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ORIGINAL PAPER

Comparative analysis of the physicochemical properties and biological activities of *Ziziphus Jujuba cv. Goutouzao* polysaccharides obtained by fractional precipitation

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Abstract

Polysaccharides are the main active constituents of *Jujuba*, yet there are few researches on *Ziziphus Jujuba cv. Goutouzao* polysaccharides. Traditional separation and purification method of *Jujuba* polysaccharides was ion-exchange column chromatography, which is complicated and time-consuming. In this study, four polysaccharide components (SJP-1, SJP-2, SJP-3, and SJP-4) were obtained from crude *Ziziphus Jujuba cv. Goutouzao* polysaccharides (SJP) by ethanol fractional precipitation which are higher convenience than traditional method. The physicochemical properties, immune activities, and antioxidant activities (free radical scavenging ability, reducing power, lipid peroxidation, metal chelating power, inhibition of protein oxidative damage, protective effects of HL-7702 cell damage) of SJP and SJP-1–SJP-4 were investigated to determine its major active constituent. These results showed that SJP-4 had the highest antioxidant and immune activities, which could be related to its monosaccharide composition (Ara, 29.5%; Man, 10.0%; Glc, 16.9%; and Gal, 28.2%) and low molecular weight (0.73×10^4 Da). These results provide a scientific basis for the comprehensive development and utilization of *Ziziphus Jujuba cv. Goutouzao* in the food, medicine, and health product industry.

Keywords *Ziziphus Jujuba cv. Goutouzao* · Polysaccharides · Fractional precipitation · Physicochemical property · Bioactivity

Abbreviations

RQP1d	*Fructus Jujubae* polysaccharides fraction
EDTA	Ethylene diamine tetraacetie acid
DMEM	Dulbecco's modified eagle medium
DEAE-cellulose	Diethylaminoethyl cellulose
BSA	Bovine serum albumin
ZP2	Jujube fruits polysaccharide

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Introduction

The abnormal increase of free radicals in the human body breaks the dynamic balance between free radical production and elimination, resulting in oxidative stress in the cellular system. This oxidative stress causes the damages of DNA, lipids, and proteins in cells and body fluids ultimately resulting in the development of various health disorders such as diabetes mellitus, cancer, Alzheimer's disease, ageing, atherosclerosis, and hypertension [1, 2]. Hence, searching and identifying natural and safe antioxidants from plant origin with highly nutritious and functional fruit, has become a research hotspot in recent years.

Ziziphus jujuba Mill., also known as jujube or Chinese date, has been widely used because of its highly nutrition

and function. China is the center of origin and the main production region of jujube. More than 90% of the world's total annual production are from China [3]. Polysaccharides are the main active constituents of Jujuba, including antioxidant activities [4], immune activities [5], and antitumor activities [5]. The biological activities of polysaccharides are closely related to their physicochemical properties and structures, which are also affected by the methods of extraction and purification. At present, hot water extraction and column chromatography are the main methods for extraction and purification of jujube polysaccharides. A homogeneous polysaccharide fraction (Ju-B-2) from *Zizyphus jujuba* Mill. cv. *jinsixiaozao* Hort with immuno-enhancing activity, composed of galacturonic acid, was obtained by hot water extraction and DEAE column chromatography combined with SepharoseCL-6B column chromatography purification [6]. Li et al. obtained a water-soluble polysaccharide fraction ZSP3c from *Zizyphus Jujuba cv. Jinsixiaozao* by ultrasonic-assisted hot water extraction, and DEAE-SepharoseCL-6B anion-exchange column combined with SepharoseCL-6B size-exclusion chromatography purification, which was rich in pectin with a degree of esterification (DE) of 49% and had immunological activity [7]. Two homogenous polysaccharide fractions RQP1d (WM 83.8 kDa, composed of arabinose, xylose and galactose) and RQP2d (WM 123.0 kDa, composed of arabinose and xylose) from *Fructus Jujuba* with immunity activity were obtained by hot water extraction and DEAE-cellulose chromatography purification [8]. In our previous study, we compared the purification effect of polysaccharide by ethanol fractional precipitation and anion column chromatography, and found that ethanol fractional precipitation had the advantages of higher yield with high convenience, saving time, and lower material loss compared with anionic column chromatography [9]. Hence, we speculate that all the polysaccharide components from jujube may not be obtained by the traditional purification method, which result in the comprehensive and systematical evaluation on the biological activity being difficult, and the study on its structure-activity relationship will be limited.

