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Extraction optimization, characterization and immunity activity of polysaccharides from *Fructus Jujubae*

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ABSTRACT

The versatile *Fructus Jujubae* is widely used in Chinese and Korean traditional medicine. In this study, the extraction optimization, characterization and immunostimulatory activities of polysaccharides from *Fructus Jujubae* were investigated. Based on a four-variable-three-level Box–Behnken statistical design, the optimal extraction parameters were optimized as follows: extraction temperature 90 °C, extraction time 3.23 h, water to raw material ratio 33:1 and extraction 3 times. Under these conditions, the experimental yield of polysaccharides was 6.47 ± 0.26%, which was close to the predicted yield value (6.54%). The crude *Fructus Jujubae* polysaccharide was further purified by DEAE-cellulose chromatography repeatedly, and two homogenous fractions, designated as RQP1d and RQP2d with molecular weight of 83.8 and 123.0 kDa respectively, were obtained. Their structures were determined by chemical analysis, Fourier transform infrared (FT-IR) spectroscopy and scanning electron microscopy (SEM). Finally, preliminary immunological tests indicated that both RQP1d and RQP2d significantly stimulated NO production in RAW264.7 macrophages, and promoted LPS-induced splenocyte proliferation. These data implied *Fructus Jujubae* polysaccharides had the potential to be explored as novel natural immunostimulant for using in functional foods or medicine.

1. Introduction

Fructus Jujubae is the fruit of *Ziziphus jujuba* Mill belonging to the Rhamnaceae family and is favorable due to its high nutritional and medicinal values. In Traditional Chinese Medicine, *Fructus Jujubae* is used to treat several diseases such as diabetes, digestive disorders, diarrhea, skin infections, liver complaints, urinary trouble, tumors and cardiovascular diseases (Jin-Wei, Yi-Yong, Shao-Dong, & Lian-Fu, 2007; Li, Ding, & Ding, 2007; Pawlowska, Camangi, Bader, & Braca, 2009). Is effectiveness in replenishing blood (Ko et al., 2006) and protecting the hematopoietic function of bone marrow and immune organs (Wang et al., 2011) were also reported.

Nowadays, polysaccharides have attracted more and more attention for their healthy benefits and therapeutical uses with low toxicity and little side effects (Krifa, Bouhlel, Ghedira-Chekir, & Ghedira, 2013; Schepetkin & Quinn, 2006; Simas-Tosin et al., 2012). For instances, we and others have demonstrated that the polysaccharides possess antioxidant activities (Cui et al., 2013; Mu et al., 2012), and function as immunomodulatory reagents (Bafna & Mishra, 2010; Mikhaeil, Badria, Maatooq, & Amer, 2004; Zhang et al., 2012).

Response surface methodology (RSM) is an efficient mathematical and statistical technique for optimizing processes even in the presence of complex interactions, due to it allows more efficient and interpretation of experiments than other methods (Samavati & Yarmand, 2013; Zhao et al., 2011). Moreover, it is less labor and time-consuming than other methods (Yang, Chen, Zhou, & Zhang, 2013). RSM has been extensively utilized to optimize the extraction process in food and pharmaceutical research.

In this study, the aim was to optimize the process for extraction of water-soluble polysaccharides (RQP) from *Fructus Jujubae* using Response surface methodology (RSM) in which four main factors (extraction time, extraction temperature, extraction times and water to the raw material ratio) were chosen as extraction parameters. In addition, the structures of two purified fractions RQP1d

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0144-8617/

Table 1
Factors and levels of response surface method.

Independent variables	Factor level		
	−1	0	1
X_1 (extraction temperature, °C)	70	80	90
X_2 (extraction time, h)	2	3	4
X_3 (number of extraction)	1	2	3
X_4 (water to raw material ratio)	20:1	30:1	40:1

and RQP2d were reported and their immunomodulatory activities were evaluated *in vitro*.

2. Materials and methods

2.1. Materials

The *Fructus Jujubae* was collected from Ruoqiang, Xinjiang Uygur Autonomous Region, China. Trifluoroacetic acid (TFA), acetic anhydride, sodium borohydride ($NaBH_4$), D-glucose, and galacturonic acid were purchased from Aladdin Reagent Int (Shanghai, China). 3-(4,5-Dimethyl tiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) was obtained from Sigma Chemical Co. Ltd. The RAW264.7 cell line was a kindly gift from Prof. Xuebo Liu in Northwest A&F University. All other reagents were of analytical grade available.

2.2. Preparation of crude RQP

The *Fructus Jujubae* powder was immerged and washed with ethanol to deactivate the steroidal saponins and remove some soluble materials, including free sugars, amino acids and some phenols. The dried residue was extracted with water. The aqueous solution was condensed and a final concentration of 80% ethanol was added to precipitate polymers. Then the precipitates were collected by centrifugation, washed with ethanol, acetone and lyophilized. The water-soluble polysaccharides designated as RQP were obtained. The polysaccharide yield (%) was calculated using the following equation:

$$\text{Polysaccharide yield } \% = \frac{\text{Polysaccharides weight (g)}}{\text{Raw material weight (g)}} \times 100\% \quad (1)$$

2.3. Box–Behnken design

On the basis of single-factor test, the preliminary range of extraction variables was determined. A Box–Behnken design (BBD) with four independent variables (X_1, extraction temperature; X_2, extraction time; X_3, number of extraction; X_4, water to raw material ratio) at three levels was performed. As shown in Table 1, the center point values and ranges of four independent variables were detailed. A design matrix comprising of 29 experimental runs was constructed. The second degree polynomial model was as follows:

$$Y = \sum A_0 + \sum_{i=1}^{4} A_i X_i + \sum_{i=1}^{4} A_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} A_{ij} X_i X_j \quad (2)$$

where Y was the response value, A_0, A_i, A_{ii}, A_{ij}, were the regression coefficients of variables for intercept, linearity, square and interaction terms, respectively, while X_i and X_j were the independent variables ($i \neq j$).

2.4. Separation and purification of the polysaccharides

The RQP was dissolved in distilled water, centrifuged at 3000 × *g* for 10 min to remove insoluble materials. The sample was applied to a DEAE-cellulose column (2.4 cm × 60 cm) which was sequentially eluted with distilled water for 3000 mL at 0.5 mL/min, followed by 0.1, 0.2 M NaCl at a flow rate of 0.5 mL/min. Each fraction was collected and assayed for carbohydrate content by phenol–sulfuric acid method. The water eluate, RQP1 and the 0.1 M NaCl eluate, RQP2 were further purified with DEAE-cellulose chromatography individually. The main polysaccharides fraction (RQP1d and RQP2d) were collected, dialyzed and lyophilized.

