Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Optimization of Steaming Process for Polysaccharides from *Panax notoginseng* by Box-Behnken Response Surface Methodology and Comparison of Immunomodulatory Effects of Raw and Steamed *Panax notoginseng* Polysaccharides

Na Xing, Zhihong Zhang[1], Donghui Peng, Yuxin Li, Xue Wang, Rui Wang, Yanshan He[1], Yuanning Zeng[1], Haixue Kuang, Qiuhong Wang[1]

Department of Chinese Medicine Processing, Key Laboratory of Chinese Materia Medica, Heilongjiang University of Chinese Medicine, Ministry of Education, Harbin, [1]Department of Chinese Medicine Processing, School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou, China

Submitted: 26-Jan-2021 Revised: 16-Jun-2021 Accepted: 26-Jul-2021 Published: 24-Jan-2022

ABSTRACT

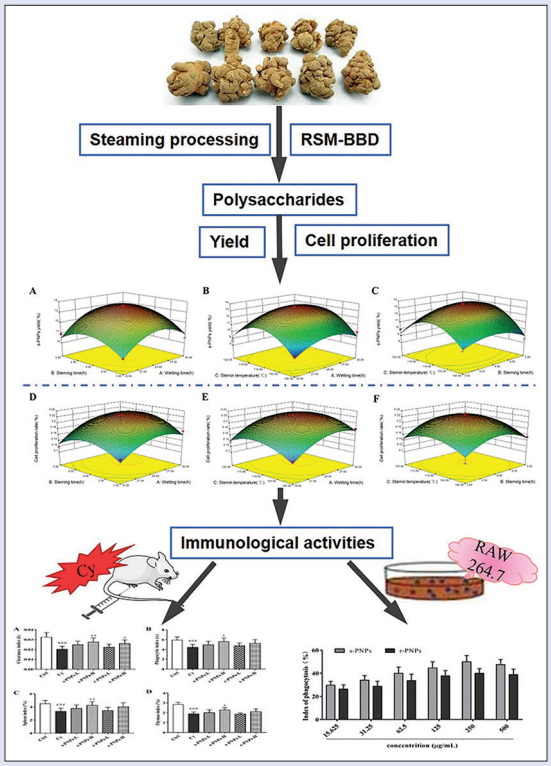
Aim: We aimed to optimize the steaming process of *Pana notoginseng* using response surface methodology (RSM). **Materials and Methods:** The yield and immune activity of steamed *P. notoginseng* polysaccharides (s-PNPs) was used as the indicators to optimize the steaming parameters: wetting time, steaming time, and temperature. After this, chromatogram and spectroscopy were used to analyze the effect of steaming on the structure of polysaccharides. Then, our study evaluated the immunological activities between s-PNPs and raw *P. notoginseng* polysaccharides (r-PNPs) *in vivo* and *in vitro*. **Results:** The optimal parameters of processing technology were as follows: wetting time was 25 h, steaming time was 4 h, and steaming temperature was 110°C. The s-PNPs yield reached a maximum of 13.67 ± 0.39%, and the cell proliferation rate was 22.87 ± 0.015% under these conditions. The chemical analysis method showed that the polysaccharide content was slightly increased, the protein content was decreased and the monosaccharide composition analysis indicated that the glucose content in s-PNPs was significantly increased than r-PNPs. Moreover, Fourier transform infrared spectroscopy analysis showed that the functional groups of polysaccharides changed partially between s-PNPs and r-PNPs. In addition, the immunological activity of s-PNPs was better than that of r-PNPs *in vivo* and *in vitro*. **Conclusion:** These studies showed that polysaccharides content was increased, and the immune function of s-PNPs was enhanced after applying steaming, making this approach meaningful for the development and application of *P. notoginseng* with its immunomodulatory.

Key words: Immunological activity, *Panax notoginseng,* polysaccharides, process technology, response surface methodology

SUMMARY

- Steaming processing is a common preparation method for *Panax notoginseng*, which showed a great potential in enhancing the yield and immune activity of steamed *P. notoginseng* polysaccharides. Moreover, response surface methodology-Box-Behnken design was indicated to be a reliable technique for optimizing the conditions of parameters of steaming processing, including wetting time, steamed temperature, and steamed time.

Abbreviations used: ANOVA: Analysis of variance; BBD-RSM: Box-Behnken design response surface methodology; BSA: Bovine serum albumin; CCK-8: Cholecystokinin-octopeptide; FBS: Fetal bovine serum; FTIR: Fourier transform infrared spectroscopy; HPLC: High-performance liquid chromatography; LPS: Lipopolysaccharide; PBS: Phosphate-buffered solution; *P. notoginseng*: *Panax notoginseng*; PNPs: *Panax notoginseng* polysaccharide; R^2: Regression coefficient; RPMI: Roswell park memorial institute.



Correspondence:

Prof. Qiuhong Wang,
School of Traditional Chinese Medicine,
Guangdong Pharmaceutical University,
510006 Guangzhou, China.
E-mail: qhwang668@sina.com
DOI: 10.4103/pm.pm_42_21

Access this article online
Website: www.phcog.com
Quick Response Code:

Cite this article as: Xing N, Zhang Z, Peng D, Li Y, Wang X, Wang R, *et al.* Optimization of steaming process for polysaccharides from *Panax notoginseng* by box-behnken response surface methodology and comparison of immunomodulatory effects of raw and steamed *Panax notoginseng* polysaccharides. Phcog Mag 2021;17:743-51.

INTRODUCTION

Panax notoginseng, the root of *P. notoginseng* (Burk.) F. H. Chen, one of the precious and important Traditional Chinese Medicines (TCMs), has the functions of dispersing blood stasis and stopping bleeding, detaching swelling and alleviating pain and Qi and blood invigorating.[1] It was a TCM for promoting blood circulation and removing blood stasis. At present, two kinds of decoction pieces, raw and steamed *P. notoginseng*, are mainly used in clinic. Steamed *P. notoginseng*, a dosage form of *P. notoginseng* mixed with water vapor by a TCM processing method, was used instead of raw *P. notoginseng* to strengthen the efficacy of tonifying Qi and enhance the immune system.[2,3] However, studies in the literature on *P. notoginseng* are mainly concerned on the raw form.[4-6] In recent years, an increasing number of researches have been focused on the small molecule components of Steamed *P. notoginseng*.[3,7,8] However, few attentions have been focused on the macromolecular polysaccharides from the steamed *P. notoginseng* polysaccharide (s-PNPs) which are the main active ingredients of *P. notoginseng* with various biological activities, including antitumor,[9] antioxidant, anti-aging,[10,11] and immunomodulatory properties.[12,13] Some studies have reported that the polysaccharides content was significantly increased after processing.[14] It is inferred that the steaming process technology has a significant impact on the polysaccharides yield.

To optimize the parameters for s-PNPs, the Response Surface Methodology (RSM), a novel method comprised of mathematical and statistical techniques, was used to identify complex quantitative relations between parameters and responses.[15] Considering its high efficiency and ease of operation, the Box–Behnken design (BBD) is the best type of RSM that is commonly used to optimize the technical parameters.[16,17] Therefore, in this study, we selected BBD to optimize the process conditions of steaming for s-PNPs, including the wetting time, steaming time, and steaming temperature. Besides, s-PNPs have been reported to have several physiological and health effects, such as anti-complementary activity, inducing the production of interferon-γ and tumor necrosis factor-α, which may be closely related to immune regulation.[18] Thus, the polysaccharides yield and *in vitro* immune activity were comprehensively evaluated to determine the best steaming conditions. The results were expected to provide a scientific foundation and technical support for further study of *P. notoginseng*, especially s-PNPs.

