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# Extraction, purification, characterization and antioxidant activities of polysaccharides from *Zizyphus jujuba cv. Linzexiaozao*

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ABSTRACT

The extraction process, purification and characterization analyses of polysaccharides (LZJP) in *Ziziphus jujuba* planted in Linze County, Gansu Province were investigated, respectively. The results showed a maximum polysaccharide yield of 5.72% was achieved at a solid/liquid ratio of 1:20 g/mL for 90 min at 80 °C. Two homogenous acidic polysaccharides (LZJP3 and LZJP4) were purified successively by DEAE-52 cellulose and Sephadex G-100 column chromatography. LZJP3 is composed of one polymer with galactose while LZJP4 is made up of two different kinds of polymers with xylose and glucose by size-exclusion chromatograph combined with multi-angle laser photometer (HPSEC-LLS) and gas chromatography (GC) analysis. LZJP3 and LZJP4 were β-pyran polysaccharides with a large number of molecular globular aggregates by FT-IR (Fourier-transform infrared) and AFM (Atomic force microscopy) analysis, and the surface morphology exhibited smooth and filamentous staggered extension in the form of rod-like aggregation with SEM (Scanning electron microscopy) determination. Meanwhile, LZJP3 and LZJP4 exhibited antioxidant activities against DPPH, hydroxyl radical, hydrogen peroxide, superoxide radical and stronger reducing power *in vitro* with the concentration increasing. The results indicated that LZJPs were worthy of being developed further as a natural antioxidant in food and medicine industries.



## 1. Introduction

Jujube (*Ziziphus jujuba Mill.*), *Ziziphus* genus, Rhamnaceae family, is widely cultivated in the subtropical and tropical regions in Australia, southern and eastern Asia, and Europe, especially, distributed in the Yellow River basin of China [1]. Ancient Chinese books on herbal medicine long considered the jujube to be one of the five most valuable fruits (Huangdi Neijing, 475–221 BCE) and also an excellent herbal medicine (Shen-nong Bencao Jing 300 BCE–200 CE) [2]. Modern medical studies have shown that jujube is rich in active polysaccharides, cyclic adenosine phosphate, pentacyclic triterpene compounds and other bioactive substances [3]. Daily consumption of jujube could help to calm the mind, improve the quality of sleep and prolong the life-span [4]. Polysaccharides had been found to have bioactivities in the immune system [5, 6], anti-tumor [7], anti-virus [8], anti-ulcer [9], and anti-oxidation [10]. More and more researchers have focused on the extraction, purification, bio-function of polysaccharides from different jujube cultivars [11–15].

Various methods have been used to improve the extraction efficiency of polysaccharide from different jujubes including treatment with enzymes, microwave and high power ultrasound by Response surface methodology (RSM) [15–21]. Hot extraction is the classical and most convenient extraction method, and widely used in industry [26]. Liu [20] optimized the hot water extraction conditions of jujube polysaccharides from Changzao and obtained the optimal extraction conditions with a maximum yield of 8.02%, including an extraction time of 5 h, extraction temperature of 90 °C, solid/liquid ratio of 1:11. Zhao et al. [21] extracted the water-soluble pectic polysaccharides composed with uronic acid, arabinose and galactose from Chinese Jujube (*Ziziphus jujuba Mill. cv. Dongzao*) with hot water. Rostami et al. [16] optimized the microwave extraction conditions for maximizing the yield of jujube polysaccharides from the capital of Southern Khorasan province in Eastern Iran using RSM. Ultrasound-assisted aqueous two-phase extraction (UAATPE) performed *via* a one-step procedure was applied to extract polysaccharides from *Ziziphus jujuba cv. Muzao* by Li et al. [11].

RSM has been widely used in statistics and mathematical optimization of various factors with Central composite design (CCD), Doehlert

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design (DD) and Box-Behnken design (BBD) [22], which can simulate a true limit state surface by fitting a response surface through a series of deterministic "experiments", and simultaneously consider the relationship between various influencing factors to optimize the experimental results of multi-influencing factors [23–25].

In our previous study, the main components of *Ziziphus jujuba cv. Linzexiaoza* planted in Linze County, Gansu province, China, were determined including total sugar, crude protein, and inorganic elements, the mean of sugar content reached at 72% to 80% with the higher content compared with the others 22 kinds of famous jujubes such as Jinsixiaozao, Junzao, Huizao *etc.* [27]. The jujube average weight is about 4.3 g with the longitudinal and transverse diameter of about 2.3 × 1.8 cm, which indicates that the fruit is small, suitable for the development of traditional Chinese medicine preparations. In this paper, the optimum hot-extraction conditions of polysaccharides from *Ziziphus jujube cv. Linzexiaozao* were obtained by the Box-Behnken response surface method. Meanwhile, purification, monosaccharide composition, molecular weight, physicochemical property and surface structure of the polysaccharides was studied by DEAE-52 cellulose and Sephadex G-100 columns, size-exclusion chromatography combined with multi-angle laser photometer (HPSEC-LLS), Gas chromatography (GC), Fourier transform infrared spectrum (FT-IR), Atomic force microscope (AFM), and Scanning electron microscopy (SEM), respectively. Moreover, the antioxidant activity of LZJP *in vitro* was also evaluated.

## 2. Materials and methods

### 2.1. Materials

Jujube fruits were provided by Zeyuan agricultural technology Co., Ltd., Linze County, Zhangye City, Gansu Province, China. After natural drying, they were crushed into powder with the pulverizer (Tianjin Taisite Instrument Co. Ltd., Tianjin, China), then sifted through a 60 mesh sieve for polysaccharide extraction.

### 2.2. Extraction process

Each sample of jujube fruit powder (5.0 g) was mixed with distilled water under the predesigned solid/liquid ratio, temperature and time in a water-bath (HH-4, Beijing Kowei Yongxing Instrument Co. Ltd., Beijing, China) with the extraction of polysaccharides, the mixture was centrifuged at 3900 r/min for 10 min to collect the supernatant. The supernatant was concentrated to 200 mL using rotary evaporation (R-1001VW, Zhengzhou Greatwall Scientific Industrial and Trade Co. Ltd., Henan, China) and then precipitated with the final concentration of 80% (v/v) ethanol and kept for 12 h at 4 °C. The precipitate was collected by centrifugation (TD5A-WS, Changsha Xiangyi Centrifuge Instrument Co. Ltd., Hunan, China) at 3900 r/min for 10 min, and lyophilized (FDU-1200, Ai Lang Instrument Co. Ltd., Shanghai, China) to obtain crude polysaccharides named LZJP. Total polysaccharide was detected by the phenol sulfuric acid method. The content of LZJP was calculated according to equation of linear regression ($Y = 1.312x - 0.0161$, $R^2 = 0.9979$) based on the standard curve whose horizontal coordinate and vertical coordinate denoted the concentration of glucose (mg/mL) and $OD_{485}$, respectively. The LZJP yield (%) was calculated using the following equation:

$$\text{LZJP yeild}(\%) = \frac{C \times V \times N}{W \times 1000} \quad (1)$$

where C is the concentration of LZJP calculated by the calibrated regression equation (mg/mL); N is the dilution factor; V is the total volume of extraction solution (mL); and W is the weight of jujube fruit powder (g).

#### 2.2.1. Single factor experimental design

Every extraction process was performed one variable with the other two constant tested factors. In detail, the tested factors included the solid/liquid ratio (1:10, 1:15, 1:20, 1:25 g/mL), extraction temperature (60, 70, 80, 90 °C) and extraction duration (30, 60, 90 min). The content of LZJP was measured based on the abovementioned method.

#### 2.2.2. Box-Behnken design and statistical analysis

According to the results of single factor experiment, Box-Behnken design with three independent variables of solid/liquid ratio (A), extraction temperature (B) and extraction duration (C) was applied to further optimize the hot water extraction conditions of LZJP at three levels. The complete quadratic equation was used as follows:

$$Y = \beta 0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta ii X_i^2 + \sum_{i=1}^{3} \sum_{i=1}^{3} \beta ij X_i \beta i X j \quad (2)$$

where Y denotes the response function, $X_i$ and $X_j$ were the independent variables, and $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ represented the constant, linear coefficient, second-order interaction and quadratic coefficient of the model, respectively.

