/10.1080/21691401.2019.1666863. PMid:31581847.

Zhao, P., & Zhang, T. T. (2015). Preparation of Carboxymethyl Lonicera japonica Polysaccharides and its antioxidant ability. *Natural Product Research and Development*, 27, 114-119.

Zhao, X. H., Qian, L., Yin, D. L., & Zhou, Y. (2014). Hypolipidemic effect of the polysaccharides extracted from pumpkin by cellulase-assisted method on mice. *International Journal of Biological Macromolecules*, 64, 137-138. http://dx.doi.org/10.1016/j.ijbiomac.2013.12.001. PMid:24325857.

Zhu, H., Liu, C., Hou, J., Long, H., Wang, B., Guo, D., Lei, M., & Wu, W. (2019). Gastrodia elata Blume Polysaccharides: a review of their acquisition, analysis, modification, and pharmacological activities. *Molecules (Basel, Switzerland)*, 24(13), 2436. http://dx.doi.org/10.3390/molecules24132436. PMid:31269719.

Supplementary Material

Supplementary material accompanies this paper.

Supplemental Table 1 Levels and code of extraction variables used in Box-Behnken design

Supplemental Table 2 Box–Behnken experimental design and the results for extraction yield of crude PJPS

Supplemental Table 3 Analysis of variance of the experimental results of the BBD in extraction of crude PJPS

This material is available as part of the online article from https://www.scielo.br/j/cta