MATERIALS AND METHODS

Plant materials and reagents

Dried roots of *P. notoginseng* were purchased from Harbin, Heilongjiang, China. A voucher specimen was deposited at the Key Laboratory of Chinese Materia Medica, Heilongjiang University of Chinese Medicine, Harbin, China. 2,4-Dinitrofluorobenzene (DNFB) and cholecystokinin octopeptide (CCK-8) were obtained from Phar Mingen products (San Diego, CA, USA), RPMI 1640 medium was obtained from Gibco-BRL (Gaithersburg, MA, USA), fetal bovine serum (FBS) was from Gibco-BRL (Grand Island, NY, USA), Penicillin, streptomycin, lipopolysaccharide (LPS), D-Mannose (Man), L-rhamnose (Rha), D-glucose (Glc), D-galactose (Gal), D-xylose (Xyl), L-fucose (Fuc) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Unless otherwise stated, all of other reagents were of analytical grade.

Cells and animals

RAW264.7 cells (a murine macrophage line) were purchased from the Cell Resource Center, Shanghai Institute of Life Sciences, Chinese Academy of Sciences (Shanghai, MD, CN.). RAW264.7 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, under 5% CO_2, at 37°C, in a CO_2 incubator (Inco153, Memmert, Schwabach, Germany).

SPF BALB/c mice (half male and female), aged 6–8 weeks (22 ± 2 g) were purchased from Guangdong Medical Laboratory Animal Center (Guang Zhou, China [SYXK (Yue) 2018-0002]). The animals were maintained with free access to food and water in the temperature at 25 ± 2°C on a 12 h light/dark cycle and humidity at 60 ± 10%. All animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Guangdong Pharmaceutical University and approved by the Animal Ethics Committee of Guangdong Pharmaceutical University.

Steaming methods of *Panax notoginseng*

Evenly sized root of *P. notoginseng* was put into the container, then 40% distilled water (V/W) was added into container, stirring to make the surface of *P. notoginseng* completely contact with water for wetting. After it and finally the soaked *P. notoginseng* roots were steamed in a high-pressure steam boiler (Hirayama Manufacturing Corp., Saitama, Japan) high-pressure cooker for steaming processing after steaming, drying, and crushing.

Extraction and refining of *Panax notoginseng* polysaccharides

The coarse powders of *P. notoginseng* (steamed or raw) were submitted to sequential extraction as follows: Coarse powders (500 g) were refluxed with 5 Vol. of 80% ethanol 3 times for 2 h and each time. The residue was dried in air and then extracted three times with 30 Vol. of distilled water for 2 h and each time. The aqueous extract solution was filtered and concentrated to 1/5 volume and precipitated with 80% ethanol overnight. The precipitate was dissolved in distilled water to remove protein by Sevag method (n-butanol and chloroform mixed solution extraction).[19] The resulting aqueous fraction was dialyzed (cut-off Mw 3500 Da) and precipitated again by adding a 4-fold volume of 80% ethanol. After centrifugation, the precipitated was washed with anhydrous ethanol and acetone, and then dissolved in distilled water and lyophilized to acquire the PNPs. The polysaccharides yield (%) was calculated using the following Eq. (1).

$$Yield\ (\%) = W/W_m \times 100\% \quad (1)$$

W is the weight (g) of polysaccharides and W_m is the weight (g) of crude *P. notoginseng* powder.

Measurement of Immunomodulatory Activities. s-PNPs was dissolved in RPMI 1640 medium at a final concentration of 1.0 mg/mL and the solution was sterilized by filtration through 0.22 μm membrane filter. RAW264.7 cells were seeded into 96 well plates at 5×10^4 cells/mL for 24 h, then incubated with 100 μL of s-PNPs (250 μg/mL) for 24 h. Cell viability was determined using a Cell Counting Kit-8 (CCK-8), according to the manufacturer's instructions, using a microplate reader (Epoch2, BioTek, Winooski, USA) at a wavelength of 450 nm. The cell proliferation rate was calculated according to the following equation:

$$\text{Cell proliferation rate } (\%) = [\ 1\text{-}(A_1\text{-}A_2)/A_0] \times 100\% \quad (2)$$

Single-factor experiments

The main factors of the polysaccharides yield in the steamed processing of *P. notoginseng* are wetting time, steam time, and temperature. To explore the impact of various factors on the polysaccharides yield of steamed processing of *P. notoginseng*, the yield and immune activity of s-PNPs were used as indicators and wetting time, steam time and temperature were investigated as factors arranged in a single-factor test.

Response surface experimental design

Based on the single factor experiments, three independent variables (*A*, wetting time; *B*, steam time; and *C*, steam temperature) at three levels were employed for the optimization of the steamed processing of *P. notoginseng* to obtain the best yield of PNPs and immune activity by BBD-RSM. The range of independent variables and the corresponding response values are shown in Table 1. The range of the independent variables, the levels of the independent variables, and the results of the whole design consisting of 17 test points performed in random order are presented in Table 2.

The lack of fit statistics, *P* value, and *F*-value obtained from the analysis of variance (ANOVA) were calculated by Design-Expert version 8.0.6 software to evaluate the adequacy of the model.[20]

Analysis of monosaccharides of *Panax notoginseng* polysaccharides

After that, the polysaccharides, uronic acid, and protein content of polysaccharides of steamed *P. notoginseng* (s-PNPs) and raw *P. notoginseng* (r-PNPs) were determined by the phenol–sulfuric acid method,[21] sulfuric acid carbazole method,[22] and Bradford method,[23] respectively. D-glucose, D-glucuronic acid, and bovine serum albumin (BSA) were used to construct a standard curve, respectively. Three replicate data were obtained.

The monosaccharide composition of the s-PNPs and r-PNPs samples was analyzed by high-performance liquid chromatography (HPLC) system (2695 HPLC, Waters Co., Ltd. USA) with Diamonsil C-$_{18}$ column (250 mm × 4.6 mm, 5 μm) under the mobile phase of water and acetonitrile in a ratio of 20:80 (v/v, %, 0–70 min) at a flow rate of 1 mL/min and ultraviolet absorbance of the effluent was monitored at 245 nm. The s-PNPs and r-PNPs samples preparation were as follows: polysaccharides sample (10 mg) was dissolved in 2 mL 2.0 M trifluoroacetic acid (TFA) and hydrolyzed at 120°C for 160 min. Then dried TFA with methanol after cooled down to room temperature, the dry residuum was dissolved in distilled water for the following experiments. 1 mL sample solution, 500 μL of 0.5M PMP methanol solution and 500 μL of 0.3 M sodium hydroxide solution were mixed and incubated at 70°C for 60 min. Then, the samples were cooled down at the room temperature for 10 min and neutralized with 100 μL (0.3 M) hydrochloric acid (HCl) solution. The resulting solution was extracted with equal volumes of isoamyl acetate (twice) and chloroform. The chloroform was discarded by centrifugation to obtain the upper aqueous phase, followed by filtering with 0.45 μm microporous membrane. Moreover, the supernatant was centrifuged for HPLC analysis. Six kinds of monosaccharides (Man, Rha, Glc, Gal, Ara, and Xyl) were prepared as separate stock solutions with purified water to different concentrations (187.5, 375, 750, 1500, and 3000 μM). The derivatization procedure of hydrolysate samples and standard samples must be carried out simultaneously under the same conditions.