Ziziphus Jujuba cv. Goutouzao, one of the main varieties of Chinese jujube, is produced in Yan'an, Shaanxi Province. Polysaccharides are one of the main active ingredients, yet there are few researches on *Ziziphus Jujuba cv. Goutouzao* polysaccharides and no researches on separation and purification with ethanol fractional precipitation, which limited the exploitation and utilization of jujube.

In this study, the crude polysaccharides (SJP) and four different polysaccharide components, i.e. SJP-1 to SJP-4 of *Ziziphus Jujuba cv. Goutouzao* was obtained by using hot water extraction and ethanol fractional precipitation. The physicochemical properties of SJP, SJP-1, SJP-2, SJP-3, and SJP-4 were characterized. Moreover, immunological and antioxidant activities of SJP and SJP-1, SJP-2, SJP-3, and SJP-4 were also studied in vitro, and the most active components were determined. This study lays a foundation for further studies on the structure-activity relationship and the utilisation of *Ziziphus Jujuba cv. Goutouzao* polysaccharides.

Materials and methods

Materials and chemicals

Ziziphus Jujuba cv. Goutouzao was purchased from Zaoyuan, Baota District, Yan'an City, Shaanxi Province, China. Monosaccharide standards (L-rhamnose, L-fucose, D-mannose, D-xylose, D-arabinose, D-galactose, D-glucose, D-glucuronic acid, and D-galacturonic acid), dextran standards (1, 5, 12, 25, 50, 80, 150, 270 kDa) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). DMEM was purchased from Hyclone (Logan, UT, USA). HL-7702 cells and macrophages were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Fetal bovine serum (FBS) was purchased from Shanghai Yikesai Biological products Co., Ltd (Shanghai, China). Tetrazolium blue (MTT), penicillin streptomycin mixture (100 ×), and lipopolysaccharide (LPS) were purchased from Beijing Solebao Technology Co., Ltd (Beijing, China). All other reagents were of analytical grade.

Polysaccharides preparation

Extraction of crude polysaccharides (SJP)

The seeds of *Ziziphus Jujuba cv. Goutouzao* fruits was removed and then oven-dried at 60 °C for 48 h until completely dry. The sarcocarp with skin in *Ziziphus Jujuba cv. Goutouzao* were pulverized, and the powder was collected. Ground dried powder (100 g) was extracted twice with water (1:10, w/v) at 90 °C for 2 h, and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected, concentrated, alcohol-precipitated [1:4, v/v], deproteinized using the Sevage method, dialyzed, and freeze dried to obtain the *Ziziphus Jujuba cv. Goutouzao* crude polysaccharides (SJP).

Preparation of SJP-1–SJP-4

Graded ethanol precipitation was performed using a method reported in our previous study [9]. Crude polysaccharides (1%) were precipitated with 30% anhydrous ethanol (v/v) at 4 °C for 12 h; the sample was then centrifuged at 10,000 rpm for 10 min. The precipitate was collected and lyophilized to obtain SJP-1. Absolute ethanol was added to the remaining supernatant until its concentration was 50%, and the precipitate was collected and lyophilized to obtain SJP-2.

Ethanol was added to the remaining supernatant until the concentration was 70%, and the precipitate was collected to obtain SJP-3. Ethanol was continually added to the remaining supernatant until its concentration was 80%; the precipitate was collected to obtain SJP-4.

Monosaccharide composition analysis

The monosaccharide composition of the polysaccharide samples was evaluated by a simultaneous detection method for neutral and acidic sugars [10]. Polysaccharide samples (5 mg) were hydrolyzed in 2 mL of 2 M trifluoroacetic acid (TFA) at 121 °C for 2 h. After the solution was adjusted to neutral pH with NaOH, Na_2CO_3 powder was added to protect the carboxyl group. After 45 min, $NaBH_4$ was added and the solution was left to react for 1.5 h. Excess Na^+ were removed from the solution, the solution was vacuum dried, acetylated, dissolved in dichloromethane, and extracted with double distilled water. The samples were subjected to Gas-chromatography. Gas chromatography column and program settings were used as described by Chen et al. [11].