2.5. Characterization of RQP1d and RQP2d

2.5.1. Chemical composition analysis

Neutral carbohydrate content was determined by the phenol-sulphuric acid method (Masuko et al., 2005), with D-glucose as standard. Uronic acid content was measured according to m-hydroxybiphenyl-sulphuric acid method and galacturonic acid was used as the standard (Blumenkrantz & Asboe-Hansen, 1973). Protein content was evaluated by Bradford's method (Bradford, 1976) with bovine serum albumin (BSA) as standard. Neutral monosaccharide compositions were analyzed by GC after conversion of the hydrolysate into alditol acetates as described (Duan, Wang, Dong, Fang, & Li, 2003).

2.5.2. Molecular weight determination of RQP1d and RQP2d

The molecular of RQP1d and RQP2d were evaluated by high performance gel permeation chromatography (HPGPC), as described previously (Duan, Zheng, Dong, & Fang, 2004; Zhang et al., 2013). Briefly, three columns (Waters Ultrahydrogel 250, 1000 and 2000; 30 cm × 7.8 mm; 6 m particles) in series were calibrated with T-series Dextrans (molecular weights of 5.2, 10, 48.6, 668, 2000 kDa). The columns were maintained and the mobile phase was sodium acetate (3 mM) with flow rate of 0.5 mL/min. A 100 μL aliquot was injected for each run. The calibration curve of $\log(M_w)$ *vs.* elution time (T) is:

$$\log(M_w) = -0.1171T + 10.45 \quad (3)$$

2.5.3. FT-IR analysis

The organic functional groups of the two polysaccharides were identified using an FTIR spectrophotometer (BRUKER TENSOR 27, BRUKER, Germany). The polysaccharides were pressed into KBr powder and then pressed into a disk. The spectrum were recorded within 4000–400 cm^{-1} (Wu, Zhu, Zhang, Yang, & Zhou, 2012).

2.5.4. SEM analysis

The scanning electron microscope (SEM) images of the two polysaccharides were obtained by field emission scanning electron microscope (FESEM, S-4800, Hitachi, Japan). The dried samples were coated with a thin layer of gold under reduced pressure. They were examined at a 10 kV acceleration voltage under a high vacuum condition, as well as image magnification of 10K×, 100K× and 300K×.

2.6. Assay of immunological activities

Nitrite accumulation was used as an indicator of NO production (Green et al., 1982). RAW264.7 cells (1×10^6/well) were incubated in 96-well plates with PBS (for the control group) or different concentrations of polysaccharides at 37 °C for 24 h. Nitrite in culture supernatants was measured by a Griess reagent and $NaNO_2$ was used as a standard (Zhang et al., 2011).

Lymphocyte proliferations were determined using the colorimetric MTT assay as described previously (Mosmann, 1983). Spleen cells (2×10^6/mL) were seeded into a 96-well plate in the presence of mitogen ConA (5 μg/mL) or LPS (5 μg/mL). The polysaccharide samples in various concentrations (0, 10, 100, 200 μg/mL) were added. After incubation for 48 h at 37 °C in a humidified 5% CO_2

atmosphere, 20 μL of MTT (5 mg/mL) was added into each well, followed by incubation for 4 h, and then 150 μL of DMSO was added into each well to dissolve the precipitation completely. The light absorbance was measured at 570 nm using an ELISA reader after 10 min.

The percentage of proliferation was calculated using the following equation:

$$\text{Proliferation } (\%) = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{control}})}{\text{OD}_{\text{control}}} \times 100 \quad (4)$$

2.7. Statistical analysis

Data are reported as the mean ± SD of three measurements. Design-Expert (Version 8.0, Stat-Ease) was used for the experimental design and the regression analysis of experimental data. The regression coefficients were determined by analysis of variance (ANOVA). The scientific statistic software GraphPad Prism 5.01 was used to evaluate the significance of differences between groups. $p < 0.05$ was regarded as significant.

3. Results and discussion

3.1. Extraction yield of RQP

3.1.1. Effect of extraction temperatures on extraction yield of RQP

To evaluate different extraction temperature (60, 70, 80, 90 and 100 °C) on the extraction yield of RQP, the other three factors were set as follows: extraction time 1 h, number of extraction 2 times and water to raw material 10:1. As shown in Fig. 1A, the extraction yield of RQP increased with the increasing extraction temperature and reached the peak value at 100 °C. This tendency was probably due to the increase of the polysaccharide solubility in the solvent at the higher temperature (Samavati, 2013). Since increasing temperature will result in cost for the extraction process, 70–90 °C was considered in the BBD experiments.

3.1.2. Effect of extraction time on extraction yield of RQP

As reported elsewhere, a long extraction time had a favorable effects on the production of polysaccharides (XuJie & Wei, 2008; Zhongdong, Guohua, Yunchang, & Kennedy, 2006). The effect of different extraction time (1, 2, 3, 4, 5 h) on extraction yield of RQP was shown in Fig. 1B, when the extraction temperature, water to raw material ratio and number of extraction were fixed at 80 °C, 10:1 and 2 times, respectively. The variance of extraction yield was relatively rapid when extraction time varied from 1 to 3 h. The extraction yield of RQP increased slowly with increasing time, and reached a maximum at 5 h. This might be due to the time requirement of the exposure of the RQP to the release water where the liquid penetrated into the dried powdered material, dissolved the RQP and subsequently diffused out from the material (Ye & Jiang, 2011). For saving of energy and lowering of cost 3 h was selected as the center point of extraction time in the BBD experiments.

3.1.3. Effect of number of extraction on extraction yield of RQP

In this study, the effect of number of extraction (1, 2, 3, 4, 5 times) on extraction yield of RQP was investigated, when the other three factors (extraction temperature, extraction time, water to raw material ratio and number of extraction) were fixed at 80 °C, 3 h and 10:1, respectively. As listed in Fig. 1C, it was found that twice of extraction had obviously higher extraction yield than once of extraction, and no longer significantly increased when the extraction times was above twice.

3.1.4. Effect of water to raw material ratio on extraction yield of RQP

Water to raw material ratio, as another important extraction parameter could affect the extraction yield dramatically (Li et al., 2013). If water to raw material ratio is too low, RQP in raw material cannot be completely extracted up. On the contrary, if ratio of water to raw material is too high, it will lead to a higher process cost. In the present experiments, the water to raw material ratio was set at 10:1, 20:1, 30:1, 40:1 and 50:1, while the other factors were given as the follows: extraction temperature 80 °C, extraction time 2 h and extraction number 2 times. It could be founded that the extraction yield of RQP increased obviously with increasing ratio of water to raw material, reached the peak value at 30:1, and remained almost constantly.