Table 1: Codes and level of variables chosen for the Box–Behnken design

Symbol	Factors	Variable	Coded levels		
			−1	0	1
A	Wetting time (h)	X_1	18	24	30
B	Steaming time (h)	X_2	3	4	5
C	Steaming temperature (°C)	X_3	100	110	120

Table 2: Box–Behnken design matrix and the experimental data for the responses

Test group	A/h (X_1)	B/h (X_2)	C/$_Z$ (X_3)	Yield s-PNPs (%)	Cell proliferation rate (%)
1	18 (−1)	5 (1)	110 (0)	10.89	11.68
2	24 (0)	4 (0)	110 (0)	13.67	21.32
3	24 (0)	4 (0)	110 (0)	13.12	22.64
4	24 (0)	5 (1)	120 (1)	11.55	19.51
5	24 (0)	5 (1)	100 (−1)	8.51	15.24
6	18 (−1)	4 (0)	100 (−1)	8.74	12.66
7	30 (1)	4 (0)	120 (1)	10.67	18.15
8	24 (0)	4 (0)	110 (0)	13.41	21.01
9	18 (−1)	4 (0)	120 (1)	8.67	13.69
10	24 (0)	3 (−1)	120 (1)	9.93	14.37
11	30 (1)	3 (−1)	110 (0)	10.53	17.25
12	30 (1)	5 (1)	110 (0)	12.11	18.24
13	24 (0)	3 (−1)	100 (−1)	9.12	11.81
14	18 (−1)	3 (−1)	110 (0)	10.12	13.04
15	24 (0)	4 (0)	110 (0)	13.52	21.88
16	30 (1)	4 (0)	100 (−1)	9.95	18.08
17	24 (0)	4 (0)	110 (0)	13.32	22.87

s-PNP: Steamed-*Panax notoginseng* polysaccharides

Fourier transform infrared spectroscopy analysis of *Panax notoginseng* polysaccharides

Fourier transform infrared spectroscopy (FTIR) spectra of the s-PNPs and r-PNPs samples were identified using a FTIR spectrophotometer (TENSOR37, FTIR, Bruker, Germany) in the 4000-400 cm^{-1} frequency range through the potassium bromide (KBr) pressed-disc technique.[24]

Assay of neutral red

RAW264.7 cells (1 × 10^5/mL) were incubated in 96-well plates for 24 h at 37°C. Cells were exposed to various concentrations of s-PNPs and r-PNPs (15.625, 31.25, 62.5, 125, 250, and 500 μg/mL) and LPS (1 μg/mL) and vehicle for 24 h. Then, the supernatant was removed and 100 μl/well of 0.1% neutral red solution (100 mg neutral red/100 mL physiological saline) were added to each well and incubated for 3 h. Then, the cells were washed three times with warm PBS and 200 μL cell lysis solution (acetic acid, ethanol = 1:1) were treated for 30 min, which was measured as the absorbance value at 540 nm using a microplate reader (Epoch2, BioTek, Winooski, USA). The phagocytic index was calculated according to the following equation:

$$\text{Phagocytic index (\%)} = (A_{\text{medicated}}/A_{\text{blank}} - 1) \times 100\% \quad (3)$$

Assay of carbon clearance

BALB/c mice were allocated randomly into seven groups with 10 rats in each group, control group (Ctrl), model group (cyclophosphamide, Cy) and Cy + s-PNPs-L, Cy + s-PNPs-H, Cy + r-PNPs-L and Cy + r-PNPs-L. Ctrl and Cy mice, oral gavage with 0.2 mL of 0.9% saline solution for 7 days, polysaccharides groups, oral gavage with s-PNPs and r-PNPs solutions for 7 days with different doses of 50 mg/kg and 200 mg/kg, respectively. All mice, except for Ctrl, were subcutaneously injected with Cy (80 mg/kg, 0.1 ml/10 g) on the 1st, 3rd, and 5th days to replicate immunosuppression models.

All mice were injected with Indian ink (0.01 mL/g) through caudal vein after 24 h of last administration. Blood samples were collected from the ophthalmic vein at intervals of 2 (T_1) and 12 (T_2) min, a 20 μl sample was mixed with 2 mL 0.1% sodium carbonate solution (Na_2CO_3,) and placed for 2 h to measure absorbance (OD) at 680 nm. Then, the mice were sacrificed by cervical dislocation. Organ index, carbon clearance index (K), and phagocytic index (α) were calculated as following formula:

$$K = (logOD1 - logOD2)/(T2 - T1) \quad (4)$$

$$\alpha = (K^{1/3} \times \text{body wt of animal})/(\text{liver wt} + \text{spleen wt}) \quad (5)$$

$$\textit{organ index (\%)} = \textit{organ}\ \text{mass/weight} \times 100\% \quad (6)$$

OD_1 and OD_2 are the absorbance of blood collected at 2 min and at 12 min; T_1 and T_2 are the time point of blood collection at 2 min and at 12 min.

Statistical analysis

The BBD-RSM results of the response surface design were analyzed using the Design-Expert 8.0.6 software (trial version, State-Ease Inc., Minneapolis, MN, USA). The data were presented as mean ± SEM and analyzed by GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). Moreover, statistical comparisons among groups were examined using the two-tailed unpaired *t*-test. Statistical significance was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Single-factor experiments for wetting time on the steaming process

As shown in Figure 1a and d, the wetting time on yield and immune activity of s-PNPs in the steaming process was investigated. The yield of s-PNPs increased with wetting time until 24 h and began to decrease and the maximum extraction yield was 13.38 ± 0.401% at 24 h. The proliferation rate of RAW264.7 increased from 8.70 ± 0.013% to 21.31 ± 0.012% when the wetting time increased from 6 to 24 h and then presented a slight downward trend when the time exceeded 24 h. The maximum values (13.38 ± 0.401% and 21.31 ± 0.012%) were reached when the wetting time was 24 h. Therefore, 24 h was selected as the center point of wetting time in the following RSM experiments.

Single factor experiments for steaming time on the steaming process

The yield and immune activity of s-PNPs steamed by different steaming time from 1 h to 5 h is shown in Figure 1b and e. The wetting time and steaming temperature was fixed at 24 h and 100°C, respectively. The yields of s-PNPs increased from 7.37 ± 0.405% to 13.58 ± 0.520% with the steaming time increasing from 1 to 4 h. Then, it tended to go steady. The proliferation rate of RAW264.7 increases rapidly with the increasing steaming time from 1 to 4 h, then the proliferation rate of RAW264.7 tended stabilization from 4 to 5 h. The optimum yield (13.58 ± 0.520%) of s-PNPs was obtained when the steaming time was 4 h. At the same steaming time, the cell proliferation rate reached to a maximum value, which was 22.65 ± 0.014%. Thus, the optimized teaming time range was 3–5 h.