### 2.3. Isolation and purification of LZJP

LZJP was dissolved with distilled water, and removed the protein by sevage method, loaded on a DEAE-52 cellulose column (2.6 cm × 20 cm) with the LZJP of 50 mg/mL, and stepwise eluted with 0, 0.1, 0.3, 0.5 mol/L sodium chloride (NaCl) solution at a flow rate of 1 mL/min, respectively. Eluate (5 mL/tube) was collected and further purified with the distilled water by Sephadex G-100 column chromatography at a flow rate of 0.8 mL/min. The LZJP content of fractions was determined by the phenol sulfuric acid method, and the main purified polysaccharides were named LZJPs for further study.

### 2.4. Characterization of LZJPs

#### 2.4.1. Physical and chemical properties analyses

Color and solubility of LZJP were respectively observed in various solvents (hot and cold water, ethanol, methanol, and acetone). Starchy polysaccharide was determined by iodine reaction [28]. Reducing sugar was determined by Fehling reagent [29]. Uronic acids was assayed by the sulfuric acid–carbazole reaction according to the procedure outlined by Bitter et al. [30]. Protein was measured by Coomassie brilliant blue reaction [31]. Each experiment was performed in triplicate.

#### 2.4.2. Molecular weight analysis

The molecular weight (Mw) of LZJPs was determined according to Wang et al. [32] by size-exclusion chromatography combined with multi-angle laser photometer (HPSEC-LLS) (λ = 690 nm; DAWN EOS, Wyatt Technology Co., USA), which was coupled with Ultrahydrogel™ column (7.8 × 300 mm, Waters, USA). An Optilab refractometer (Dawn, Wyatt Technology Co., USA) was connected, simultaneously. LZJPs were prepared with the concentration of 0.5 mg/mL, and optical clarification of the LZJPs was achieved by filtrating them into a scattering cell. The injection volume was 20 μL with the flow rate of 1 mL/min. A specific refractive index increments (dn/dc) value of the LZJPs in distilled water determined at 633 nm (25 °C) was 0.135 mL/g. Astra Software was used for data analysis.

#### 2.4.3. Analysis of monosaccharide compositions

LZJPs (20 mg) was hydrolyzed with 20 mL of 1.0 M trifluoroacetic acid (TFA) at 120 °C for 6 h in a sealed glass tube, then neutralized, washed and dried, respectively. Acetylation was then carried out using the trifluoroacetic anhydride reagent [33]. The derivatives were analyzed by gas chromatography (GC) with DB-5 chromatographic column

(0.2 mm × 35 m × 0.25 μm, American Agilent) equipped with a flame-ionization detector (FID). Seven standard sugars (lyxose rhamnose, arabinose, xylose, mannose, glucose and galactose) were converted to their acetylated derivatives and analyzed. The GC operation was performed as $N_2$ of 15 mL/min, injection temperature of 210 °C, detector temperature of 300 °C, column temperature programmed from 180 to 220 °C at 10 °C/min.

*2.4.4. Infrared spectra analysis*

LZJPs (5 mg) was grounded with KBr powder and then pressed into pellets for FT-IR measurement (Nexus 670, America) in the frequency range of 400–4000 $cm^{-1}$ at a resolution of 0.09 $cm^{-1}$ [34].

*2.4.5. Atomic force microscopy (AFM) analysis*

LZJPs aqueous solution (1 μg/mL) were dropped onto the surface of a mica sample carrier and allowed to dry at room temperature, and then imaged in air using atomic force microscopy.

*2.4.6. Scanning electron microscopy (SEM) analysis*

LZJPs were individually coated with a thin layer of gold under reduced pressure, and then examined using a scanning electron microscope (JSM-5600LV, American Kevex Company, America) with an acceleration voltage of 20 kV under a high vacuum condition.

*2.5. Determination of antioxidant activities*

*2.5.1. The total reducing power*

The total reducing power of LZJPs was determined according to the method of Yildirim et al. [35] with slight modifications. 1 mL of LZJPs with the different concentrations (0.2–1.0 mg/mL) was mixed with 0.2 mL of potassium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of 1% potassium ferricyanide solution. The reaction mixtures were incubated at 50 °C for 20 min, and 1.0 mL of 10% trichloroacetic acid (TCA) was added into the reaction mixtures, then centrifuged at 3000 rpm for 10 min at room temperature. Finally, 1.5 mL of the supernatant solution was mixed with 3.0 mL of distilled water and 0.2 mL of 0.1% ferric chloride, and the absorbance of the mixture solutions was measured at 700 nm after incubation for 10 min. Three replicates were carried out for each test sample.

*2.5.2. DPPH radical scavenging activity*

DPPH radical scavenging activity of LZJPs was measured according to Brand-Williams et al. [36] with necessary modifications. Briefly, 1.0 mL of different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) of LZJPs prepared in deionized water mixed with 1.0 mL DPPH-ethanol solution. The mixture reaction solution incubated at 37 °C for 30 min, and measured at 517 nm in a spectrophotometer (Carry50, American, Varian) with deionized water as the negative control. Ascorbic acid (Vc) and butylated hydroxytoluene (BHT) were used as the positive controls. The scavenging activity of DPPH radicals was calculated by the following equation:

$$\text{DPPH radical scavenging activity } (\%) = [1-(Abs_1-Abs_2)/Abs_0] \times 100 \quad (3)$$

where $Abs_0$ was the absorbance of the negative control, $Abs_1$ was the absorbance of a mixture of DPPH solution with the sample and $Abs_2$ was the absorbance of the sample only (ethanol instead of DPPH-ethanol solution).

*2.5.3. Hydroxyl radical scavenging activity*

The hydroxyl radical scavenging activity was assayed based on the Fenton reaction [37] with slight modifications. 2 mL sample solution was incubated with 2 mL salicylate (6 mM), 2 mL $FeSO_4$ (6 mM) and 2 mL $H_2O_2$ (6 mM) at 37 °C for 30 min and the absorbance at 510 nm was determined. The deionized water was used as blank control. Ascorbic acid (Vc) and BHT was served as blank and positive controls. The scavenging activity of hydroxyl radical was calculated by the following equation:

$$\text{Hydroxyl radical scavening activity } (\%) = [1-(Abs_1-Abs_2)/Abs_0] \times 100 \quad (4)$$

where $Abs_0$ is the absorbance of the negative control, $Abs_1$ is the absorbance of the test sample mixed with reaction solution and $Abs_2$ is the absorbance of the sample only (deionized water instead of hydroxyl radical generating system solution).

*2.5.4. Superoxide anion scavenging activity*

The superoxide anion scavenging activity of LZJPs was determined according to a previously reported method [38]. The LZJPs was dissolved in deionized water at various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). A solution of Tris–HCl (0.05 M, pH 8.2, 5 mL) and 1 mL of sample solution was incubated for 20 min at 25 °C. Subsequently, 1, 2, 3-phentriol (3 mM, 0.3 mL) was added into the reaction mixture followed by an incubation at 25 °C for 5 min. The reaction was terminated with hydrochloride solution (HCl, 10 mM, 1 mL). Vitamin C and BHT diluted in the deionized water was used as positive controls, and the absorbance was determined at 325 nm. The scavenging superoxide anion activity percentage was calculated according to the following equation:

$$\text{Superoxide anion scavening activity } (\%) = 1-(As/Ac) \times 100 \quad (5)$$

where As represents the absorbance of the reaction mixture containing the LZJPs, and Ac is the absorbance of the control.

*2.5.5. Hydrogen peroxide scavenging activity*

LZJPs were dissolved in deionized water at various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). 5 mL of LZJPs solution was added into 5 mL $H_2O_2$ (10 mmol/L) prepared with phosphate buffer solution (pH = 7.40). Subsequently, the absorbance was detected at 230 nm, and Vitamin C aqueous solution was used as positive controls. The scavenging hydrogen peroxide activity was measured according to the following equation:

$$\text{Hydrogen peroxide scavening activity } (\%) = [Abs_0-(Abs_1-Abs_2)/Abs_0] \times 100 \quad (6)$$

where $Abs_0$ denotes the absorbance of the negative control, $Abs_1$ denotes the absorbance of the test sample mixed with reaction solution and $Abs_2$ denotes the absorbance of the sample only (deionized water instead of hydroxyl radical generating system solution).