According to the single-parameter study, we adopted extraction temperature 70–90 °C extraction time 2–4 h, number of extraction 2–4 and water to raw material ratio 20:1–40:1 for RSM experiments.

3.1.5. Optimization of extraction parameters of polysaccharides

3.1.5.1. Statistical analysis and the model fitting. The BBD in the experimental design consisted of four factors, three levels and three replicates and the five center point runs were carried out to measure the process stability and inherent variability. The experimental conditions and the results of 29 runs with BBD design were presented in Table 2. As shown in Table 2, the extraction yield of RQP values (%) varied from 2.02% to 6.45%.

The results of extraction yield affected by four factors were fitted with a second order polynomial equation, and the values of regression coefficients were calculated. The effects of three variables were highly significant on extraction yield of RQP (Table 3). The extraction yield value could be expressed by the following second order polynomial equations:

$$\begin{aligned} Y = {} & 4.37 + 1.58X_1 + 0.66X_2 + 0.27X_3 + 0.19X_4 + 0.10X_1X_2 \\ & + 0.25X_1X_3 + 0.17X_1X_4 + 0.080X_2X_3 + 0.17X_2X_4 + 0.095X_3X_4 \\ & - 0.21X_1^2 - 0.33X_2^2 - 0.27X_3^2 - 0.46X_4^2 \end{aligned} \quad (5)$$

The statistical significance of regression equation was checked by *F*-test, and ANOVA for response surface quadratic polynomial model were summarized in Table 3. The results of high model *F*-value (125.54) and low *p*-value ($p < 0.0001$) indicated that the models were highly significant. The determination coefficient (R^2) for model (0.9921) was close to 1.0, which represented the satisfactory correlation between actual and predicted values. The adjusted R^2 (Adj. R^2) value was 0.9842, which meant most variation (>98%) of the extraction yield could be predicted by the model, and less than 2% variations could not be explained by the models. The lack-of-fit measured the failure of the model to represent the data in the experimental domain at points which were not included in the regression. The *F*-value of 0.56 and *p*-value of 0.7889 for extraction yield, which implied that the lack of fit was insignificant relative to the pure error due to noise. Adequate precision compared the range of the predicted values at the design points to the average prediction error. Ratio greater than 4 indicated adequate model discrimination. In this research, the value was well above 4.

It can be seen from Table 3 that linear coefficients (X_1, X_2, X_3), quadratic term coefficient (X_1^2, X_2^2, X_3^2, X_4^2) and cross product coefficients (X_1X_3, X_1X_4, X_2X_3) were significant with very small *p* values ($p < 0.05$). The other term coefficients were not significant ($p > 0.05$).

3.1.5.2. Analysis of response surfaces. The 3D response surface and 2D contour plots are the graphical representations of regression

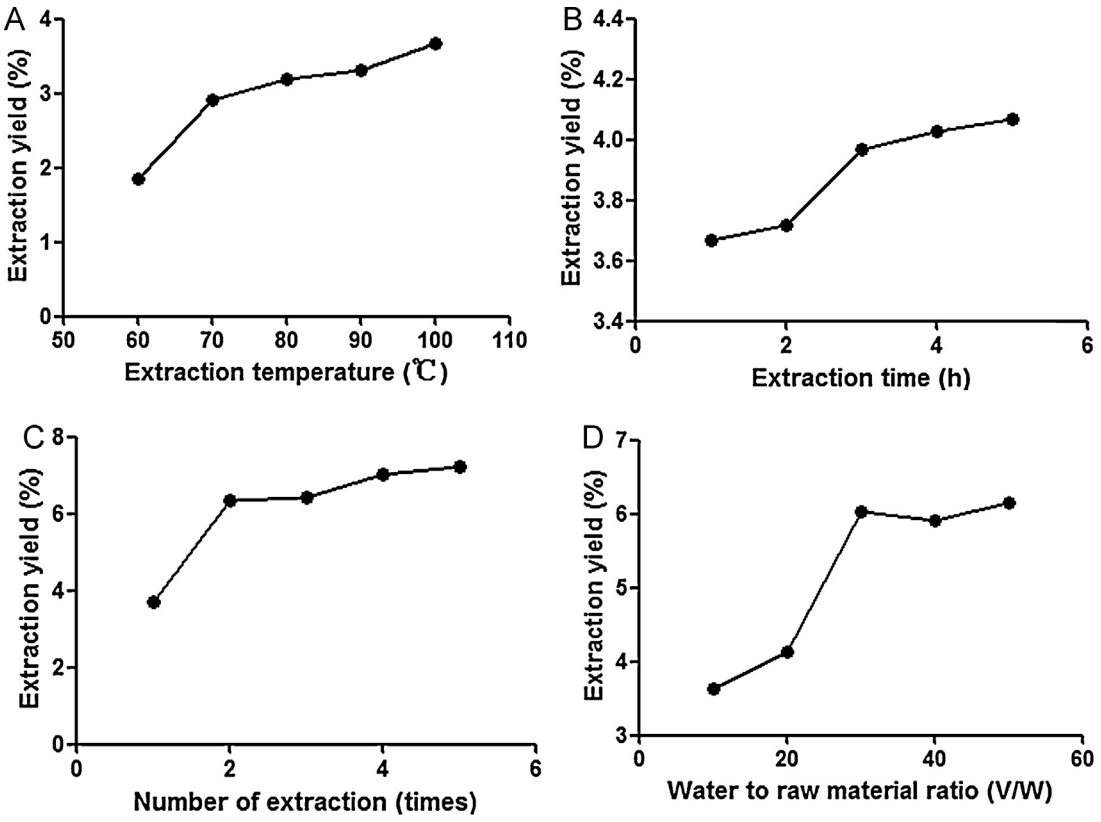

Fig. 1. Effects of different extraction temperature (A), extraction time (B), number of extraction (C) and water to raw material ratio (D) on extraction yield of RQP.

Table 2
Box–Behnken experiment design and the results (means of triplicate tests) for extraction yield of RQP.