Single factor experiments for steaming temperature on the steaming process

In the steaming process, the steaming temperature was performed using ranging from 90°C to 130°C with the other steaming parameters as follows: 24 h of wetting time and 4 h of steaming time. The s-PNPs yield increased as the steaming temperature ascended from 90°C to 110°C, and then, the s-PNPs yield presented gradually descended from 110°C to 130°C [Figure 1c]. The cell proliferation rate increased rapidly when the steaming temperature ranged from 90°C to 110°C and then dropped gradually when the steaming temperature ascended from 110°C to 130°C [Figure 1f], which showed a similar tendency with the s-PNPs yield and the maximum yield (12.46 ± 0.572%) and cell proliferation rate (21.03 ± 0.015%) were reached when the steaming temperature was performed 110°C. According to the results in Figure 1c and f, 100°C–120°C were chosen as the range of steaming temperature for the following experiments in the steaming process.

According to the single-parameter study, we adopted steaming time 18–30 h; steaming time 3–5 h, and steaming temperature 100°C–120°C for RSM experiments.

Statistical analysis and the model fitting

At present, the RSM is widely recommended in optimization research due to its characteristics of optimizing processing conditions, reducing development costs and improving product quality.[25] BBD is an effective statistical method for obtaining optimal levels of two or more treatment variables.[26] RSM with BBD was employed to optimize the processing technology of steamed *P. notoginseng*. According to the experimental design, the results of 17 trial points tested in a random order are presented in Table 3. After the steaming process of *P. notoginseng*, the percentage yield of s-PNPs ranged from 8.51% to 13.67% and the proliferation rate of RAW264.7 ranged from 11.68% to 22.87%. The maximum yield of s-PNPs (13.67%) and proliferation rate of RAW264.7 (22.87%) was obtained at the following experimental conditions: 25 h of wetting time,

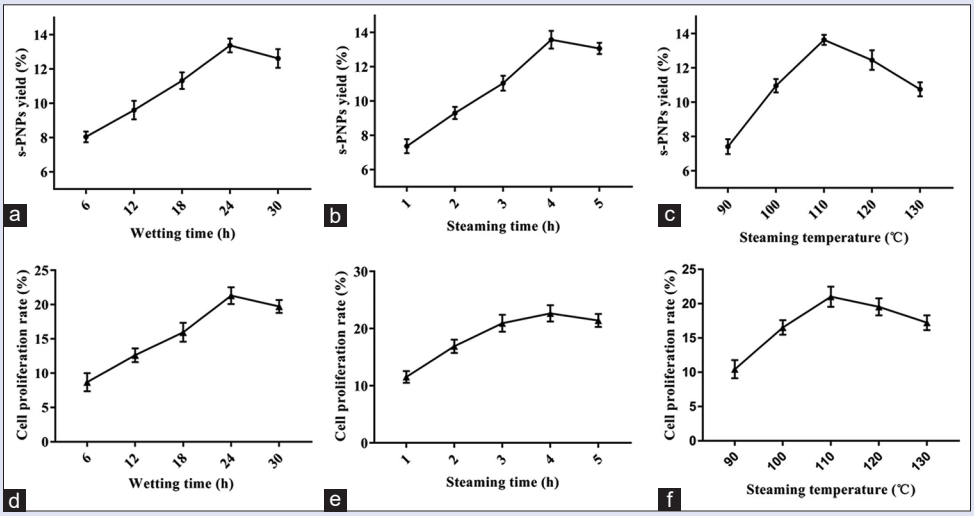


Figure 1: Effect of different steam parameters on the steamed *P. notoginseng* polysaccharides yield (a-c) and cell proliferation rate on RAW264.7 (b-d). (a and d) Effect of wetting time, (b and e) steaming temperature (c and f) steaming time ($n = 3$)

Table 3: The analysis of variance results for the response surface quadratic models for the yield and immune activity of steamed *Panax notoginseng* polysaccharides

Factor	Sum of squares	Degrees of freedom	Mean square	*F*	*P* (Pro>*F*)
Yield of s-PNPs					
Model	52.30	9	5.81	20.46	0.0003
A	2.93	1	2.93	10.31	0.0148
B	1.41	1	1.41	4.97	0.0611
C	2.53	1	2.53	8.91	0.0204
AB	0.16	1	0.16	0.58	0.4721
AC	0.16	1	0.16	0.55	0.4827
BC	1.24	1	1.24	4.38	0.0747
A^2	8.05	1	8.05	28.34	0.0011
B^2	5.21	1	5.21	18.35	0.0036
C^2	26.69	1	26.69	93.96	<0.0001
Residual	1.99	7	0.28		
Lack of fit	1.82	3	0.61	14.09	0.0136
Pure error	0.17	4	0.043		
Cor total	54.29	16			
R^2, R^2adj, CV%			0.9634, 0.9163, 4.82		
Immune activity of s-PNPs					
Model	0.023	9	2.533×10^{-3}	10.55	0.0026
A	5.330×10^{-3}	1	5.330×10^{-3}	22.21	0.0022
B	8.405×10^{-4}	1	8.405×10^{-4}	3.50	0.1035
C	7.861×10^{-4}	1	7.861×10^{-4}	3.28	0.1133
AB	7.381×10^{-4}	1	7.381×10^{-4}	0.58	0.4730
AC	2.304×10^{-5}	1	2.304×10^{-5}	0.096	0.7657
BC	7.310×10^{-5}	1	7.310×10^{-5}	0.30	0.5982
A^2	4.419×10^{-3}	1	4.419×10^{-3}	18.41	0.0036
B^2	5.616×10^{-3}	1	5.616×10^{-3}	23.40	0.0019
C^2	3.941×10^{-3}	1	3.941×10^{-3}	16.42	0.0049
Residual	1.680×10^{-3}	7	2.400×10^{-4}		
Lack of fit	1.419×10^{-3}	3	4.731×10^{-4}	7.26	0.0428
Pure error	2.608×10^{-4}	4	6.519×10^{-5}		
Cor total	0.024	16			
R^2, R^2adj, CV%			0.9314, 0.8431, 8.98		

CV: Coefficient of variation; s-PNPs: Steamed-*Panax notoginseng* polysaccharides

4 h of steaming time, 110°C of steaming temperature. The multiple regression analysis was applied for statistical analysis and the predicted response *Y* for the s-PNPs yield and cell proliferation rate could be obtained by the following second-order polynomial equation:

s-PNPs yield = 13.41 + 0.61 *A* + 0.42 *B* + 0.56 *C* + 0.20 *AB* + 0.20 *AC*– 0.56 *BC*– 1.38 *A2*-1.11 *B2*-2.52 *C*2.

Cell proliferation rate = 0.22 + 0.026 *A* + 0.010 *B* + 9.913 × 10-3 *C* + 5.875 × 10-3 *AB*– 2.400 × 10-3 *AC* + 4.275 × 10-3 *BC*– 0.032 *A2*-0.037 *B2*-0.031 *C*2.

Where *A*, *B,* and *C* are the coded values of the wetting time, steaming time, and steaming temperature, respectively.