## 3. Results and discussion

*3.1. Single factor experimental analysis*

*3.1.1. Effects of different solid/liquid ratios on extraction yield of LZJP*

The yield of LZJP was determined with the different ratios of raw material to extraction solvent at 80 °C for 90 min. As shown in Fig. 1A, when the ratio increased from 1:10 g/mL to 1:25 g/mL, the yield of polysaccharide significantly increased ($p < 0.05$) from 3.37% to 4.45% and reached at a maximum value of 5.04 ± 0.16% at 1:20 g/mL, then dropped with higher solid/liquid ratios. Higher solid/liquid ratios could make concentration difference increase between extraction solvent and internal tissues of raw material, thus significantly facilitating diffusion of LZJP and resulting in an increase of LZJP yield [39]. However, excessive extraction solvent also need assimilate cavitations energy from the extraction process, leading to a lower yield [40]. Therefore, 20 g/mL was chosen as the optimal solid/liquid ratios.

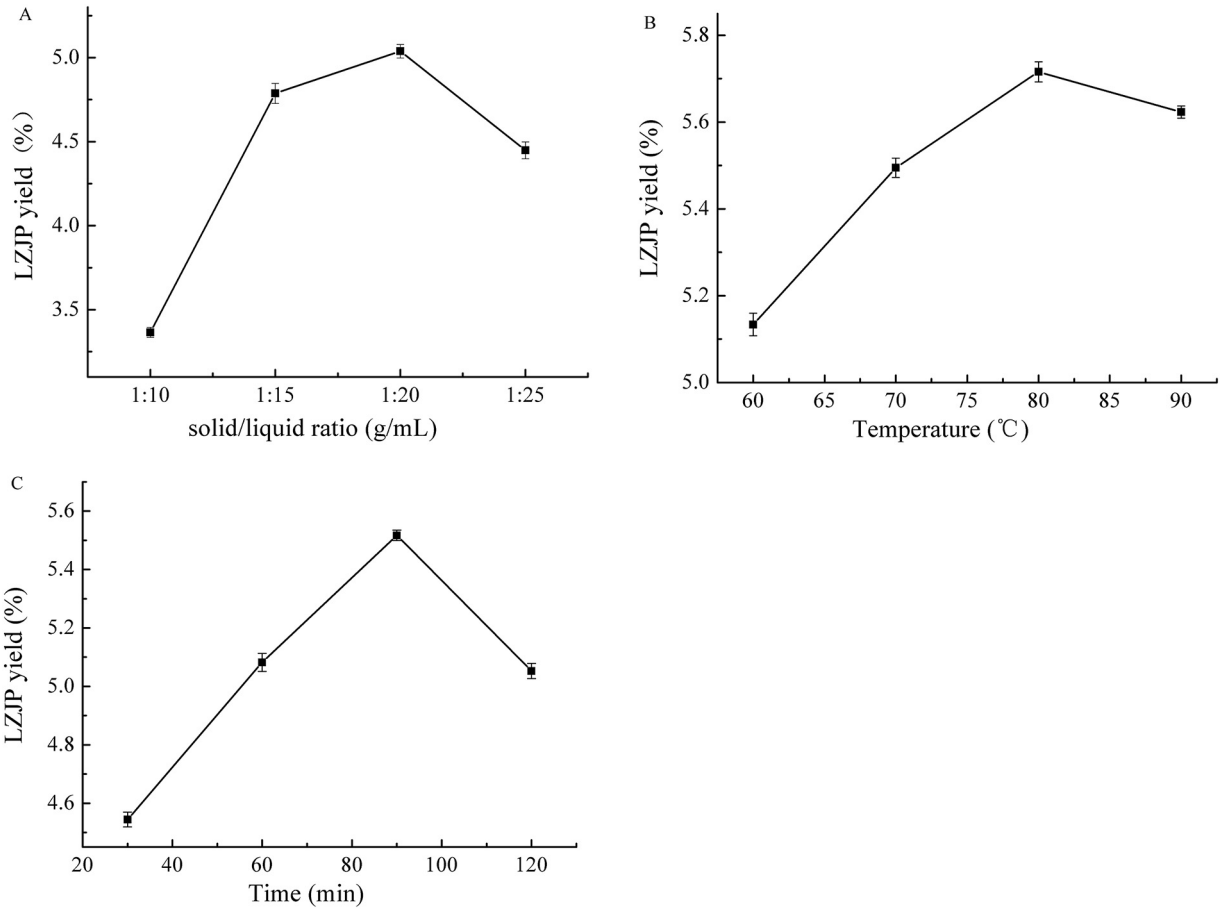


**Fig. 1.** Effect of different extraction parameters on the yield of LZJP. (A. solid/liquid ratio g/mL; B. temperature °C, C. time, min).

*3.1.2. Effect of different temperatures on extraction yield of LZJP*

Solid/liquid ratios and extraction time were maintained at 1:20 g/mL and 90 min, respectively. When extraction temperature increased from 60 °C to 80 °C, the extraction yield of LZJP significantly increased to (5.72 ± 0.09) % ($p < 0.01$) (Fig. 1B) and decreased until extraction temperature exceeded 80 °C. The results could be attributed to the structure of LZJP be damaged and degraded at high temperature. Meanwhile, the cost of energy also increased in the extraction procession [41]. Hence, 80 °C was evaluated as the optimal extraction temperature.

*3.1.3. Effect of different extraction time on extraction yield of LZJP*

The effects of different extraction time on yield of LZJP were considered with solid/liquid ratios and extraction temperatures being fixed at 1:20 g/mL and 80 °C, respectively. As shown in Fig. 1C, when extraction time increased from 30 min to 90 min, the yield of LZJP increased significantly with the highest extraction yield (5.52 ± 0.13%) at 90 min ($p < 0.01$). An increased extraction time can strengthen the mass transmission, release and diffusion of LZJP for entering into the solvent and make the extraction procession become easier and quicker. Nevertheless, excessive extraction durations (>90 min) also may lead to the degradation or conversion of the polysaccharide [42]. Therefore, 90-min-long treatment process was deemed to be the optimal extraction duration.

*3.2. Optimization of extraction conditions of LZJP*

*3.2.1. Statistical analysis and the model fitting*

Based on the single factor experiment, a total of 20 experimental points for optimizing the three individual parameters and three levels in the BBD, including solid/liquid ratio (A), extraction temperature (B) and extraction duration (C), were shown in Table 1. The extraction yield of LZJP under different conditions was shown in Table 1, and the data were analyzed using the Design Expert Software (Version 8.05). Y represented the yield of LZJP and was expressed by the second-order polynomial equation as follows:

$$Y = 5.56 + 0.15A + 0.22B + 0.11C - 0.027AB - 0.10AC - 0.044BC - 0.1A^2 - 0.11B^2 - 0.05C^2 \quad (7)$$

A summary of the analysis of variance and the adequacy of the models were shown in Table 2. The value of the determination coefficient ($R^2 = 0.9056$) and the degree of fitting (>90%) resulting from Analysis of the variance (ANOVA) of the quadratic regression model indicated that the model was adequate for prediction within the range of experimental variables and had small experiment error. The p-value was used as a tool to check the significance of each coefficient, and the smaller the p-value and the more significant the corresponding coefficient was [43]. Table 2 showed that three independent variables (A, B, C), and two quadratic terms ($A^2$, and $B^2$) were very significant affected the yield of LZJP with small p-values ($p < 0.01$), and the other terms were not significantly on the extraction yield. According to the F value, the order of the various factors effecting on the yield was obtained as follow extraction temperature > solid/liquid ratio > extraction duration. The deduced optimum extraction condition was $A_2B_2C_2$ displayed in Table 2, the solid/liquid ratio, extraction temperature and extraction duration were 1:19.35 g/mL, 79.68 °C and 89.36 min, respectively. Taking into account the actual extraction conditions, the