Run	X_1 Temperature	X_2 Time	X_3 Number	X_4 Ratio	Extraction yield %	
					Experimental	Predicted
1	−1	−1	0	0	2.02	2.03
2	1	−1	0	0	5.07	5.07
3	−1	1	0	0	2.76	2.63
4	1	1	0	0	6.04	5.89
5	0	0	−1	−1	3.69	3.66
6	0	0	1	−1	4.69	4.54
7	0	0	−1	1	3.89	3.90
8	0	0	1	1	4.75	4.64
9	−1	0	0	−1	2.58	2.57
10	1	0	0	−1	5.37	5.38
11	−1	0	0	1	2.39	2.40
12	1	0	0	1	5.87	5.90
13	0	−1	−1	0	3.28	3.11
14	0	1	−1	0	4.13	4.14
15	0	−1	1	0	4.23	4.24
16	0	1	1	0	4.45	4.63
17	−1	0	−1	0	2.43	2.52
18	1	0	−1	0	5.02	5.10
19	−1	0	1	0	2.72	2.76
20	1	0	1	0	6.45	6.48
21	0	−1	0	−1	3.31	3.41
22	0	1	0	−1	4.1	4.17
23	0	−1	0	1	3.59	3.63
24	0	1	0	1	4.28	4.29
25	0	0	0	0	4.48	4.79
26	0	0	0	0	4.88	4.79
27	0	0	0	0	4.89	4.79
28	0	0	0	0	4.87	4.79
29	0	0	0	0	4.81	4.79

Table 3
ANOVA for response surface quadratic model for the yield of RQP.

Source	Sum of squares	df	Mean square	*F* value	Prob > *F*
Model	36.61	14	2.61	125.54	<0.0001
X_1	29.83	1	29.83	1432.22	<0.0001
X_2	1.51	1	1.51	72.61	<0.0001
X_3	1.96	1	1.96	94.11	<0.0001
X_4	0.088	1	0.088	4.24	0.0585
$X_1\ X_2$	0.013	1	0.013	0.63	0.4388
$X_1\ X_3$	0.32	1	0.32	15.6	0.0015
$X_1\ X_4$	0.12	1	0.12	5.71	0.0314
$X_2\ X_3$	0.099	1	0.099	4.76	0.0466
$X_2\ X_4$	2.50×10^{-3}	1	2.50×10^{-3}	0.12	0.7342
$X_3\ X_4$	4.90×10^{-3}	1	4.90×10^{-3}	0.24	0.6352
X_1^2	0.79	1	0.79	37.99	<0.0001
X_2^2	1.83	1	1.83	88.06	<0.0001
X_3^2	0.32	1	0.32	15.49	0.0015
X_4^2	0.91	1	0.91	43.91	<0.0001
Residual	0.29	14	0.021		
Lack of fit	0.17	10	0.017	0.56	0.7889
Pure error	0.12	4	0.03		
Cor total	36.9	28			
R^2	0.9921				
Adj R^2	0.9842				
Pred R^2	0.9682				
Adeq precision	42.861				
C.V.%	3.46				

equation. They provide a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. Circular contour plot indicates that the interactions between the corresponding variables are negligible, while elliptical contour plot indicates that the interactions between the corresponding variables are significant (Yang et al., 2013). In this study, the results of extraction yield of RQP affected by extraction temperature, extraction time, number of extraction cycle and water to raw material ratio were presented in Figs. 2 and 3.

Figs. 2A and 3A, which demonstrated the extraction yield of RQP as a function of extraction temperature and time at fixed number of extraction cycle (2) and water to raw material ratio (30:1), indicated that the extraction yield increased rapidly with increase of extraction temperature from 70 °C to 90 °C. However, with the extraction time from 2 h to 4 h, the extraction yield only had a small increase.

The extraction yield of RQP affected by different extraction temperature and number of extraction cycle was shown in Figs. 2B and 3B, when the extraction time and water to raw material ratio were fixed at 3 h and 30:1 respectively. It appeared that the extraction yield of RQP increased with the increase in number of extraction cycle from 1 to 3. The extraction yield of RQP was found to increase rapidly following the increase of extraction temperature from 70 °C to 90 °C. The maximum extraction yield of RQP was achieved when extraction temperature and number of extraction cycle were set around 90 °C and 3 times, respectively.

Figs. 2C and 3C showed the 3D response surface plot and the contour plot at different extraction temperature and water to raw material ratio at fixed extraction time (3 h) and number of extraction cycle (2). The maximum extraction yield of RQP could be achieved when extraction temperature and ratio of water to raw material were kept at 90 °C and 30:1, respectively.

Figs. 2D and 3D illustrated the 3D response surface plot and the contour plot at varying extraction time and number of extraction cycle at fixed extraction temperature (80 °C) and water to raw material ratio (30:1). The extraction yield of RQP increased with the increasing extraction time from 2 h to 3 h and reached the maximum value when the number of extraction at the threshold level of 3.

In Figs. 2E and 3E, when the 3D response surface plot and the contour plot were developed for the extraction yield of RQP with varying extraction time and water to raw material ratio at fixed extraction temperature (80 °C) and number of extraction cycle (2 times). The yield of RQP increased rapidly with the extraction time from 2 h to 3 h as well as water to raw material ratio from 20:1 to 30:1. The maximum value reached rapidly at an extraction time around 3 h, but beyond this ratio, extraction yield of RQP did not further increase.

The 3D response surface plot and the contour plot based on the independent variable number of extraction and ratio of water to the raw material were shown in Figs. 2F and 3F, while the other two independent variables, extraction time and temperature were kept at 2 h and 80 °C, respectively. An increase in the extraction yield of RQP could be significantly achieved with the increasing of extraction number. The extraction yield of RQP increased with the ratio of water to raw material from 20:1 to 30:1, while a further increase of water to raw material ratio would not increase the extraction yield of RQP anymore.

3.1.6. *Verification of predictive model*

In order to validate the adequacy of the model equations, verification experiment was carried out under the optimal conditions: extraction temperature 90 °C, extraction time 3.23 h, extraction 3 times and ratio of water to raw material 33:1. A mean value of 6.47 ± 0.26% ($n = 5$), obtained from real experiments was close to the predicted yield value (6.54%). These data indicated that there was no significant difference between experimental and predicted

Table 4
Chemical compositions of RQP1d and RQP2d.