ANOVA was used for response surface analyze quadratic and the statistical summary is shown in Table 3. The *F* values of the model were 20.46 and 10.55, respectively, that means each model was significant and able to fit the data properly.[27] The co-efficient of determination (R^2) was the proportion of variability in the data explained by or accounted for by the model.[28] For the quadratic regression model, the R^2 values were 0.9634 and 0.9314, respectively. These indicated that the general availability and accuracy of the polynomial model were adequate and could explain 96.34% and 93.14% of the variation. The value of adjusted determination coefficient (R_{adj}^2) was 0.9163 and 0.8431, respectively. The high values of R_{adj}^2 confirmed that the model has a good fitting degree. At the same time, the coefficient of variation value (4.82% and 8.98%) was relatively low, indicating a high degree of precision, reliability, and reproducibility of the model.[29]

According to Table 3, we could find that the linear coefficients (*A* and *C*) and the quadratic term coefficients (*A*2, *B*2 *and C*2) were significant with *P* values ($P < 0.05$), whereas the linear coefficients (*B*) and cross-product coefficients (*AB, AC, and BC*) had no significant influence ($P > 0.05$) on the yield of s-PNPs. From an analysis of the influence on cell proliferation rate of s-PNPs, Table 3 showed that linear coefficients (*A*) and quadratic coefficients (*A*2, *B,*2*and C*2) were significant ($P < 0.05$), while linear coefficients (*B* and *C*) and cross product coefficients (*AB, AC, and BC*) had no significant difference ($P > 0.05$). It concluded from the data presented in Table 1 that the independent variable (A), the interaction terms (*A*2, *B*2*and C*2) significantly affected the yield and cell proliferation rate of s-PNPs.

Response surface plots analysis

Then, we evaluate the influence of the interaction between wetting time and steaming time on the s-PNPs yield [Figure 2a]. The yield of polysaccharides s-PNPs was increased with the increase of the preparation time and the peak value was at 25.49 h. In addition, when the steaming time was from 3 to 4.25 h, the s-PNPs yield always increased and then decreased with further steaming time. As shown in Figure 2b, the dependence of the s-PNPs yield on the varying wetting time and steaming temperature was measured and found that the yield increased as the wetting time increased from 18 to 25.49 h and the steaming temperature increased from 100 to 111.48°C, but the yield decreased for more than 25.49 h and 111.48°C. Figure 2c shows the effect of the interaction of steaming time and steaming temperature on the s-PNPs yield. The s-PNPs yield was increased with steaming time increased from 3 to 4.25 h. After the steaming time reaches 4.25 h, the curve tends to be stable. The s-PNPs yield increased in the

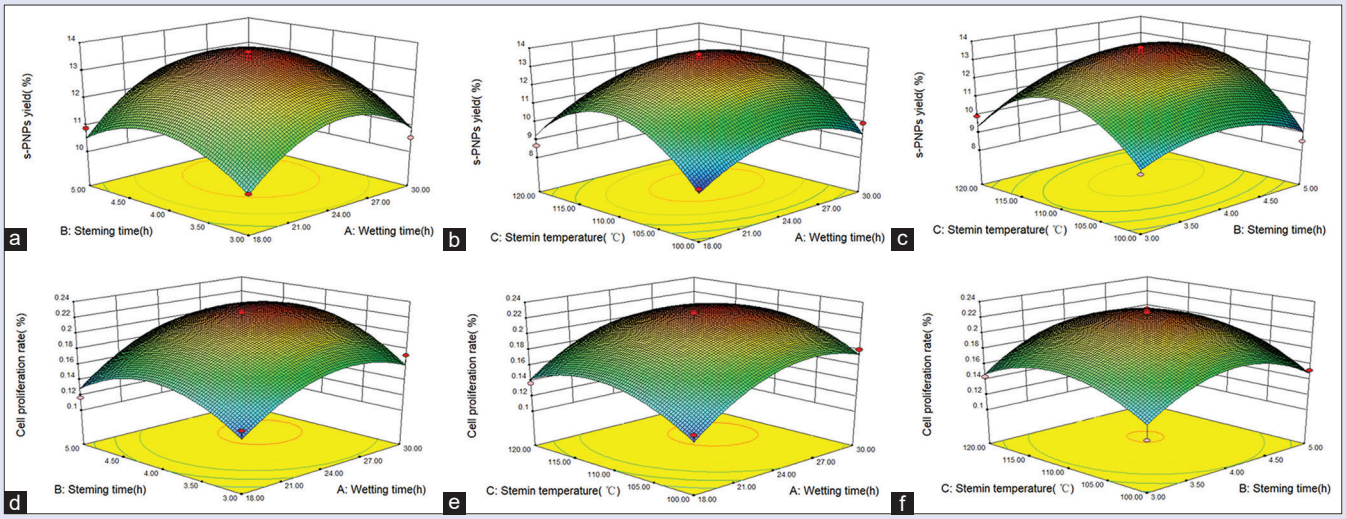


Figure 2: Response surface plots (3D) showing effect of variables on the steamed *P. notoginseng* polysaccharides yield (a-c) and cell proliferation rate of RAW264.7 (d-f). Wetting time, (a and d), Steaming time (b and e), Steaming temperature (c and f)

temperature range of 100°C–111.48°C and showed a downward trend over 111.48°C.

As shown in Figure 2d, within a certain range of wetting time, the cell proliferation rate of s-PNPs increases with the increase of the wetting time and steaming time and the maximum value was at 25.99 h of wetting time and 4.21 h of steaming time. As shown in Figure 2e, it was apparent that the cell proliferation rate for s-PNPs reached maximum when the wetting time was 25.99 h and the steaming temperature was 111.53°C, respectively. From Figure 2f, we evaluated the effect of the interaction between steaming time and steaming temperature on the cell proliferation rate for s-PNPs and found that the cell proliferation rate for s-PNPs had a maximum value at 4.21 h of steaming time and 111.53°C of steaming temperature.

According to these results, the optimal modified conditions for the steaming process of *P. notoginseng* were as follows: the wetting time was 25 h, the steaming time was 4 h, and the steaming temperature was 110°C. Under this condition, the actual s-PNPs yield, and cell proliferation rate were 13.36 ± 0.43% and 22.03 ± 0.014%, which were similar to the predicted value 13.67 ± 0.39% and 22.87 ± 0.015%. This similarity indicates that the steaming regression model is highly significant and can be used to predict the yield and immune activity of s-PNPs.

Monosaccharide composition

The changes of polysaccharides, uronic acid, and protein before and after processing were compared by chemical analysis method, and these results showed that the sugar, uronic acid, and protein contents of the s-PNPs were 75.6 ± 2.37, 25.3 ± 2.09, and 11.3 ± 0.91 (w/w), while r-PNPs were 69.7 ± 2.05, 28.8 ± 2.17, and 19.9 ± 1.01 (w/w), respectively. The results suggested that the polysaccharides content was significantly increased, and the protein content was reduced after steaming.

The analysis results of monosaccharide composition are shown in Figure 3. The chromatograms of 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives of six kinds of standard monosaccharide samples are shown in Figure 3c. These six kinds of monosaccharide standards were well separated, and the peak sequence was as follows: 1: Man; 2: Rha; 3: Glc; 4: Gal; 5: Xyl; 6: Fuc. And their corresponding molar percentages (mol%) were 0.75%, 1.69%, 162.62%, 65.56%, 12.40%, and 2.29% in r-PNPs sample and 0.56%, 1.34%, 218.91%, 70.91%, 10.62%, and 2.44% in s-PNPs sample, respectively [Figure 3a and b]. These results also showed that the Glc content in the polysaccharides of *P. notoginseng* increased significantly after steaming.