Molecular weight determination

The molecular weights of SJP and SJP-1-SJP-4 were determined by high-performance gel-permeation chromatography (HPGPC) using a high-performance liquid chromatography (LC-2010 A) system (Shimadzu) equipped with a differential detector, RID-20 A, and a TSK-Gel G4000PWxL (7.5 mm × 30.0 cm) column [12]. The mobile phase used for the experiments was 0.02 M phosphate buffer salt (pH 6.0) with a 0.3 mL/min flow rate. Dextran standards with the following molecular weights were used in the experiment: 1, 5, 12, 25, 50, 80, 150, and 270 kDa. The injection volume of each sample (concentration: 2.0 mg/mL) was 20 µL. The molecular weight linear regression equation $y = -0.1794x + 9.932$ and $R^2 = 0.995$ was used to calculate the relative molecular weight of each polysaccharide component.

Sugar and protein content determination

The phenol sulfuric acid method was used to determine the sugar content of each polysaccharide component [13], and bicinchoninic acid (BCA) assays were used to determine the protein content of each component [14].

Antioxidant activity analysis

Free radical scavenging ability

DPPH radical scavenging activity of each polysaccharide component was determined according to a previously published method by Li et al. [15]. Hydroxyl radical scavenging ability was determined according to the methods by Liu et al. and Chang et al. [16, 17]. H_2O_2 radical scavenging ability was determined according to the method by Liu et al. [16].

Reducing power and metal chelating power

Fe^{3+} reducing ability was determined according to the methods of Oyaizu [18]. The absorbance was measured at 700 nm.

Metal chelating power was determined according to the method by Liu et al. [19]. EDTA was used as the contrast, distilled water was used as the blank, and the absorbance of each component was measured at 562 nm.

Lipid peroxidation and protein damage inhibition assays

Lipid peroxidation inhibition assay was performed according to the methods published by Wu et al. [20]. The lipid peroxidation reaction was initiated by the mixture of free radicals and phospholipids in the Fe^{2+}-Vc system and sample inhibition rates were determined.

The inhibitory effect of polysaccharide components on free radical-induced protein oxidative damage was determined according to the method by Qi et al. [21]. The protein oxidative damage system was established, and the degree of protein oxidation was measured by Coomassie brilliant blue staining to obtain the optimal damage concentration. Then, the inhibitory effects of polysaccharides on oxidative damage of proteins were determined.

Determination of protective effects of HL-7702 cell damage

Antioxidant activity of the polysaccharide components in the cells was determined using the methods published by Mario et al. and Zhuang et al. [22, 23]. HL-7702 cells were thawed in a water bath at 37 °C and transferred to a Petri dish. The cells were cultured in complete DMEM containing 10% FBS, 0.6% penicillin, and streptomycin at 37 °C in 5% CO_2 for 24 h. The effect of each polysaccharide component on the vitality of normal human hepatocytes was evaluated using the MTT method. An H_2O_2 induced injury model of HL-7702 cells was also established, and the HL-7702 cells (1×10^5 cells/well) were inoculated in a 96-well plate. After 12 h of culture, the supernatant was discarded and 100 µL of DMEM containing 1 mM H_2O_2 was added to the cells, and they were incubated at 37 °C for 12 h. Then, the medium was removed and 100 µL (6.25–100 µg/mL) polysaccharide solution prepared in DMEM was added to each well for 24 h. The protective effects of polysaccharides from *Ziziphus Jujuba cv. Goutouzao* on H_2O_2-damaged HL-7702 cells were then detected by the MTT method.

Immunomodulatory activity analysis

Determination of viability of macrophages

Macrophage culture was prepared as described in the section of protectiving effects of HL-7702 cell damage. Viability of macrophages was assessed using the MTT method.

Phagocytic capacity measurement of macrophages

The effects of polysaccharide samples on phagocytic ability of macrophages were investigated by the neutral red test using the method described by Li et al. with slight modifications [24]. DMEM was used as the blank control, and DMEM with LPS was used as the positive control. Macrophages (1×10^5 cells/well) were inoculated in a 96-well plate; the medium was removed after 12 h, and the cells were treated with 100 µL of different concentrations (6.25–100 µg/mL) of polysaccharide solution prepared in DMEM. After culturing for 24 h, the supernatant was discarded, and 100 µL of neutral red saline solution was added to each well for 4 h. Then, the neutral red was removed, and the cells were washed four times with PBS until no excess red coloration remained; 100 µL of the cell lysate was added and the absorbance of each well was measured at 540 nm.