Sample	Neutral carbohydrate (%)	Uronic acid (%)	Protein %	Molecular weight
RQP1d	29.8 ± 4.668	63.27 ± 2.131	0	83,775.3
RQP2d	50.8 ± 10.17	15.82 ± 0.752	17.05 ± 1.393	122,985.5

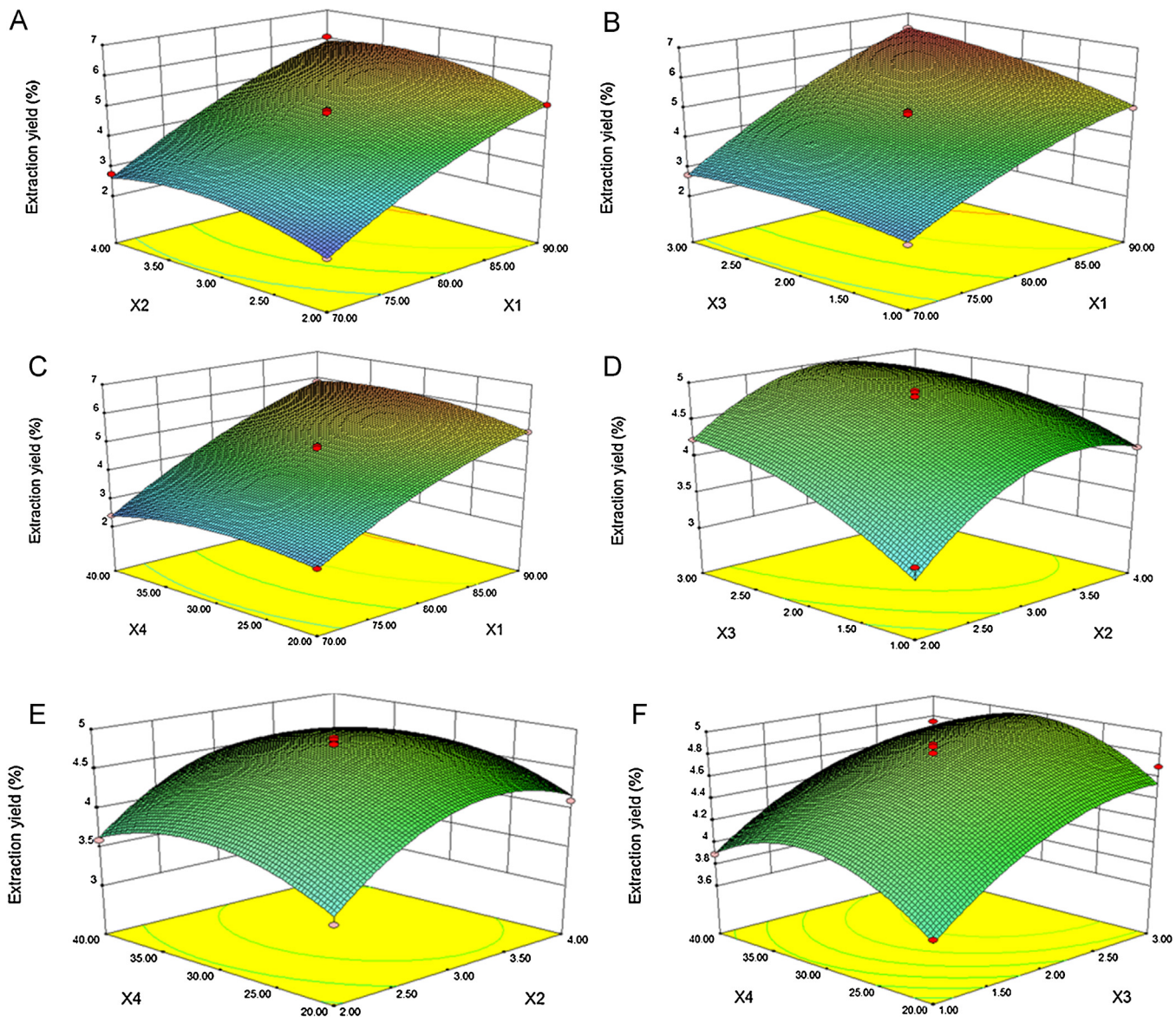

Fig. 2. Response surface plots showing the effect of the extraction temperature (X_1), extraction time (X_2), number of extraction (X_3) and water to raw material ratio (X_4) on the extraction yield of RQP.

values, suggesting that the response model was adequate for reflecting the expected optimization.

3.2. Structural features of RQP1d and RQP2d

3.2.1. Basic properties of RQP1d and RQP2d

RQP was repeatedly subjected to DEAE-cellulose chromatography, which yielded two homogenous fractions, RQP1d and RQP2d. Its molecular weight was estimated to be 83,775.3 Da and 122,985.5 Da, respectively. The chemical compositions of RQP1d and RQP2d were listed in Table 4. RQP1d was free of protein and contained large amount of uronic acid. In contrast, RQP2d was predominant in neutral carbohydrates, along with 17.05% protein.

To determine the monosaccharide composition, RQP1d and RQP2d were hydrolyzed by trifluoracetic acid and the hydrolytes were further acetylated for gas chromatography (GC) analysis. As shown in Table 5, RQP1d was mainly composed of arabinose, xylose and galactose while RQP2d contained arabinose and xylose predominantly.

Table 5
Neutral monosaccharide composition of RQP1d and RQP2d.

Samples	Rha	Ara	Xyl	Gal	Glc	Man
RQP1d	9.763	23.439	18.765	31.145	2.072	4.391
RQP2d	3.165	32.7763	35.721	7.821	5.503	0.347

3.2.2. FTIR analysis

FTIR spectroscopy is used for the qualitative measurement of organic functional groups (Wu et al., 2012). FTIR spectra of RQP1d and RQP2d were shown in Fig. 4. The band around 3427 cm^{-1} indicated O–H stretching vibration. The absorbance peaks nearly 2938 cm^{-1} may attribute to the symmetric C–H vibration decreased of the polysaccharides. Two distinct absorbance peaks around 1742 cm^{-1} and 1615 cm^{-1} may be due to the presence of uronic acids in the polysaccharides. The peaks toward 1000–1200 cm^{-1} suggested the presence of C–O–C and C–O–H, indicating the presence of pyranose.