Fourier transform infrared spectroscopy spectroscopy

To further characterize s-PNPs and r-PNPs and identify the fundamental group present in its structure, FTIR spectra analysis was carried out. As Figure 3d, the strong peaks at 3401 and 3419 cm^{-1} were the characteristic signals of the hydroxyl groups which due to inter-and intramolecular interactions of the polysaccharide chains. The weak peaks at 2931 and 2934 cm^{-1} were caused by the C–H anti-symmetrical stretching vibration of methyl group. The band at around 1619 and 1604 cm^{-1} was regarded as the nonesterified carbonyl group carbon–oxygen double bond (C = O) asymmetric stretching vibration, which indicated the presence of characteristic peaks of uronic acid and bound water absorption peak and the C = O absorption peak of r-PNPs was higher than that of s-PNPs.[30] The absorption peaks at 1411 and 1420 cm^{-1} were derived from the stretching vibration of C-H and represented the deformations of CH_2 groups.[31] The absorption peak at 1250 and 1241 cm^{-1} demonstrated C = O stretching vibration. The particular bands at approximately 1200-1000 cm^{-1}, suggesting the pyranose form of sugar.[32] In addition, r-PNPs contained the α-configuration as demonstrated by the bands at 825 cm^{-1}, r-PNPs existed absorption at 1328 cm^{-1} indication of the presence of a carboxyl group with symmetrical C = O stretching vibration, 959 and 896 cm^{-1} were representative of β-dominating configuration in pyranose and 825 cm^{-1} was an indication of the presence of α-glycoside bond in the polysaccharides.[33] The absorption peak at 849 cm^{-1} was representative of α-configuration bond in s-PNPs.[34] Besides, the absorption bands at 765 and 762 cm^{-1} were taken as the typical pattern for a glucopyranose derivative. Based on the above results, it could be concluded that s-PNPs mainly contained α-glycosidic bonds of sugar units and r-PNPs mainly contained two glycosidic linkages of α-and β-configuration.

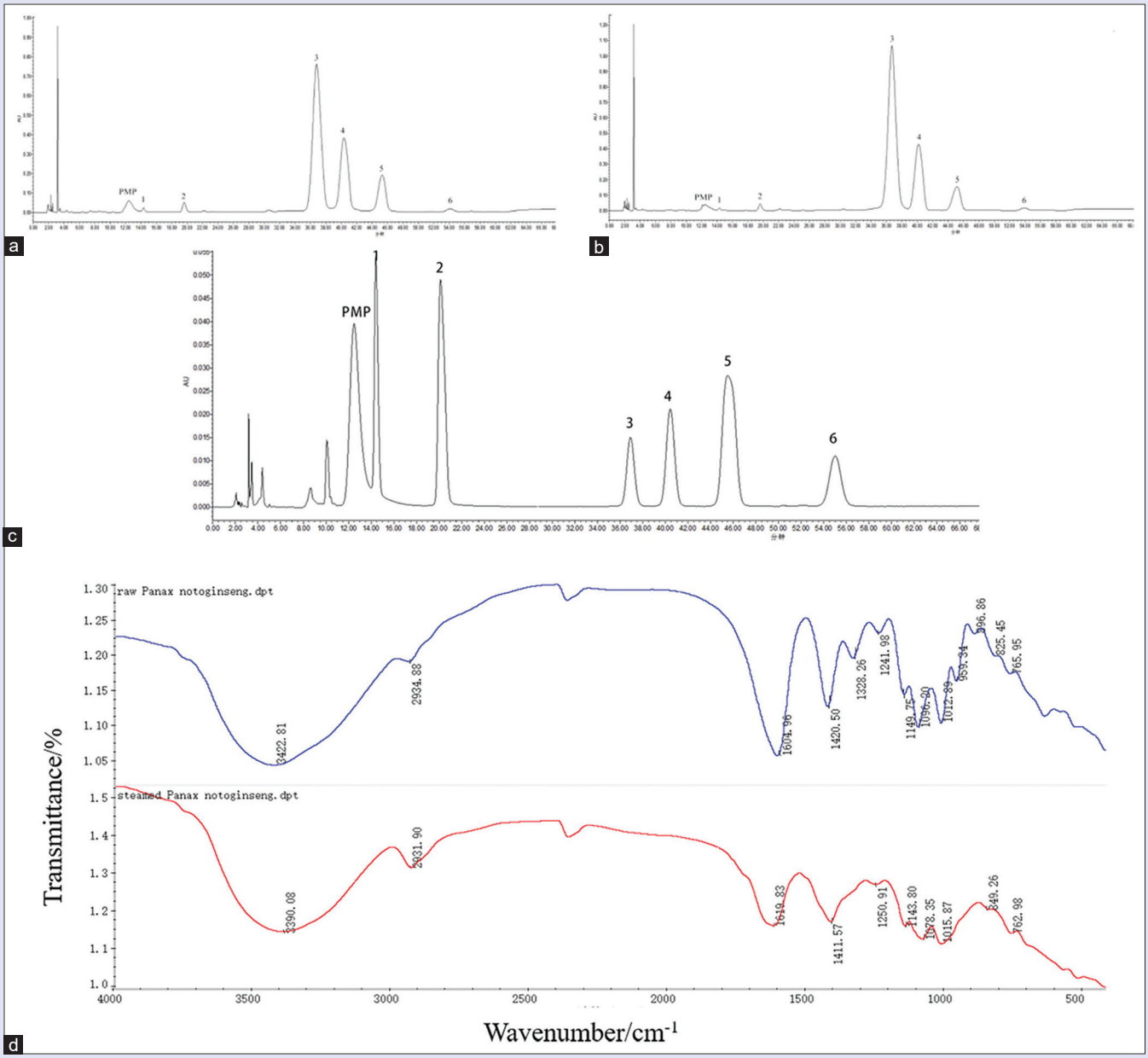


Figure 3: (a-c) Chromatography of monosaccharides of steamed *P. notoginseng* polysaccharides and raw *P. notoginseng* polysaccharides, (a) raw *P. notoginseng* polysaccharides, (b) steamed *P. notoginseng* polysaccharides; (c) six standard monosaccharides. 1: Man; 2: Rha; 3: Glc; 4: Gal; 5: Xyl; 6: Fuc. (d) Fourier transform infrared spectroscopy spectroscopy of steamed *P. notoginseng* polysaccharides and raw *P. notoginseng* polysaccharides between 400 and 4000 cm^{-1}, the blue line represents raw *P. notoginseng* polysaccharides and the red line represents steamed *P. notoginseng* polysaccharides

Effect of *Panax notoginseng* polysaccharides on the phagocytosis of RAW264.7 cells

The phagocytic function of resting macrophages was measured by detecting the size of phagocytic activity.[35] The neutral red method is a common and simple method to quickly screen the components that regulate the phagocytic function of macrophages. The experimental results showed [Table 4] that s-PNPs and r-PNPs (15.62, 31.25, 62.5, 125, 250, and 500 μg/mL) incubation in RAW264.7 cells for 24 h could promote the activation of macrophages and improve phagocytosis of neutral red. With the increase of s-PNPs or r-PNPs concentration, the phagocytosis of macrophages also increased, but the proliferation activity gradually decreases at high concentrations. It was found that the phagocytosis index of the s-PNPs group was higher than that of the r-PNPs group (s-PNPs, 49.86% vs. r-PNPs, 40.92%), suggesting that the polysaccharides and protein content of s-PNPs and the different functional groups might affect the phagocytic activity of macrophages [Figure 4]. These results indicated that PNPs could activate macrophages, promote macrophages differentiation and maturation, and significantly enhance their phagocytic ability. Moreover, the effect of s-PNPs was better than that of r-PNPs.