Determination of NO production

The cells were treated as described in the section of phagocytic ability determination. After being cultured for 24 h, 50 µL of the cell supernatant was transferred into a new 96-well plate, and the effect of polysaccharide components on NO secretion was detected using the Griess reagent method.

Acid phosphatase activity assay

The cells were inoculated as described in the section of phagocytic ability determination. Acid phosphatase activity was evaluated as described by Liu et al. [25]. After being cultured for 24 h, the supernatant was aspirated, and 25 µL of 1% Triton X-100 was added along with 150 µL of 1 mg/mL p-nitrophenyl phosphate solution and incubated for 1 h. Finally, 50 µL of NaOH solution was added.

Results

Preparation of crude polysaccharides and fractional precipitated components

As shown in Fig. 1, crude polysaccharides (SJP) were obtained from the dried powder of *Ziziphus Jujuba cv. Goutouzao* sarcocarp with skin by water extraction, alcohol precipitation, deproteinization, and dialysis. The four polysaccharide components (SJP-1, SJP-2, SJP-3, and SJP-4) were obtained by stepwise precipitation with 30%, 50%, 70%, and 80% (v/v) ethanol, respectively. The yield, total sugar content, and protein content of each polysaccharide component are shown in Table S1. The yields of SJP, SJP-1, SJP-2, SJP-3, and SJP-4 was 2.7%, 1.68%, 0.36%, 0.21%, and 0.19%, respectively. The sugar contents of SJP, SJP-1, SJP-2, SJP-3, and SJP-4 was 56.71% ± 1.004, 64.21% ± 1.023, 60.51% ± 1.013, 66.53% ± 1.004, 69.27% ± 1.010, respectively. The protein contents of 18.01% ± 0.813, 18.07% ± 0.962, 21.91% ± 0.375, 22.34% ± 0.284, 27.09% ± 0.281, respectively. SJP-4 had higher sugar content and protein content than that of other components.

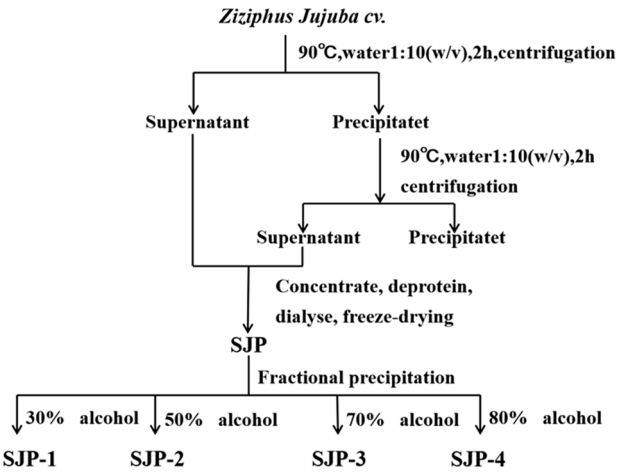

Fig. 1 Flow chart of crude polysaccharide (SJP) from *Ziziphus Jujuba cv. Goutouzao* and its fractional precipitated components preparation

Monosaccharide composition analysis

As shown in Fig. 2 A, SJP was mainly composed of Ara (22.1%), Glc (7.5%), Gal (10.9%), and GalA (49.7%). There were significant differences in the monosaccharide composition of its four fractional components. SJP-1 was mainly composed of GalA (82.5%); SJP-2 was mainly composed of Rha (16.5%), Ara (35.4%), Glc (9.4%), and Gal (38.7%) and did not contain GalA; SJP-3 was mainly composed of Ara (22.9%), Glc (13.1%), and Gal (43.1%); SJP-4 was mainly composed of Ara (29.5%), Man (10.0%), Glc (16.9%), and Gal (28.2%). Both SJP-3 and SJP-4 contained trace amount of GalA.