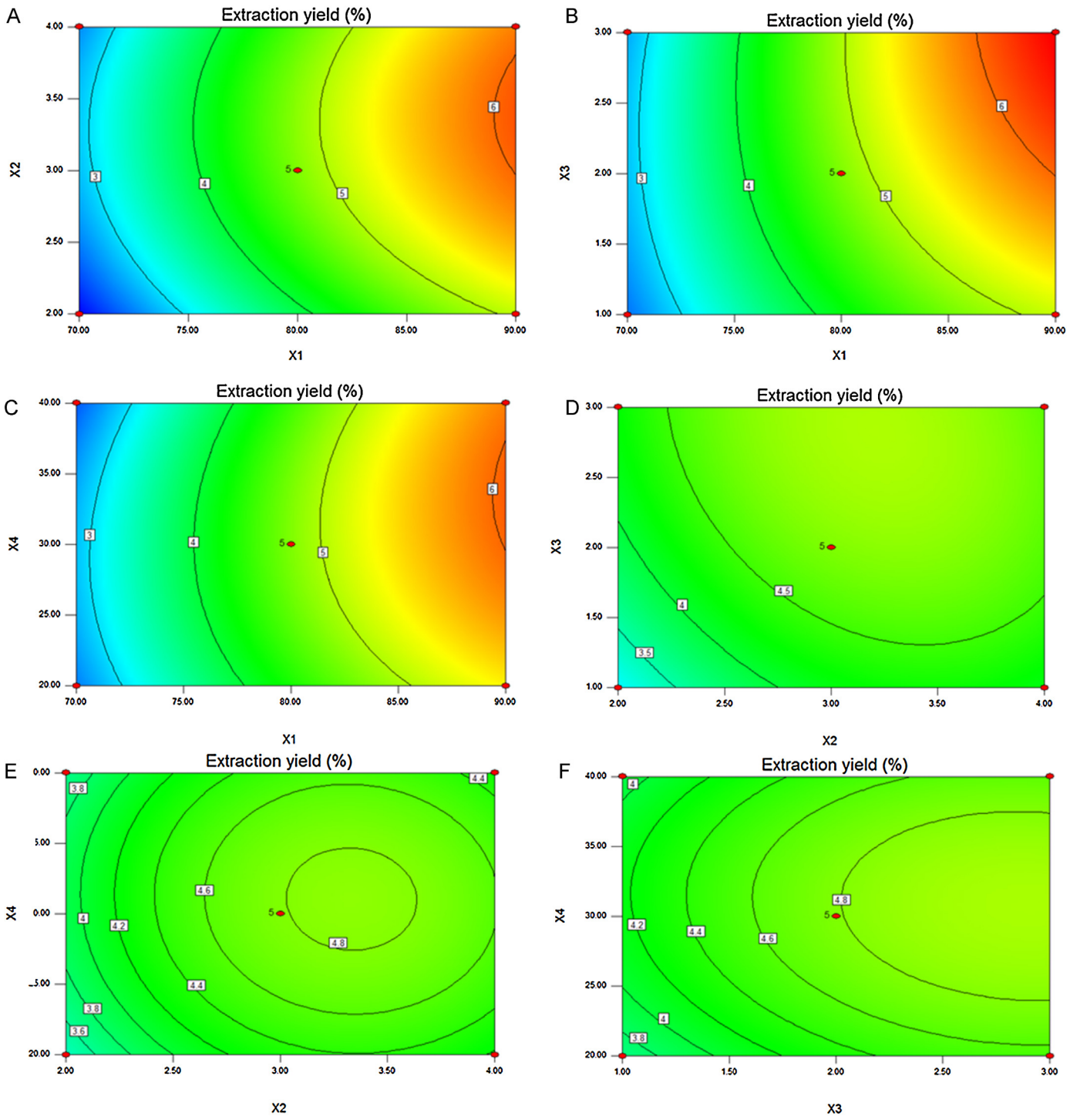

Fig. 3. Contour plots showing the effect of the extraction temperature (X_1), extraction time (X_2), number of extraction (X_3) and water to raw material ratio (X_4) on the extraction yield of RQP.

3.2.3. SEM analysis

The SEM images of the two polysaccharides were shown in Fig. 5. The surface of RQP1d and RQP2d were quite similar, and appeared to be rough with characteristic of large wrinkles.

3.3. Immunological activities of RQP1d and RQP2d

The immune system is the human's ultimate defense against infectious diseases, tumor and cancer growth. The immunologic action of polysaccharides may begin with activating major subsets of immune cells such as lymphocytes and macrophages (Chen et al., 2012a). Therefore, we tried to explore effects of RQP1d and RQP2d on the activation of macrophages and lymphocyte proliferation.

3.3.1. Effect of RQP1d and RQP2d on RAW264.7 cells NO production

NO is a gaseous molecule synthesized from L-arginine by nitric oxide synthase (NOS) and it acts as a cytotoxic agent in pathological processes, an inducer or suppressor of apoptosis and an immunoregulator (Coleman, 2001). NO has been identified as a