Effect of polysaccharides on carbon clearance test in mice

Nonspecific immunity is an important part of the body's immune system and the phagocytic ability of mononuclear macrophages is one of the indicators to measure the body's nonspecific immune function.[36]

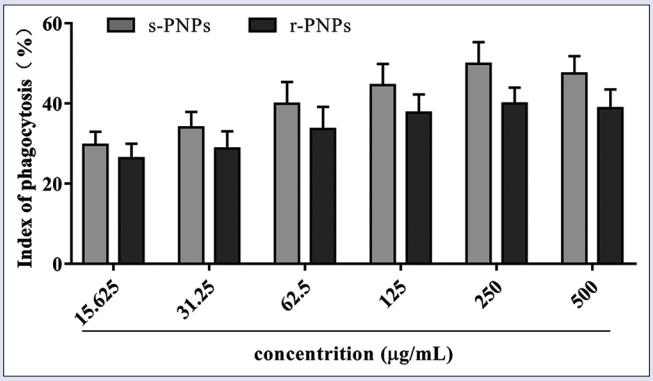


Figure 4: Comparison of steamed *P. notoginseng* polysaccharides and raw *P. notoginseng* polysaccharides on the phagocytosis of RAW264.7 cells

Table 4: Phagocytic activity of RAW264.7 cells stimulated with steamed-*Panax notoginseng* polysaccharides and raw-*Panax notoginseng* polysaccharides

Group	Dose/μg·L^{-1}	Index of phagocytosis (%)
Positive control (LPS)	1.00	53.79±5.18
s-PNPs	15.62	29.68±3.29
	31.25	34.03±3.89
	62.50	39.94±5.43
	125	44.55±5.31
	250	49.86±5.47
	500	47.46±4.35
r-PNPs	15.62	26.31±3.68
	31.25	28.77±4.29
	62.50	33.61±5.58
	125	37.70±4.55
	250	40.92±4.51
	500	39.99±4.00

PNP: *Panax notoginseng* polysaccharides; s-PNP: Steamed-PNP; r-PNPs: Raw-PNP; LPS: Lipopolysaccharide

The clear index of macrophages can reflect the strength of phagocytic function, which is positively correlated with immune activity. The immunological effects of s-PNPs and r-PNPs on carbon clearance test showed that the clearance index *k* and phagocytic index α were decreased in the cyclophosphamide (Cy) group compared to the Control (Ctrl) group [Figure 5a and b, $P < 0.001$], indicating that the mouse' immunocompromised model was successfully established. However, compared to the Cy group, there was a marked increase in the clearance index *k* in the high dose of s-PNPs (s-PNPs-H) group and high dose of r-PNPs (r-PNPs-H) group ($P < 0.01$, $P < 0.05$, respectively), phagocytic index α in high dose of s-PNPs (s-PNPs-H) group was also significant increased ($P < 0.05$). As shown in Figure 5c and d, spleen index and thymus index were decreased in the Cy group compared with the Ctrl group ($P < 0.001$). Treatment with s-PNPs-H could drastically increase the spleen index and thymus index to varying degrees ($P < 0.01$, $P < 0.05$, respectively); however, r-PNPs-H only showed an enhanced trend without significance ($P > 0.05$). Compared with r-PNPs and s-PNPs at the same concentration, the effects of s-PNPs were better than that of r-PNPs.

CONCLUSION

In summary, we have optimized stable and reliable processing parameters with wetting time 25 h, steaming time 4 h, and steaming temperature 110°C by BBD along with RSM. In addition, the immunomodulatory activity of *P. notoginseng* polysaccharides was enhanced *in vivo* and *in vitro* after steaming, which may be due to the changes in the structure of the polysaccharides during the steaming process. Based on these results, further studies on the structure and immune functions of s-PNPs should be conducted. Our study suggests that s-PNPs pretreatment is a promising therapeutic approach for patients with immune disorders.

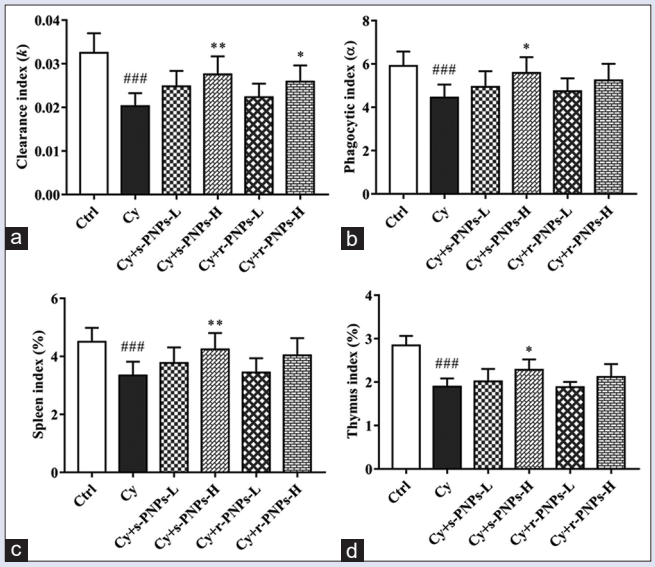


Figure 5: Effect of steamed *Panax notoginseng* polysaccharides and raw *Panax notoginseng* polysaccharides on carbon clearance test. (a) carbon clearance index ($n = 10$), (b) phagocytic index ($n = 10$). (c) Spleen index ($n = 10$). (d) Thymus index ($n = 10$). Data are shown as mean ± SD. ###$P < 0.001$ versus Ctrl group; *$P < 0.05$, **$P < 0.01$ versus Cy group

Financial support and sponsorship

This study was supported by the National Key Research and Development Program of China (2018YFC1707100), the National Science and Technology Major Projects for "Major New Drugs Innovation and Development" (2018ZX09731-001), and the National Natural Science Foundation of China (8177141630).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Wang T, Guo R, Zhou G, Zhou X, Kou Z, Sui F, *et al.* Traditional uses, botany, phytochemistry, pharmacology and toxicology of *Panax notoginseng* (Burk.) F.H. Chen: A review. J Ethnopharmacol 2016;188:234-58.
2. Wang D, Liao P, Zhu H, Chen K, Xu M, Zhang Y, *et al.* The processing of *Panax notoginseng* and the transformation of its saponin components. Food Chem 2012;132:1808-13.
3. Lau AJ, Seo BH, Woo SO, Koh HL. High-performance liquid chromatographic method with quantitative comparisons of whole chromatograms of raw and steamed *Panax notoginseng*. J Chromatogr A 2004;1057:141-9.
4. Zhao Y, Zheng J, Yu Y, Wang L. *Panax notoginseng* saponins regulate macrophage polarization under hyperglycemic condition via NF-κB signaling pathway. BioMed Res Int 2018;2018:9239354.
5. Xie W, Meng X, Zhai Y, Zhou P, Ye T, Wang Z, *et al. Panax notoginseng* saponins: A review of its mechanisms of antidepressant or anxiolytic effects and network analysis on phytochemistry and pharmacology. Molecules 2018;23:940.
6. Xu C, Wang W, Wang B, Zhang T, Cui X, Pu Y, *et al.* Analytical methods and biological activities of *Panax notoginseng* saponins: Recent trends. J Ethnopharmacol 2019;236:443-65.
7. Lau AJ, Woo SO, Koh HL. Analysis of saponins in raw and steamed *Panax notoginseng* using high-performance liquid chromatography with diode array detection. J Chromatogr A 2003;1011:77-87.