**Table 1**
Box–Behnken Design matrix (in coded level of three variables) and response values for the yield of LZJP.

| Number | A | B | C | Y |
|---|---|---|---|---|
| | Solid/liquid ratio (g/mL) | Temperature (°C) | Time (min) | LZJP yield (%) |
| 1 | 1 (1:25) | 1 (120) | 1 (90) | 5.601 |
| 2 | 0 (1:20) | 0 (90) | 0 (80) | 5.649 |
| 3 | −1 (1:15) | 1 (120) | −1 (70) | 5.054 |
| 4 | 1 (1:25) | −1 (60) | 1 (90) | 5.365 |
| 5 | 1 (1:25) | 1 (120) | −1 (70) | 5.501 |
| 6 | 1 (1:25) | −1 (60) | −1 (70) | 4.980 |
| 7 | −1 (1:15) | −1 (60) | −1 (70) | 4.651 |
| 8 | −1 (1:15) | −1 (60) | 1 (90) | 5.232 |
| 9 | 0 (1:20) | 0 (90) | 0 (80) | 5.681 |
| 10 | 0 (1:20) | 0 (90) | 0 (80) | 5.665 |
| 11 | 0 (1:20) | 0 (90) | −a | 5.443 |
| 12 | 0 (1:20) | a | 0 (80) | 5.696 |
| 13 | a | 0 (90) | 0 (80) | 5.677 |
| 14 | 0 (1:20) | 0 (90) | a | 5.531 |
| 15 | −a | 0 (90) | 0 (80) | 5.021 |
| 16 | 0 (1:20) | 0 (90) | 0 (80) | 5.625 |
| 17 | 0 (1:20) | −a | 0 (80) | 4.933 |
| 18 | 0 (1:20) | 0 (90) | 0 (80) | 5.687 |
| 19 | −1 (1:15) | 1 (120) | 1 (90) | 5.571 |
| 20 | 0 (1:20) | 0 (90) | 0 (80) | 5.634 |

a = 1.68.

optimum value was modified as solid/liquid ratio of 1:20 g/mL, extraction temperature of 80 °C and extraction time of 90 min, respectively, and the yield of LZJP was 5.72% under this condition, which was close to the predicted value 5.696%.

### 3.2.2. Optimization of the extraction procedure

The full model resulting from Eq. (7) was drawn into 3D response surface plot and contour plot to predict the relationship between the independent and dependent variables. The 3D response surface plots and 2D contour plots were obtained using the Design Expert Software (Version 8.05), and provided as graphical representations of the regression Eq. (7), which could directly reflect the relationship between experimental levels of each variable and response values. The interaction effects between the variables were reflected by the shapes of the contour plots. Circular contour plots indicated a non-significant interaction between the corresponding variables, while an elliptical contour plot indicated a significant interaction between corresponding variables

**Table 2**
Analysis of the variance (ANOVA) for the second-order polynomial model.

| Source | Sum of squares | Mean square | F-value | Prob > F | Significance |
|---|---|---|---|---|---|
| Model | 1.73 | 0.19 | 10.66 | 0.0005 | ** |
| A | 0.31 | 0.31 | 16.96 | 0.0021 | ** |
| B | 0.57 | 0.57 | 31.48 | 0.0002 | ** |
| C | 0.22 | 0.22 | 12.18 | 0.0058 | ** |
| AB | 0.00028 | 0.00028 | 0.00016 | 0.9693 | NS |
| AC | 0.047 | 0.047 | 2.16 | 0.1374 | NS |
| BC | 0.015 | 0.015 | 0.85 | 0.3795 | NS |
| $A^2$ | 0.26 | 0.26 | 14.52 | 0.0034 | ** |
| $B^2$ | 0.31 | 0.31 | 17.27 | 0.0020 | ** |
| $C^2$ | 0.11 | 0.11 | 5.91 | 0.0354 | * |
| Residual | 0.18 | 0.018 | | | |
| Lack of fit | 0.18 | 0.065 | | | |
| Pure error | 0.00315 | 0.00063 | | | |
| Cor. total | 1.91 | | | | |

NS denotes not significant.
** Denotes very significant at $p < 0.01$.
* Denotes significant at $p < 0.05$.

[44]. Among these three variables (extraction time, extraction temperature and solid/liquid ratio), when two of the variables within the experimental range were depicted in 3D surface plots, the third variable was kept constant at zero level. The effect of solid/liquid ratio (A), extraction temperature (B) and their reciprocal interaction on yield were shown in Fig. 2A. When solid/liquid ratio and extraction temperature was at lower level, the yield increased proportionally with the solid/liquid ratio and extraction temperature increasing. However, the effects of their interaction on the yield was not significant at higher level of factors, and the contour plot shown in Fig. 2B further indicated their mutual interactions were not significant. The influence of solid/liquid ratio (A) and extraction duration (C) on the extraction yield also was exhibited in the Fig. 2C. When the extraction temperature (B) was fixed at zero level, the yield initially increased and then decreased with both increase of solid/liquid ratio (A) and extraction time (C). The interactions between solid/liquid ratio (A) and extraction time (C) were non-significant according to the 2D contours (shown in Fig. 2D). The effects of extraction time and temperature on the yield of LZJP were shown in Fig. 2E. When extraction time and extraction temperature (B) was fixed at lower level, the yield increased with the extraction time (C) and extraction temperature (B) increasing, and then decreased with both increase of extraction temperature (B) and extraction time (C). The results also can be displayed in the contour plot (Fig. 2F), which indicated their mutual interactions were non-significant at higher level.

### 3.3. Isolation, purification of LZJP

DEAE-52 cellulose column in which the distilled water eluent and NaCl eluent were injected was applied for purification of LZJP. Four fractions named as LZJP1, LZJP2, LZJP3 and LZJP4 (Fig. 3) were obtained with the polysaccharides content of 6.4%, 4.4%, 51% and 3.85%, respectively. From these elution peaks, LZJP3 and LZJP4 exhibited single symmetrical narrow peaks, implying these fractions were homogeneous polysaccharides. On the basis, LZJP3 and LZJP4 were further purified through Sephadex G-100 column (Fig. 4). The results showed that both of LZJP3 and LZJP4 were single polysaccharides and the recovery rate were 81.7% and 89.2%, respectively.

### 3.4. Characterization of LZJP

#### 3.4.1. Physical and chemical properties

Crude LZJP powder was tan and easily dissolved in cold or hot water. Iodine reaction showed that LZJP didn't belong to starchy polysaccharide. Negative reaction was observed in Fehling reagent methods, indicating that LZJP wasn't reducing sugar without monosaccharide. Positive result was obtained during the ninhydrin reaction, indicating that one or more proteins existed in the sample and the content was 1.04% based on Coomassie brilliant blue reaction. The result of sulfuric acid–carbazole reaction was positive, which proved that LZJP contained uronic acids with the content of 27.47%.

#### 3.4.2. Molecular weight analysis

The molecular weights (Mw) of LZJPs were measured using size-exclusion chromatograph coupled with multi-angle laser photometer (HPSEC-LLS). As shown in Table 3, LZJP3 and LZJP4 had one and two adsorption peaks, respectively. The weight average molecular weight (Mw) and number-average molecular mass (Mn) of LZJP3 were $9.766 \times 10^4$ Da and $5.412 \times 10^4$ Da, respectively, which had little differences with Wang's report (2015). The Mw/Mn of LZJP3 was 1.805. Besides, the Mw of each adsorption peak for LZJP4 were determined to be $6.499 \times 10^3$ Da and $6.379 \times 10^2$ Da and the Mn were $4.686 \times 10^3$ Da and $3.536 \times 10^2$ Da, respectively.