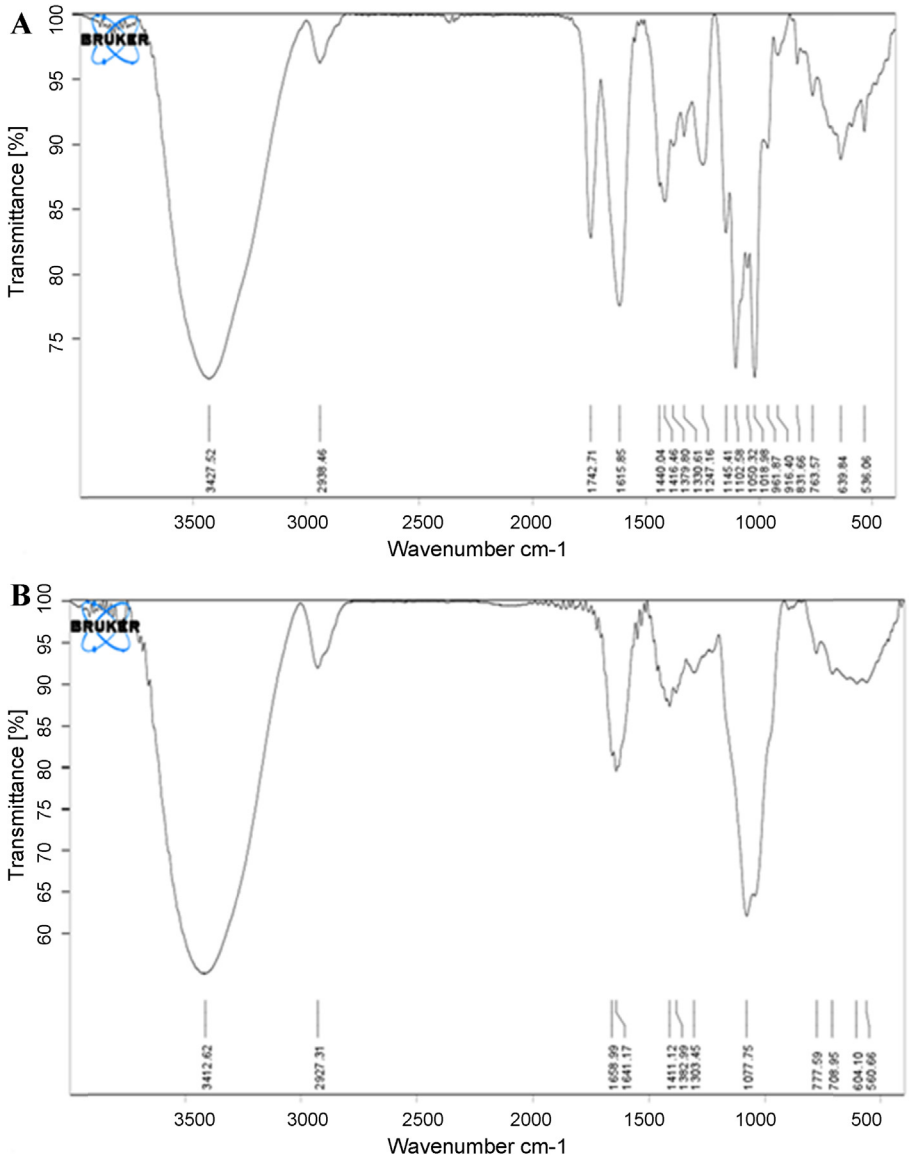

Fig. 4. Infrared spectra of polysaccharides: (A) RQP1d; (B) RQP2d.

major effector molecule produced by macrophages (Hibbs, Taintor, & Vavrin, 1987; Palmer, Ashton, & Moncada, 1988; Schepetkin, Faulkner, Nelson-Overton, Wiley, & Quinn, 2005) and was believed as a quantitative index of macrophage activation (Li & Xu, 2011). It was reported that different polysaccharides were related to different kinds of macrophage activation (Xia et al., 2012). As shown in Fig. 6, both RQP1d and RQP2d induced NO production in RAW264.7 cells in a concentration dependent manner. NO production was found to increase ($p < 0.05$) approximately 3 times compared with that of the untreated group at 48 h. The increased release of NO reflects phagocytic stimulation upon RQP1d or RQP2d. In addition, NO production was detectable after 6 h stimulation, suggesting there was a quick immune response to RQP1d and RQP2d, which suggested that both RQP1d and RQP2d could act as an immunostimulant of innate immunity. Indeed, there are various surface receptors on macrophages, which are known as pattern recognition molecules such as polysaccharides and can recognize foreign ligands during initial phases of the immune response (Gordon, 2002).

3.3.2. *Effect of RQP1d and RQP2d on splenocyte proliferation*

The immune response including both cellular and humoral immunity, characterized by T cells and B cells respectively, plays an important role in the host defense system such as anti-tumor and infectious defenses (Jeong, Koyyalamudi, Jeong, Song, & Pang, 2012; Schepetkin & Quinn, 2006). The capacity to elicit an effective T and B cells immunity could be shown by the stimulation of lymphocyte proliferation response (Chen et al., 2012b). The splenocyte proliferation is an indicator of immunoenhancement and is related to immunity improvement of T-lymphocytes or B-lymphocytes (Chen et al., 2012b; Marciani et al., 2000). Effects of RQP1d and RQP2d on splenocyte proliferation with mitogens (ConA or LPS) were shown in Fig. 7. It was demonstrated that both RQP1d and RQP2d did not promote ConA-stimulated splenocyte proliferation at concentration tested. In contrast, RQP1d at three different concentrations (10, 100, 200 μg/mL) improved LPS-induced cell proliferation. Interestingly, only a low concentration (10 μg/mL) of RQP2d had a synergistic effect with LPS. The fact that a high concentration was not essential for stronger effects, was in accordance with some other reports on the immunologic activity of plant polysaccharides (Choi, Koo, & Hwang, 2004; Raveendran Nair et al., 2004; Xia et al., 2012). The differences in immune activity of the two polysaccharides may be due to the chemical differences. It is generally known that the immunomodulating actions of polysaccharides are related to their molecular weight, chemical composition, glycosidic

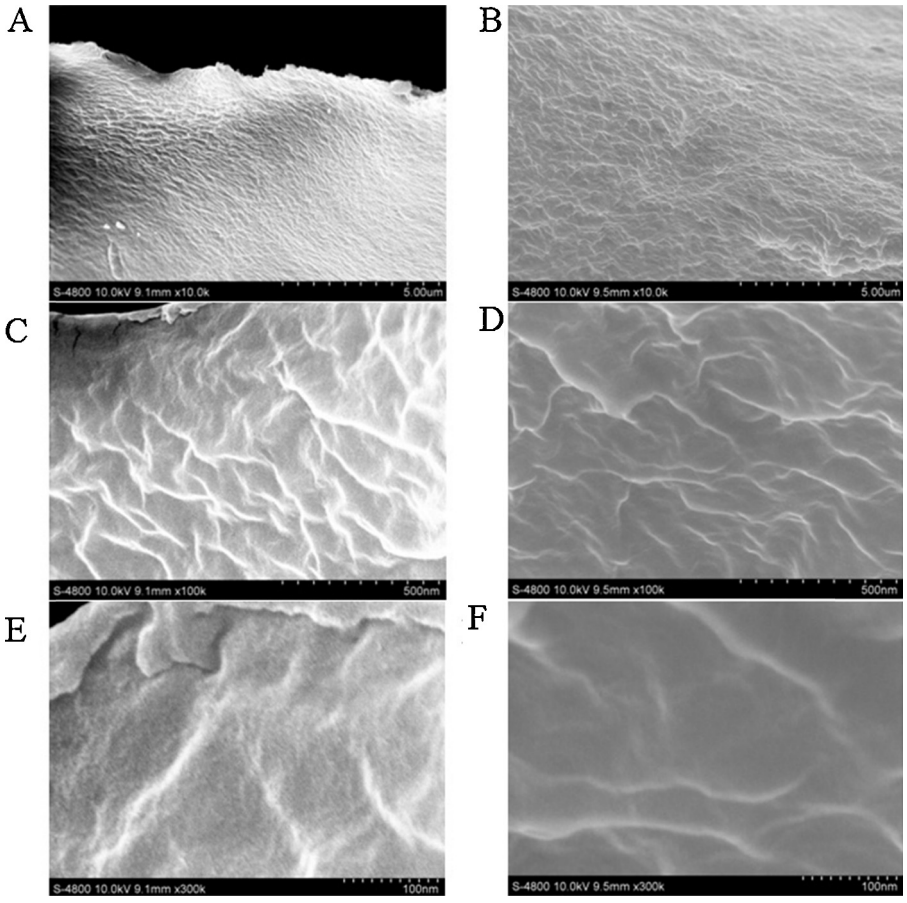

Fig. 5. Scanning electron microscope (SEM) photographs of RQP1d (A, 10K×; C, 100K×; E, 300K×) and RQP2d (B, 10K×; D, 100K×; F, 300K×).