Molecular weight determination

HPGPC was used to determine the molecular weight of each polysaccharide component. The chromatogram is

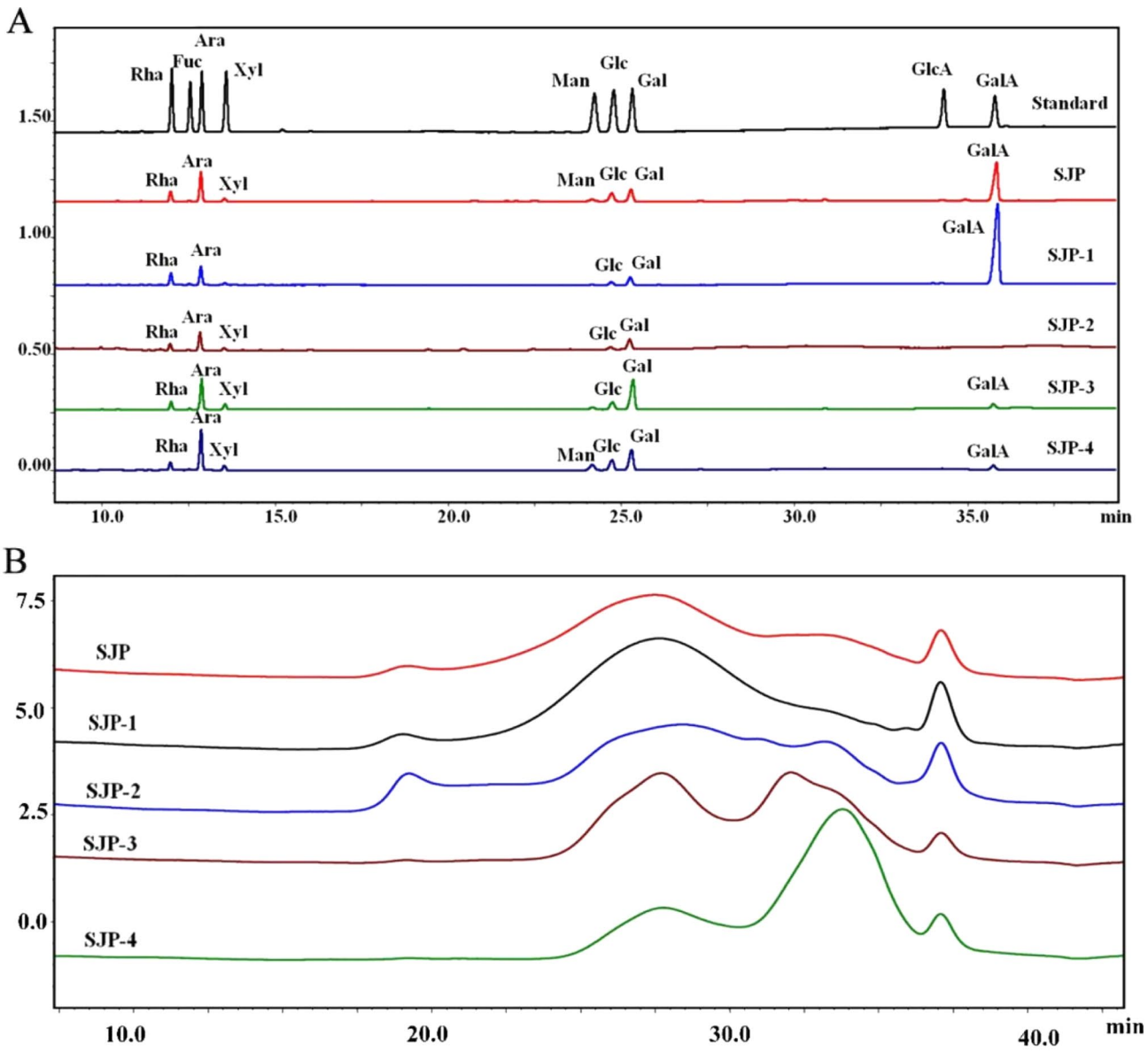

Fig. 2 The monosaccharide composition and molecular weight determination of the crude polysaccharides and its fractional precipitated components. **A** Gas chromatograms of the monosaccharide composition; **B** HPGPC chromatograms of the molecular weight determination

shown in Fig. 2B. Molecular weight distribution of SJP ranged mainly from 9.2×10^5 Da to 3.5×10^4 Da, and the average molecular weight was 9.9×10^4 Da. The molecular weight distribution of SJP-1, SJP-2, SJP-3, and SJP-4 differed significantly. The molecular weight of SJP-1 was mainly distributed at 9.2×10^4 Da. The molecular weight range of SJP-2 was wide, and there was no significant main peak. SJP-3 had two components with a narrow molecular weight distribution (9.1×10^4 Da and 1.5×10^4 Da). SJP-4 had two components with a narrow molecular weight distribution (8.7×10^4 Da and 0.73×10^4 Da); the component with 0.73×10^4 Da molecular weight was the main component.