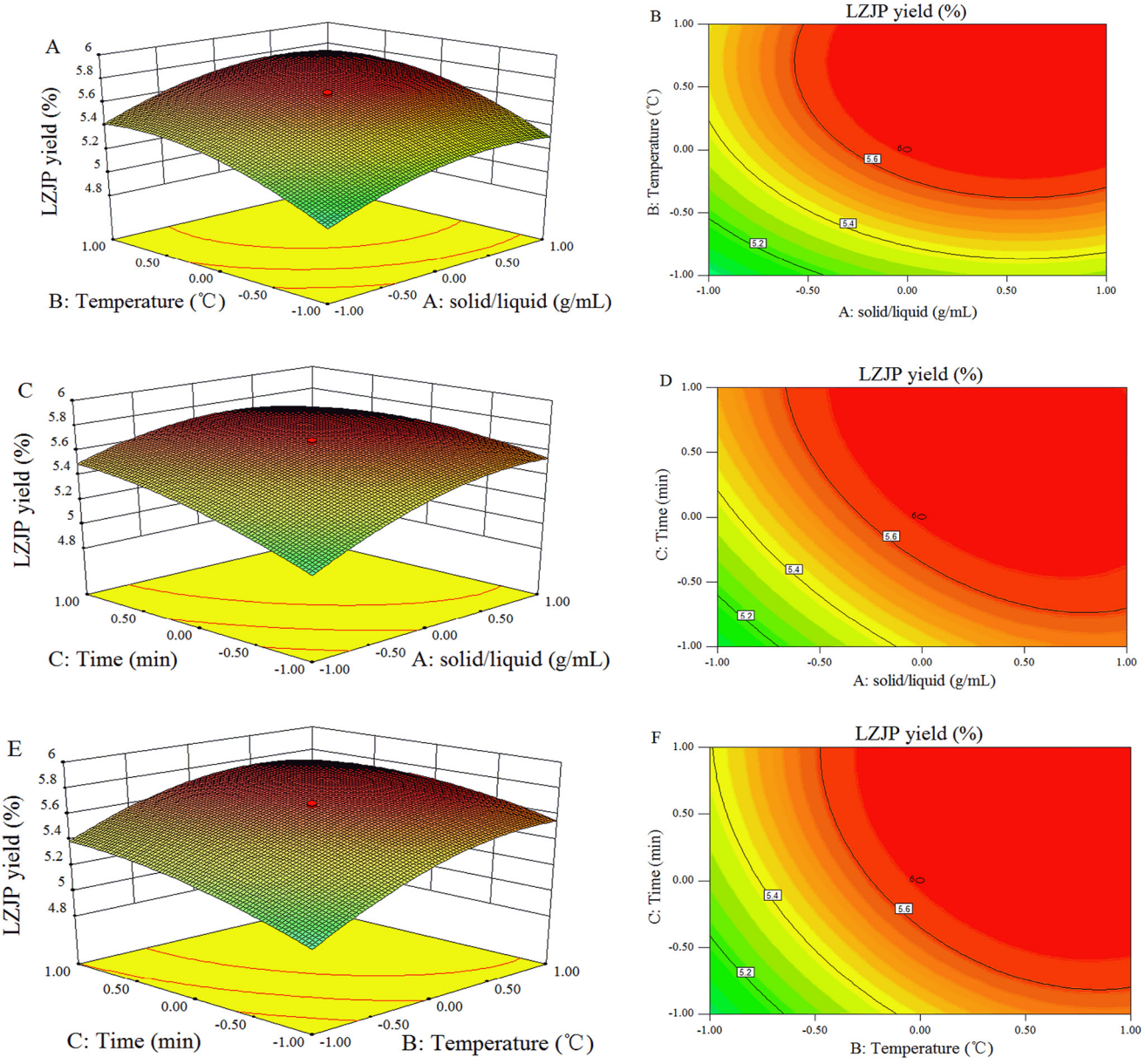


**Fig. 2.** Response surface plot (A, C and E) and contour plots (B, D and F) showing the interactive effects of solid/liquid (A), extraction temperature (B) and extraction time (C) on the extraction yield of LZJP.

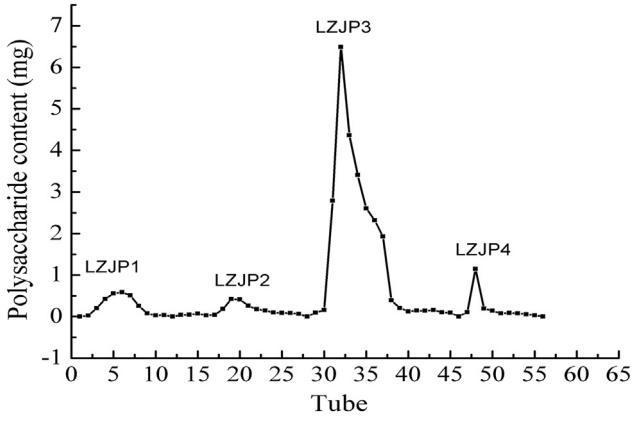


**Fig. 3.** Elution profiles of LZJP by DEAE-52 cellulose column.

*3.4.3. Monosaccharide compositions assay*

Monosaccharide compositions of LZJP3 and LZJP4 were obtained by GC. The results shown in Table 4 indicated that LZJP3 and LZJP4 had different monosaccharide compositions compared with another kinds of polysaccharides isolated from jujube fruit. LZJP3 was mainly consisted of galactose and alduronic acid with the ratio of 2.05:6.84, and LZJP4 was mainly composed of galactose, glucose and alduronic acid with the molar ratio of 16.12:3.08:8.16. Compared with other jujubes, different monosaccharide compositions of *Z. jujuba* polysaccharides have been reported as shown in Table 4, which indicated most of the polysaccharides were composed of rhamnose, arabinose, glucose, and galactose in different molar ratio. The main reasons given for this could be attributed to different raw materials and purification processes.

*3.4.4. Infrared spectra analysis*

LZJPs were characterized by FT-IR (Fig. 5). The infrared spectra of polysaccharides displayed a broad stretching intense characteristic