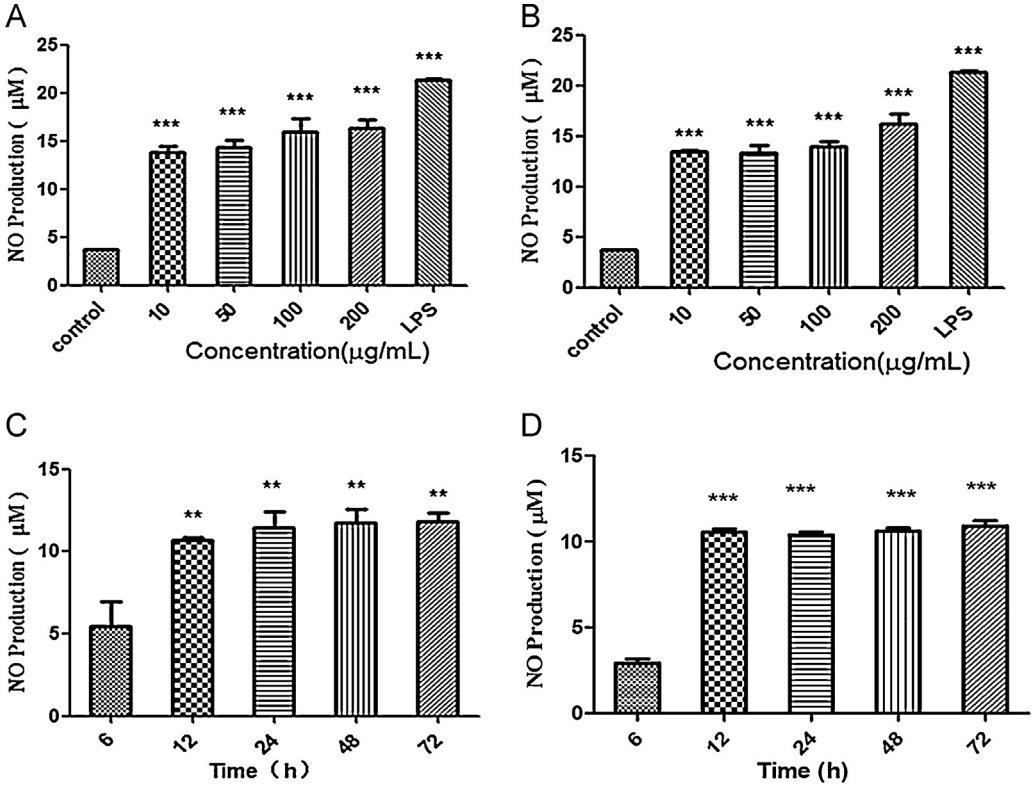

Fig. 6. Effects of RQP1d and RQP2d on NO production in RAW264.7 cells. (A and B) Cells were stimulated with RQP1d (A), RQP2d (B) at the doses of 10–200 μg/mL or LPS (1 μg/mL) for 48 h; (C and D) RAW264.7 cells were incubated with 200 μg/mL RQP1d (C) or RQP2d (D) for 6, 12, 24, 48 and 72 h. These experiments were performed three times with similar results. The significance of difference from control group (or 6 h group) was evaluated with *t*-test (***$p < 0.001$ and **$p < 0.01$).

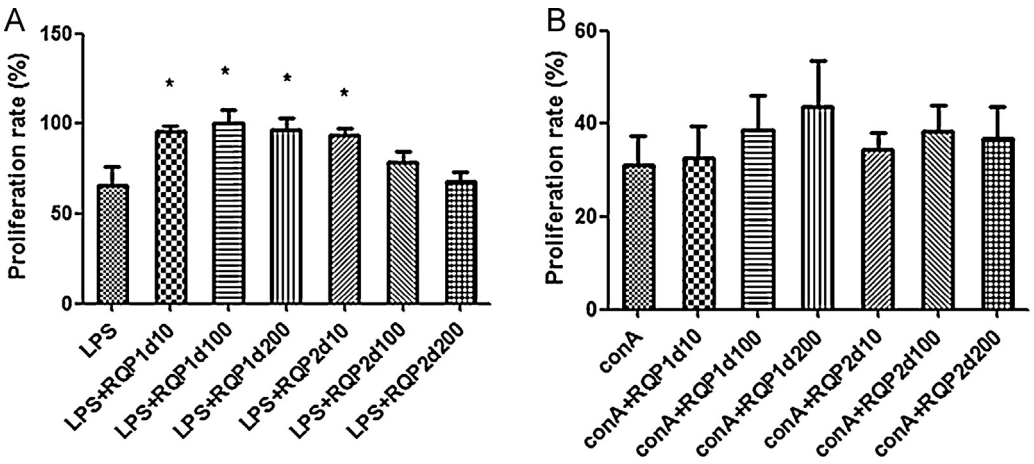

Fig. 7. Effects of RQP1d and RQP2d on splenocytes proliferation. Splenocytes were treated with different concentrations (10, 100, 200 μg/mL) of RQP1d and RQP2d in the presence of mitogens LPS (A) (5 μg/mL) or Con A (B) (5 μg/mL) for 48 h. Cell proliferation was measured using MTT test. These experiments were performed three times with similar results. Error bars represent SD. The significance of difference from LPS group was evaluated with *t*-test ($^*p < 0.05$).

linkage, conformation, degree of branching and so on (Methacanon, Madla, Kirtikara, & Prasitsil, 2005). This structural variability can profoundly affect the biological activities of these molecules (Desai, Ramkrishnan, Chintalwar, & Sainis, 2007). Our results demonstrate that RQP1d and RQP2d were different in chemical composition and molecular weight (Tables 4 and 5).

4. Conclusion

In this study, we optimized extraction parameters of water-soluble polysaccharides from *Fructus Jujubae* by RSM with a BBD design. The optimal extraction conditions for RQP were obtained as follows: extraction temperature 90 °C, extraction time 3.23 h, the ratio of water to material at 33:1 and extraction 3 times. Under this condition, the experimental yield was well agreed with the predicted value. Also, two homogenous fractions, RQP1d and RQP2d were purified by column chromatography. Chemical analysis indicated that RQP1d was free of protein and contained large amount of uronic acid while RQP2d was predominant in neutral carbohydrates with 17.05% protein. The biological tests *in vitro* indicated both RQP1d and RQP2d had a potential to be a stimulant of immune response.

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