These four components preparated by alcohol precipitation showed different physicochemical properties. Zhan et al. obtained crude polysaccharides from Xinjiang red jujube and purified them using column chromatography to obtain a polysaccharide fraction mainly composed of GalA with a yield of 0.17% [26]. Li et al. obtained ZP2a polysaccharides from red jujube using hot water extraction and purification by a DEAE-Sepharose CL-6B column. ZP2a was composed of Rha (30.23%), Ara (39.53%), Glc (6.98%), and Gal (23.25%) [27]. In our study, the SJP-1 was mainly composed of galacturonic acid (82.5%), and SJP-2 was composed of Rha (16.5%), Ara (35.4%), Glc (9.4%), and Gal (38.7%). In addition, we also obtained two additional polysaccharide

components, SJP-3, and SJP-4. Therefore, our study utilized a relatively simple method that result in higher yield with high convenience and lower material loss; thus, laying the foundation for subsequent biological activity evaluation.

Antioxidant activity analysis

Determination of free radical scavenging ability

The *Ziziphus Jujuba cv. Goutouzao* polysaccharide components scavenged the DPPH free radicals and hydroxyl free radicals (Fig. 3A and B) to varying degrees. Scavenging ability increased with increasing concentration of each component. At 1 mg/mL, the scavenging rates of SJP, SJP-1, SJP-2, SJP-3, and SJP-4 were 53.90%, 53.94%, 68.45%, 84.05%, and 91.22%, respectively. Of these, SJP-4 had the most significant antioxidant ability to scavenge DPPH free radicals ($p < 0.05$). At 2 mg/mL, the scavenging rates for hydroxyl free radicals by SJP, SJP-1, SJP-2, SJP-3, and SJP-4 were 59.62%, 48.57%, 42.52%, 29.07%, and 34.77%, respectively. Of these, SJP showed the highest hydroxyl radical scavenging ability ($p < 0.05$).

The ability of the polysaccharide components to scavenge H_2O_2 free radicals is shown in Fig. 3C. The H_2O_2 free radical scavenging ability of SJP-2, SJP-3, and SJP-4 increased with increasing concentration, whereas the scavenging effect of SJP and SJP-1 was not obvious. At 2 mg/mL, the H_2O_2 free radical scavenging rates of SJP, SJP-1, SJP-2, SJP-3 and