8. Sun S, Wang C, Tong R, Li X, Fishbein A, Wang Q, *et al.* Effects of steaming the root of *Panax notoginseng* on chemical composition and anticancer activities. Food Chem 2010;118:307-14.
9. Li H, Gu L, Zhong Y, Chen Y, Zhang L, Zhang AR, *et al.* Administration of polysaccharide from *Panax notoginseng* prolonged the survival of H22 tumor-bearing mice. Onco Targets Ther 2016;9:3433-41.
10. Feng S, Cheng H, Xu Z, Feng S, Yuan M, Huang Y, *et al.* Antioxidant and anti-aging activities and structural elucidation of polysaccharides from *Panax notoginseng* root. Process Biochem 2019;78:189-99.
11. Wu Y, Wang D. Structural characterization and DPPH radical scavenging activity of an arabinoglucogalactan from *Panax notoginseng* root. J Nat Prod 2008;71:241-5.
12. Zhu Y, Pettolino F, Mau SL, Shen YC, Chen CF, Kuo YC, *et al.* Immunoactive polysaccharide-rich fractions from *Panax notoginseng*. Planta Med 2006;72:1193-9.
13. Liu S, Yang Y, Qu Y, Guo X, Yang X, Cui X, *et al.* Structural characterization of a novel polysaccharide from *Panax notoginseng* residue and its immunomodulatory activity on bone marrow dendritic cells. Int J Biol Macromol 2020;161:797-809.
14. Wang X, Yang H, Liu L. Comparison for the research of polysaccharide in raw and prepared *Panax notoginseng*. J Henan Univ 2010;29:235-6.
15. Šumić Z, Vakula A, Tepić A, Čakarević J, Vitas J, Pavlić B. Modeling and optimization of red currants vacuum drying process by response surface methodology (RSM). Food Chem 2016;203:465-75.
16. Ferreira SL, Bruns RE, Ferreira HS, Matos GD, David JM, Brandão GC, *et al.* Box-Behnken design: An alternative for the optimization of analytical methods. Anal Chim Acta 2007;597:179-86.
17. Xie RF, Shi ZN, Li ZC, Chen PP, Li YM, Zhou X. Optimization of high pressure machine decocting process for Dachengqi Tang using HPLC fingerprints combined with the Box-Behnken experimental design. J Pharm Anal 2015;5:110-9.
18. Gao H, Wang F, Lien EJ, Trousdale MD. Immunostimulating polysaccharides from *Panax notoginseng*. Pharm Res 1996;13:1196-200.
19. Wang QH, Shu ZP, Xu BQ, Xing N, Jiao WJ, Yang BY, *et al.* Structural characterization and antioxidant activities of polysaccharides from *Citrus aurantium* L. Int J Biol Macromol 2014;67:112-23.
20. Bo R, Ma X, Feng Y, Zhu Q, Huang Y, Liu Z, *et al.* Optimization on conditions of *Lycium barbarum* polysaccharides liposome by RSM and its effects on the peritoneal macrophages function. Carbohydr Polym 2015;117:215-22.
21. Ni H, Xu S, Gu P, Wusiman A, Zhang Y, Qiu T, *et al.* Optimization of preparation conditions for CTAB-modified *Polygonatum sibiricum* polysaccharide cubosomes using the response surface methodology and their effects on splenic lymphocytes. Int J Pharm 2019;559:410-9.
22. Felz S, Vermeulen P, van Loosdrecht MC, Lin YM. Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). Water Res 2019;157:201-8.
23. Tastan O, Baysal T. Clarification of pomegranate juice with chitosan: Changes on quality characteristics during storage. Food Chem 2015;180:211-8.
24. Xue HY, Li JR, Liu YG, Gao Q, Wang XW, Zhang JW, *et al.* Optimization of the ultrafiltration-assisted extraction of Chinese yam polysaccharide using response surface methodology and its biological activity. Int J Biol Macromol 2019;121:1186-93.
25. Wang Q, Sun Y, Yang B, Wang Z, Liu Y, Cao Q, *et al.* Optimization of polysaccharides extraction from seeds of Pharbitis nil and its anti-oxidant activity. Carbohydr Polym 2014;102:460-6.
26. Wang X, Wu Y, Wang F, Yi J, Zhang Y. Optimization of polysaccharide process from *Fructus corni* with box-behnken design and antioxidant capacity. Pak J Pharm Sci 2019;32:1537-44.
27. Prakash Maran JP, Manikandan S. Response surface modeling and optimization of process parameters for aqueous extraction of pigments from prickly pear (*Opuntia ficus-indica*) fruit. Dyes Pigm 2012;95:465-72.
28. Xu Q, Shen Y, Wang H, Zhang N, Xu S, Zhang L. Application of response surface methodology to optimise extraction of flavonoids from fructus sophorae. Food Chem 2013;138:2122-9.
29. Xu L, Yu JQ, Wang XY, Xu N, Liu JL. Microwave extraction optimization using the response surface methodology of Fructus Meliae Toosendan polysaccharides and its antioxidant activity. Int J Biol Macromol 2018;118:1501-10.
30. Ji L, Jie Z, Ying X, Yue Q, Zhou Y, Sun L. Structural characterization of alkali-soluble polysaccharides from *Panax ginseng* C. A. Meyer. R Soc Open Sci 2018;5:171644.
31. Vasilieva T, Sigarev A, Kosyakov D, Ul'yanovskii N, Anikeenko E, Chuhchin D, *et al.* Formation of low molecular weight oligomers from chitin and chitosan stimulated by plasma-assisted processes. Carbohydr Polym 2017;163:54-61.
32. Malinowska E, Klimaszewska M, Strączek T, Schneider K, Kapusta C, Podsadni P, *et al.* Selenized polysaccharides – Biosynthesis and structural analysis. Carbohydr Polym 2018;198:407-17.
33. Borazjani NJ, Tabarsa M, You S, Rezaei M. Purification, molecular properties, structural characterization, and immunomodulatory activities of water soluble polysaccharides from *Sargassum angustifolium*. Int J Biol Macromol 2018;109:793-802.
34. Cai ZN, Li W, Mehmood S, Pan WJ, Wang Y, Meng FJ, *et al.* Structural characterization, *in vitro* and *in vivo* antioxidant activities of a heteropolysaccharide from the fruiting bodies of *Morchella esculenta*. Carbohydr Polym 2018;195:29-38.
35. Shu Y, Liu XB, Ma XH, Gao J, He W, Cao XY, *et al.* Immune response mechanism of mouse monocytes/macrophages treated with κ-carrageenan polysaccharide. Environ Toxicol Pharmacol 2017;53:191-8.
36. Shukla S, Mehta A, John J, Mehta P, Vyas SP, Shukla S. Immunomodulatory activities of the ethanolic extract of *Caesalpinia bonducella* seeds. J Ethnopharmacol 2009;125:252-6.