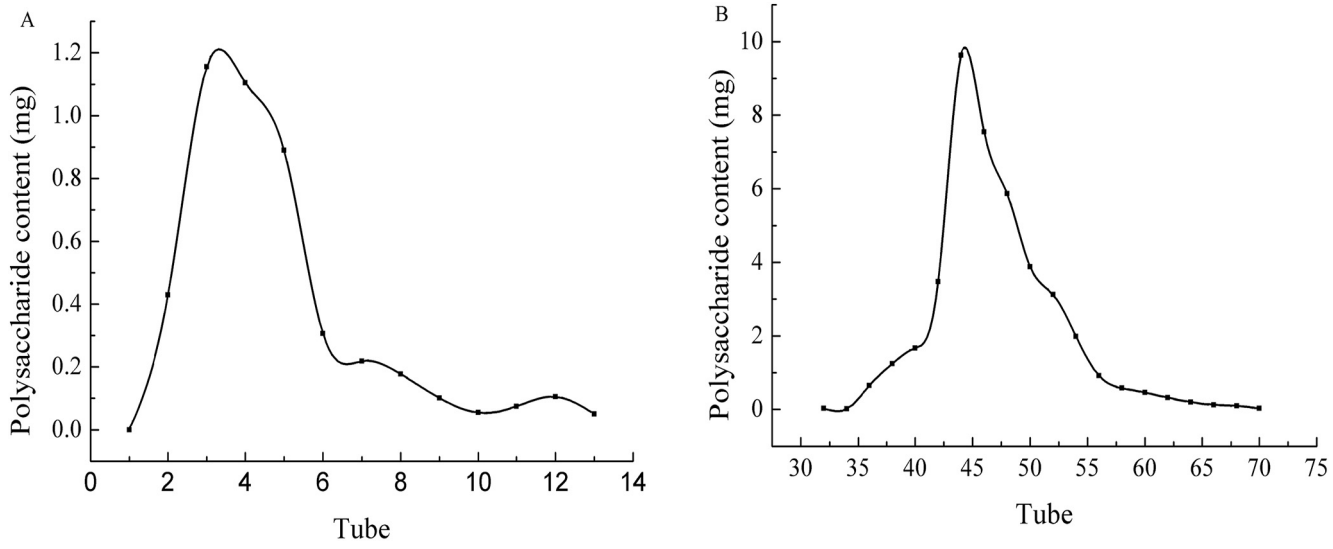


**Fig. 4.** Elution profiles of LZJP3 (A) and LZJP4 (B) by Sephadex G-100 column.

peak at around 3400 $cm^{-1}$, which was attributed to the O—H stretching vibration. The weak absorption toward 2931 $cm^{-1}$ was due to the C—H stretching vibration [45]. The relatively strong absorption peak at around 1600 $cm^{-1}$–1650 $cm^{-1}$ indicated the characteristic of C═O. The peaks at 950 $cm^{-1}$–1200 $cm^{-1}$ suggest the presence of C—O—C and C—O—H link bonds [46]. This result was consistent with the monosaccharide compositions, which indicated that the two fractions of LZJP3 and LZJP4 contained alduronic acid. The peaks at about 1630 $cm^{-1}$ and 1743 $cm^{-1}$ were attributed to the absorption of the hydrate and acetyl, respectively. One peak at 1400 $cm^{-1}$–1200 $cm^{-1}$ represented the variable angle vibration. The stretching peaks at 1103 $cm^{-1}$ suggested the presence of C—O bonds. Three peaks at 1103 $cm^{-1}$, 1049 $cm^{-1}$ and 1016 $cm^{-1}$, respectively, indicated the LZJPs had pyranoside groups and could be concluded to be kinds of β-pyranose. Meanwhile, the absorbance at around 885 $cm^{-1}$ suggested a β-D-galactose existed in LZJPs.

### 3.4.5. Atomic force microscope (AFM) analysis

The AFM images of LZJP3 and LZJP4 were shown in Fig. 6, LZJP3 and LZJP4 were observed within the same field (5 μm × 5 μm). There was only a slight difference between LZJP3 and LZJP4 in sizes and shapes and all the polysaccharide molecules were aggregate with a large number of spherical micelles and a small amount of dispersion. The result of AFM analysis illustrated that the height of the LZJP3 is 117.91 nm and the length is 7.53 nm. Similarly, the height of the LZJP4 is 117.81 nm and the length is 7.75 nm, which were far higher than the height of a single polysaccharide chain (0.1–1 nm) according to the previous report [47]. The result indicated that molecular aggregation was involved in LZJPs and also suggested the structure units of LZJPs might be branched and entangled with each other.

### 3.4.6. Scanning electron microscopy (SEM) analysis

The SEM images of the LZJPs were shown in Fig. 7. It can be seen that the texture of LZJPs was loose and displayed a soft fibrous texture at resolution of 0.09 $cm^{-1}$. The results showed that LZJP3 (Fig. 8A) had a laminated morphology with few short branched chains compared with LZJP4 (Fig. 8B) distributed throughout the surface of polysaccharide. On the contrary, the morphology of LZJP4 had many branched chains. As far as the smoothness was concerned, the morphology of LZJP3 was smoother than that of LZJP4.

**Table 3**
Characterization of LZJP3 and LZJP4 by HPSEC-LLS.

| Samples | Fractions | Mn (Da) | Mw (Da) | PD (Mw/Mn) |
|---|---|---|---|---|
| LZJP3 | Peak 1 | $5.412 \times 10^4$ | $9.766 \times 10^4$ | 1.805 |
| LZJP4 | Peak 1 | $4.686 \times 10^3$ | $6.499 \times 10^3$ | 1.387 |
| | Peak 2 | $3.536 \times 10^2$ | $6.379 \times 10^2$ | 1.804 |

## 3.5. Antioxidant activities determinations

### 3.5.1. The total reducing power analysis

The reducing power is an Important indicator to evaluate antioxidant potential by converting the oxidized form of iron ($Fe^{3+}$) to its reduced form ($Fe^{2+}$) by donating an electron [48]. The total reducing power analyses results of LZJP3, LZJP4 and Vc, as well as BHT, at different concentrations were shown in Fig. 8A. The absorbance of BHT, Vc and polysaccharide samples also increased with the concentration increasing, indicating that the total reducing power of both samples exhibited concentration-dependent. LZJP3 showed always higher antioxidant activity than LZJP4 in the same concentration and lower activity than that of BHA and Vc. The obtained results demonstrated that LZJPs can react with free radicals as an electron donor and convert them to be more stable products and thereby terminate radical chain reactions.

### 3.5.2. DPPH radical scavenging activity determination

The DPPH free radical is commonly used to evaluate the free radical scavenging activities of new antioxidants [49, 50]. Nowadays, many studies demonstrated that polysaccharides isolated from plants were able to eliminate free radicals and could therefore be categorized as natural antioxidants [50]. In the present study, the scavenging activity of the LZJPs against DPPH radicals was illustrated in Fig. 8B. LZJPs showed weaker scavenging effects against DPPH radicals than vitamin C and BHT at different concentration. The present results indicated that LZJPs could supply hydrogen atoms and the scavenging activity against DPPH radicals was enhanced with the increase of the concentrations of LZJPs.

### 3.5.3. Hydroxyl radical scavenging activity determination

Hydroxyl radicals have a free access to cell membranes and cause tissue damage. The scavenging of these specific radicals may avoid tissue injury [51]. Hydroxyl radical scavenging activities of LZJPs at different concentrations were evaluated in this study (Fig. 8C). The scavenging activity of LZJPs solution at a concentration of 1.0 mg/mL against the hydroxyl radicals reached at 13%, which was only 12% lower than that of vitamin C, which implied that LZJPs could donate electrons or hydrogens to scavenge the hydroxyl radicals with a dose-dependent manner.

**Table 4**
The polysaccharides isolated from jujube fruit (*Ziziphus jujuba Mill.*).

| Polysaccharides source | Compound name | Molecular weight (Da) | Monosaccharide composition | Reference |
|---|---|---|---|---|
| *Zizyphus jujuba cv. Linzexiaozao* | LZJP3 | $9.766 \times 10^4$ | Galactose, alduronic acid in the ratio of 2.05:6.84 | In this study |
| | LZJP4 | $6.499 \times 10^3$ | Alduronic acid, galactose, glucose with the molar ratio of 16.12:3.08:8.16 | |
| Huanghetanzao | HJP | – | Man, Rha, GalA, Glc, Gal, Ara in the ratio of 2.62:14.3:8.40:5.29:32.9:36.4 | [5] |
| *Ziziphus jujube Mill. var. spinosa* (Bunge) | PWJS | – | Man, Rha, GlcA, GalA, Glc, Xyl, Gal, Ara in the ratio of 2.03:3.47:1.05:17.64:38.59:3.36:10.44:23.16 | [6] |
| Jinsixiaozao | Ju-B-2 | $>2.0 \times 10^6$ | Rha, Ara, Gal, GalA in the ratio of 2:1:1:10.5 | [7] |
| | Ju-B-3 | $>2.0 \times 10^6$ | GalA | |
| Jinsixiaoza | ZSP1bzs | $9.3 \times 10^4$ | Glc | [10] |
| | ZSP2 | $8.6 \times 10^4$ | Rha, Ara, Glc, Gal in the ratio of 1.0:2.5:1.3:4.1 | |
| | ZSP3c | $1.6 \times 10^5$ | Rha, Ara, Gal in the ratio of 1.0:2.0:8.0 | |
| | ZSP4b | $1.4 \times 10^5$ | Rha, Ara, Man, Gal in the ratio of 13.8:4:3:8 | |
| Xinjiang Uygur Autonomous Region Fructus Jujubae | RQP1d | 83,775.3 | Rha, Ara, Xyl, Gal, Glc, Man in the ratio of 9.763:23.439:18.765:31.145:2.072:4.391 | [12] |
| | RQP2d | 122, 985.5 | Rha, Ara, Xyl, Gal, Glc, Man in the ratio of 3.165:32.7763:35.721:7.821:5.503:0.347 | |
| Junzao | ZP2a | 120,645 | Rha, Ara, Glc, Gal in the ratio of 1.3:1.7:0.3:1 | [14] |
| Shaanbeitanzao | ZSP | – | Man, Rib, Rha, Glc, GalA, Glc, Xyl, Gal, Ara in the ratio of 2.8:1.8:6.6:2.6:10.9:5.3:3.4:16.5:50.2 | [15] |
| Jujube from Southern Khorasan province in Eastern Iran | JCP-1 | $1.5 \times 10^5$ | Glc, Ara, Gal, Rha in the ratio of1.4:2.1:4.2:0.9 | [16] |
| | JCP-2 | $9.1 \times 10^4$ | Glc, Ara, Gal, Rha in the ratio of 1.2:1.8:4.1:1.1 | |
| Muzao | HJP1 | $6.762 \times 10^4$ | Man, Rha, GalpA, Glc, Gal, Ara in the ratio of 1.3:27.6:6.7:3.7:13:47.6 | [17] |
| | HJP3 | $2.936 \times 10^4$ | Man, Rha, GalpA, Glc, Gal, Ara in the ratio of 0.6:16:16.7:6.5:21:39.2 | |
| Dongzao | WSPs | – | Rha, Ara, Gal, Glc, Xyl in the ratio of 1.0:3.6:1.0:0.5:0.2 | [21] |
| Shanxi province jujube | JPC | – | Man, Rib, GlcA, GalA, Glc, Xyl, Gal and Ara in the ratio of 5.3:3.1:3.6:11.4:13.4:14.5:23.4:25.1 | [48] |