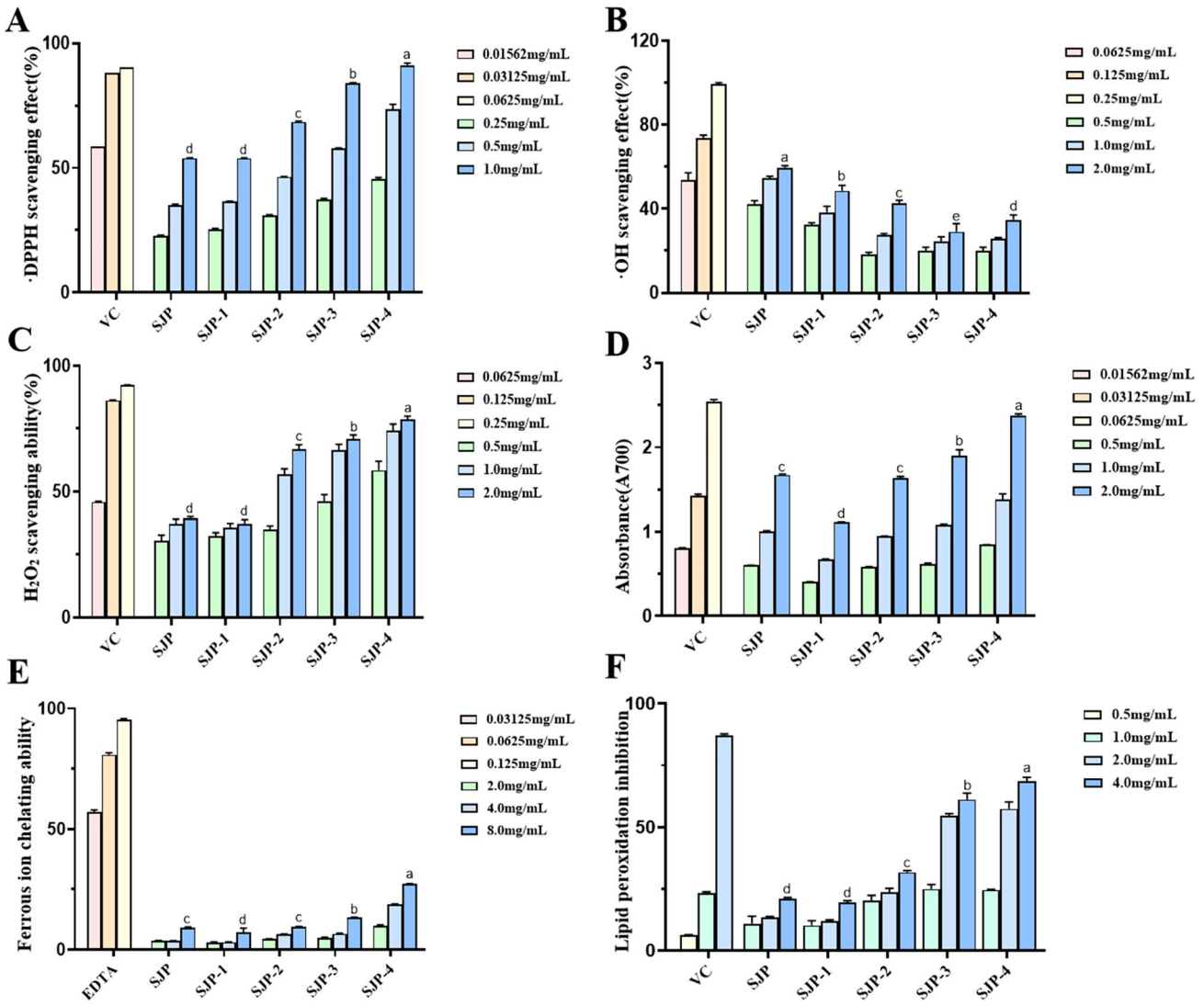

Fig. 3 Antioxidant activity of crude polysaccharides (SJP) and its fractional precipitated components. **A** DPPH free radical scavenging activity. **B** Hydroxyl radical scavenging ability. **C** Hydrogen peroxide radical scavenging ability. **D** Ferric reducing power. **E** Metal chelation ability. **F** Liposome peroxidation inhibitory ability. The data presented are means ± SD (n = 3). The same letter indicates no significant difference ($P > 0.05$), while different letters indicate significant difference ($P < 0.05$)

SJP-4 were 39.23%, 37.22%, 66.76%, 70.77% and 78.51%, respectively. The clearance rate of SJP-4 was significantly stronger than that of other components ($p < 0.05$).

Reducing force and metal chelating force determination

The reducing power of each *Ziziphus Jujuba cv. Goutouzao* polysaccharide component is shown in Fig. 3D. At 2 mg/mL, the reducing power of each polysaccharide component differed highly; the reducing power of SJP-4, SJP-3, SJP-2, SJP-1, and SJP was 2.38, 1.90, 1.63, 1.11, and 1.67, respectively. Therefore, SJP-4 showed the highest reducing power ($p < 0.05$).

The metal chelating forces of the *Ziziphus Jujuba cv. Goutouzao* polysaccharide components is shown in Fig. 3E. The chelating force of SJP-4 on Fe^{2+} increased gradually with increasing sample concentration, and its increasing trend was the most obvious compared to that of the other components. At 8 mg/mL, the metal chelation ability of SJP, SJP-1, SJP-2, SJP-3 and SJP-4 was 9.08%, 5.76%, 9.45%, 13.25%, and 27.11%, respectively. Therefore, SJP-4 had the highest metal chelation ability ($p < 0.05$).