Note: "–" represents no determination.

The results were consistent with those of the polysaccharides from Boshuzhi [52].

*3.5.4. Superoxide radical scavenging activity determination*

The superoxide anion is a weak free radical, generated by mitochondrial electron transport systems, and can create other strong free radicals that may cause various types of diseases [53]. Hence, the superoxide anion free radical scavenging activities of LZJPs were investigated *in vitro*. As illustrated in Fig. 8D, LZJPs showed obvious scavenging activity toward superoxide anion radicals in a concentration-dependent manner. The scavenging activities of LZJPs increased significantly with increasing concentrations, and displayed higher scavenging activities, especially at high concentrations. Recently, many other researchers confirmed that natural polysaccharides clearly demonstrated activities of removing superoxide anion radicals [54, 55].

*3.5.5. Hydrogen peroxide scavenging activity determination*

In this study, LZJPs and standard antioxidant (Vc) were investigated for their hydrogen peroxide scavenging ability. The hydroxyl radical scavenging activities at different concentrations were shown in Fig. 8E. The results showed that the scavenging activity of LZJP3 strengthened with the increase of the concentration, while LZJP4 had weaker activity. However, both of LZJP3 and LZJP4 performed weaker scavenging abilities to $H_2O_2$ than Vc.

## 4. Conclusion

The optimal extraction conditions of Polysaccharide from *Zizyphus jujuba cv. Linzexiaozao*. were obtained based on BBD. The yield of LZJP reached at 5.722% with the solid/liquid ratio of 1:20 (g/mL), the extraction temperature of 80 °C, and the extraction duration of 90 min. The main purified fractions LZJP3 and LZJP4 obtained by column

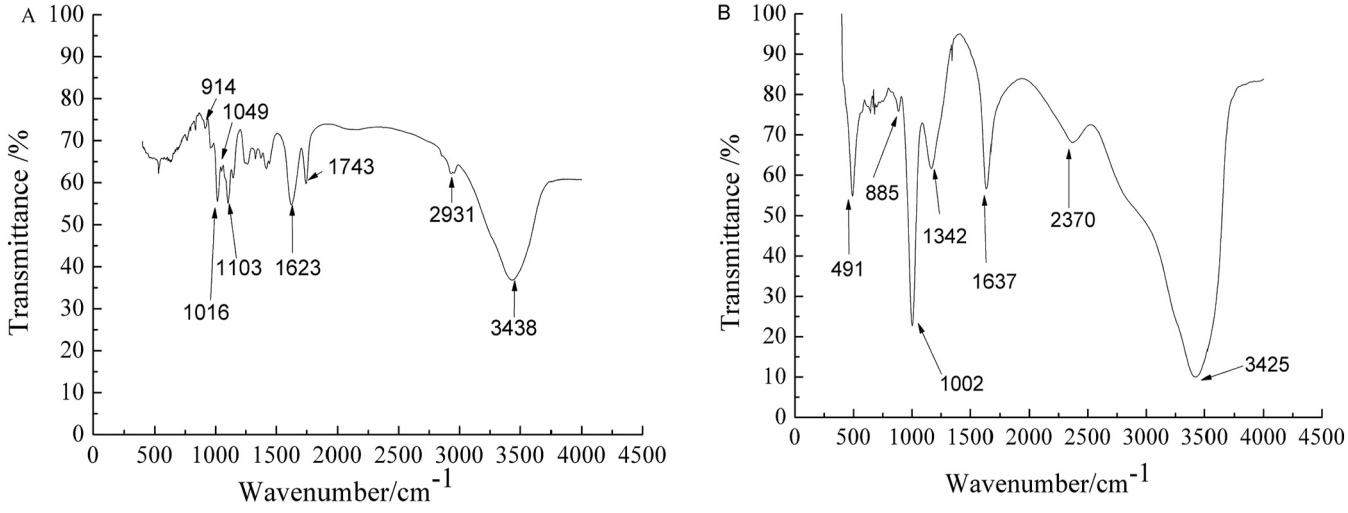


**Fig. 5.** FT-IR spectrum of LZJP3 (A) and LZJP4 (B).

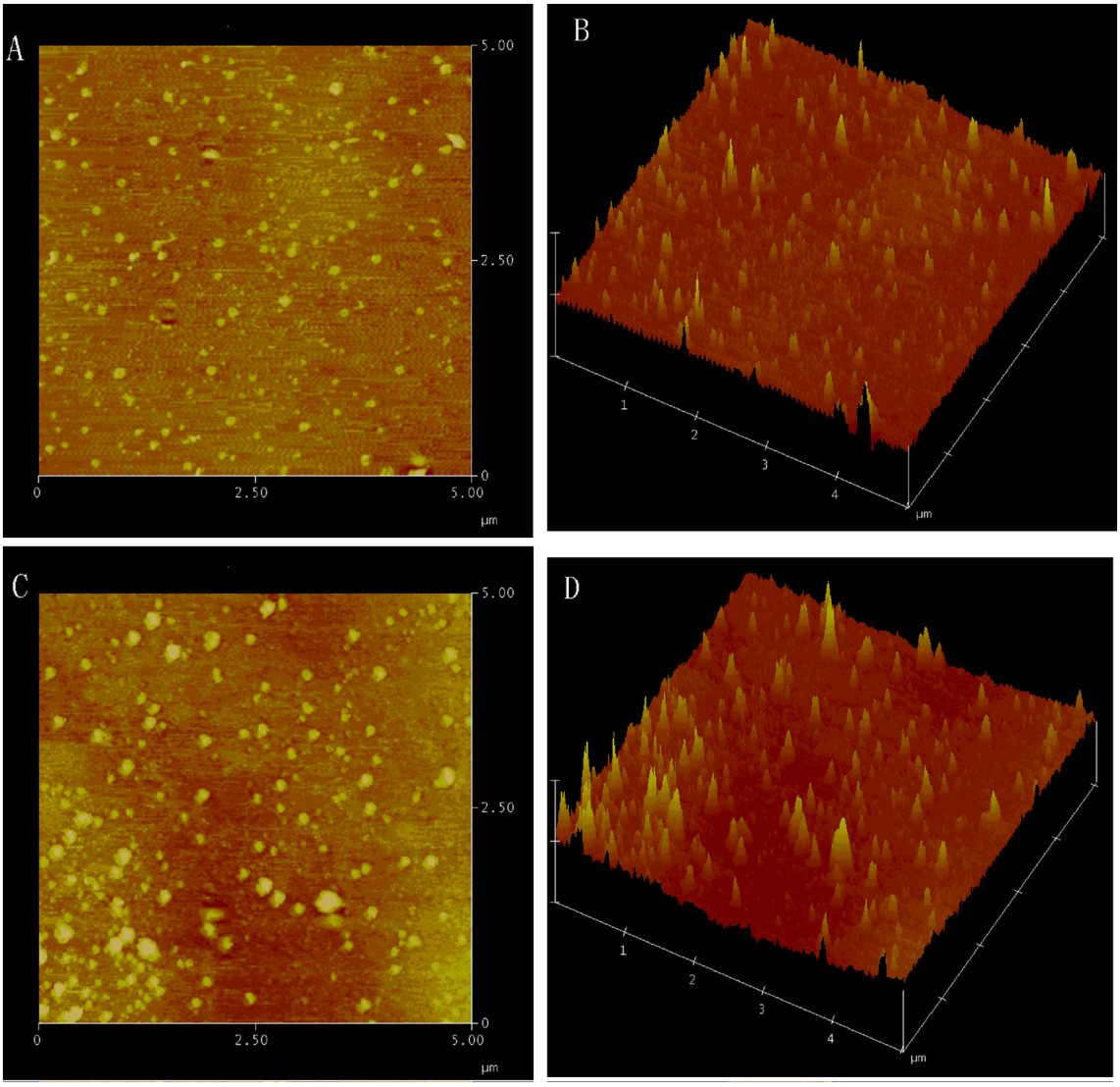


**Fig. 6.** AFM images of LZJP3 (A and B) and LZJP4 (C and D).

chromatography technology were β-pyran polysaccharide with a large number of molecular globular aggregates and a small amount of dispersion, and the surface morphology exhibited smooth and filamentous staggered extension in the form of rod-like aggregation. The molecular weight distribution further demonstrated LZJP3 and LZJP4 existed in the form of a polymer with the different monosaccharide composition.

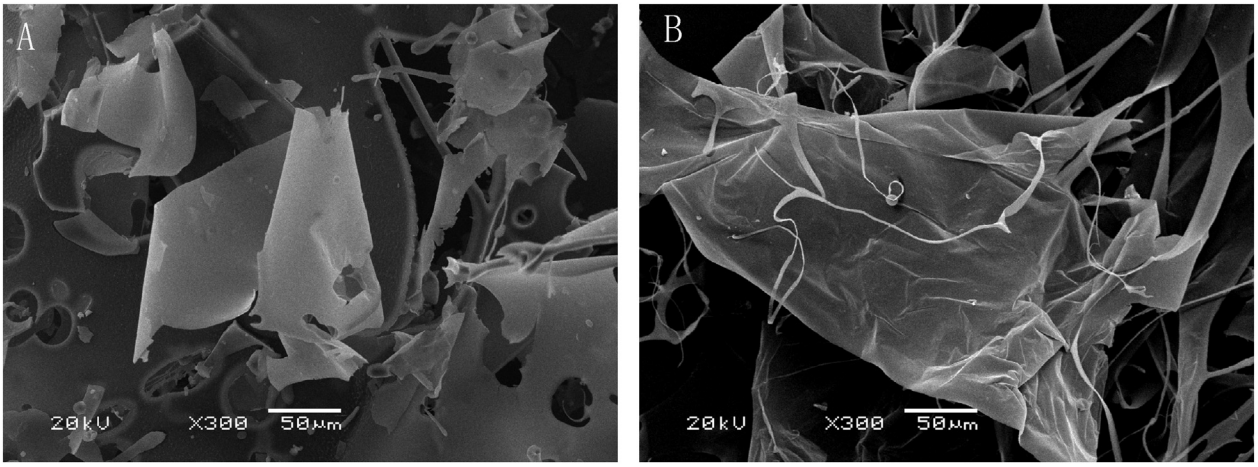


**Fig. 7.** SEM images of LZJP3 (A) and LZJP4 (B).

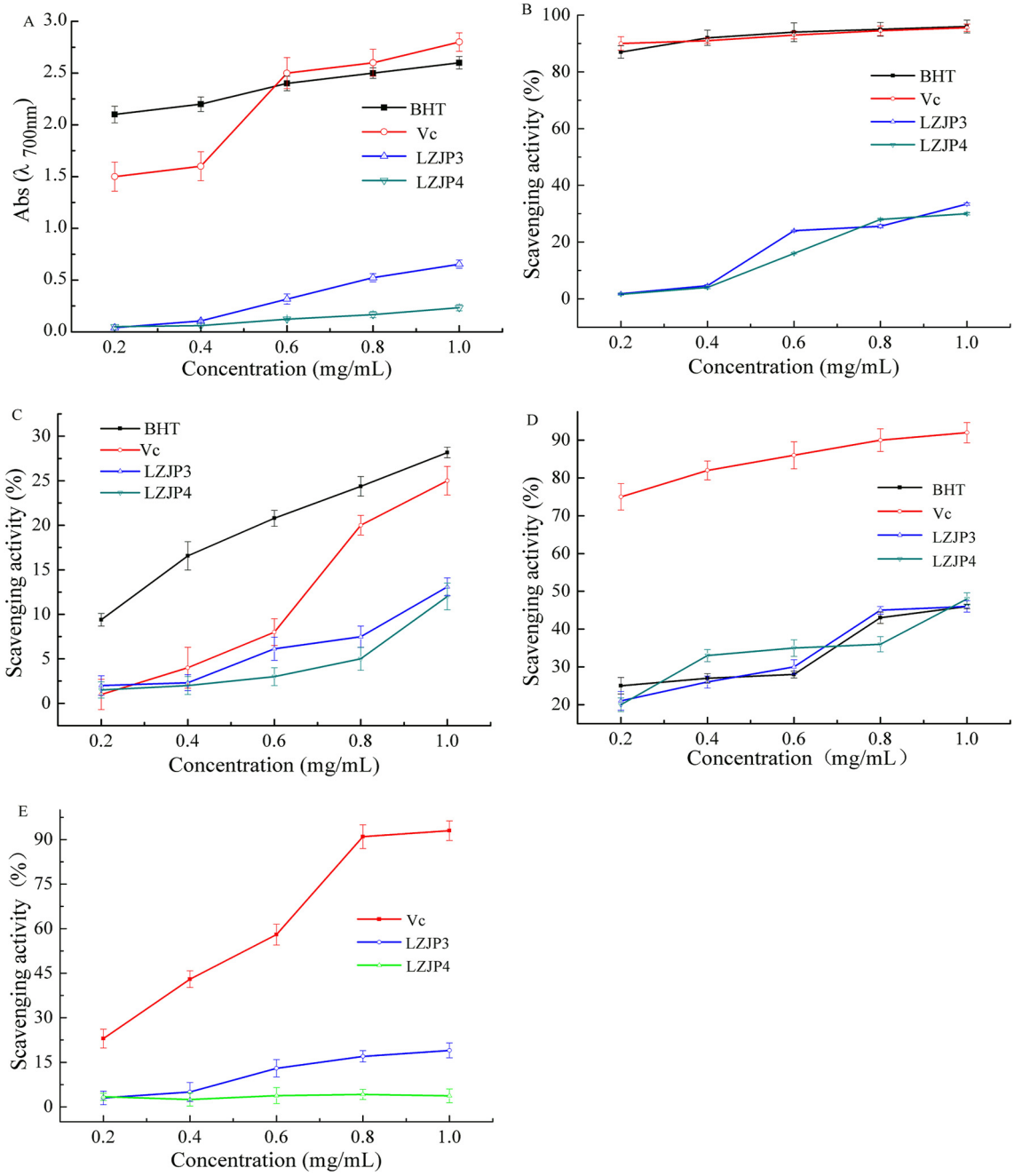


**Fig. 8.** Radical scavenging activity of LZJPs, BHT and Vc. (A. The total reducing power; B. scavenging effects on hydroxyl radicals; C. scavenging effects against hydroxyl radicals; D. scavenging effects on superoxide radicals; E. scavenging effects on hydrogen peroxide).

Meanwhile, the antioxidant activities analyses suggested that the polysaccharide exhibited considerable reducing power and antioxidant activities against DPPH, hydroxyl radical, hydrogen peroxide and superoxide radical *in vitro* in dose-dependent manners.

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