Inhibitory effect on lipid peroxidation

The inhibitory effects of *Ziziphus Jujuba cv. Goutouzao* polysaccharide components on lipid peroxidation are shown in Fig. 3F. From 0 to 4 mg/mL, the inhibition trend of SJP-4 and SJP-3 was obvious, whereas the inhibition trend of SJP, SJP-1, and SJP-2 was not obvious. At 4 mg/mL, the lipid peroxidation inhibition rate of SJP, SJP-1, SJP-2, SJP-3 was 21.11%, 19.48%, 31.71%, 61.16%, and 68.40%, respectively. Of these, SJP-4 had the highest lipid peroxidation inhibition rate ($p < 0.05$).

Protein oxidative damage

BSA protein oxidative degradation induced by Cu^{2+}/H_2O_2 is shown in Fig. S1. As the volume of the Cu^{2+}/H_2O_2 induction system increased, the oxidative degradation of BSA became more significant, and the protein bands became lighter. When the volume of Cu^{2+}/H_2O_2 was 4 μL, the oxidative degradation rate of BSA was 52.74%, and the damaging effect was nearly half. Therefore, 4 μL Cu^{2+}/H_2O_2 was used in the subsequent experiments. Figure 4 shows the inhibitory effect of the *Ziziphus Jujuba cv. Goutouzao* polysaccharide components on the free radical-induced oxidative degradation of BSA. Compared to the Cu^{2+}/H_2O_2 injury group, the gray protein bands in the polysaccharide sample treatment groups became deeper with increasing sample concentration, indicating that the inhibition of the oxidative degradation of BSA by the polysaccharides was dependent on their concentration. When the concentration of each polysaccharide component was 10 mg/mL, the inhibitory effect was the strongest; therefore, to compare the inhibitory effect of crude polysaccharides and fractional precipitated components on protein damage, this concentration was used for subsequent experiments. The BSA protein band in the SJP-4 treatment group were the darkest, and the inhibition rate of BSA oxidation degradation was 79.40%. The BSA oxidative degradation inhibition rates of the SJP, SJP-1, SJP-2, and SJP-3 polysaccharide treatment groups were 64.42%, 52.13%, 65.29%, and 57.85%, respectively. The inhibitory effect of SJP-4 on Cu^{2+}/H_2O_2-induced BSA oxidative degradation was significantly higher than that of the other four treatments ($p < 0.05$).

Antioxidant activity in cell

Figure 5A shows the effect of H_2O_2 on HL-7702 cell viability. As the H_2O_2 concentration increased, cell viability gradually decreased. When the concentration of H_2O_2 was 1 mM, cell viability was 47.52%, and the degree of cell damage was close to half. Therefore, 1 mM H_2O_2 was used to establish the cell damage model. Figure 5B shows that the *Ziziphus Jujuba cv. Goutouzao* polysaccharide components had no effect on the cell viability in the less than 100 μg/mL concentration range, so this concentration range was used in subsequent experiments. Figure 5C shows that the *Ziziphus Jujuba cv. Goutouzao* polysaccharide components protected HL-7702 cells after H_2O_2 injury to varying degrees in a dose-dependent manner. At 100 μg/mL concentration, the cell viability of SJP, SJP-1, SJP-2, SJP-3 and SJP-4 was 68.44%, 66.19%, 75.57%, 77.46% and 89.23%, respectively, indicating that SJP-4 had significantly stronger protective effect on HL-7702 cells after H_2O_2 injury than that of other components ($p < 0.05$) (Figure 5D).

Immune activity of polysaccharide components

Effect on macrophages viability

As illustrated in Fig. 6A, the effects of *Ziziphus Jujuba cv. Goutouzao* polysaccharide components on the viability of macrophages were detected by the MTT assay; *Ziziphus Jujuba cv. Goutouzao* polysaccharides increased cell viability in a concentration-dependent manner. At concentrations below 100 mg/mL, each polysaccharide component had no significant effect on the viability of macrophages. At high concentration (800 mg/mL), each polysaccharide component significantly enhanced the viability of macrophages. The highest cell viability (116.35%) was obtained using SJP-4. To ensure that subsequent experiments were performed at the same level of cell viability with no significant effects on other indicators, polysaccharide samples with a concentration below 100 mg/mL were selected.

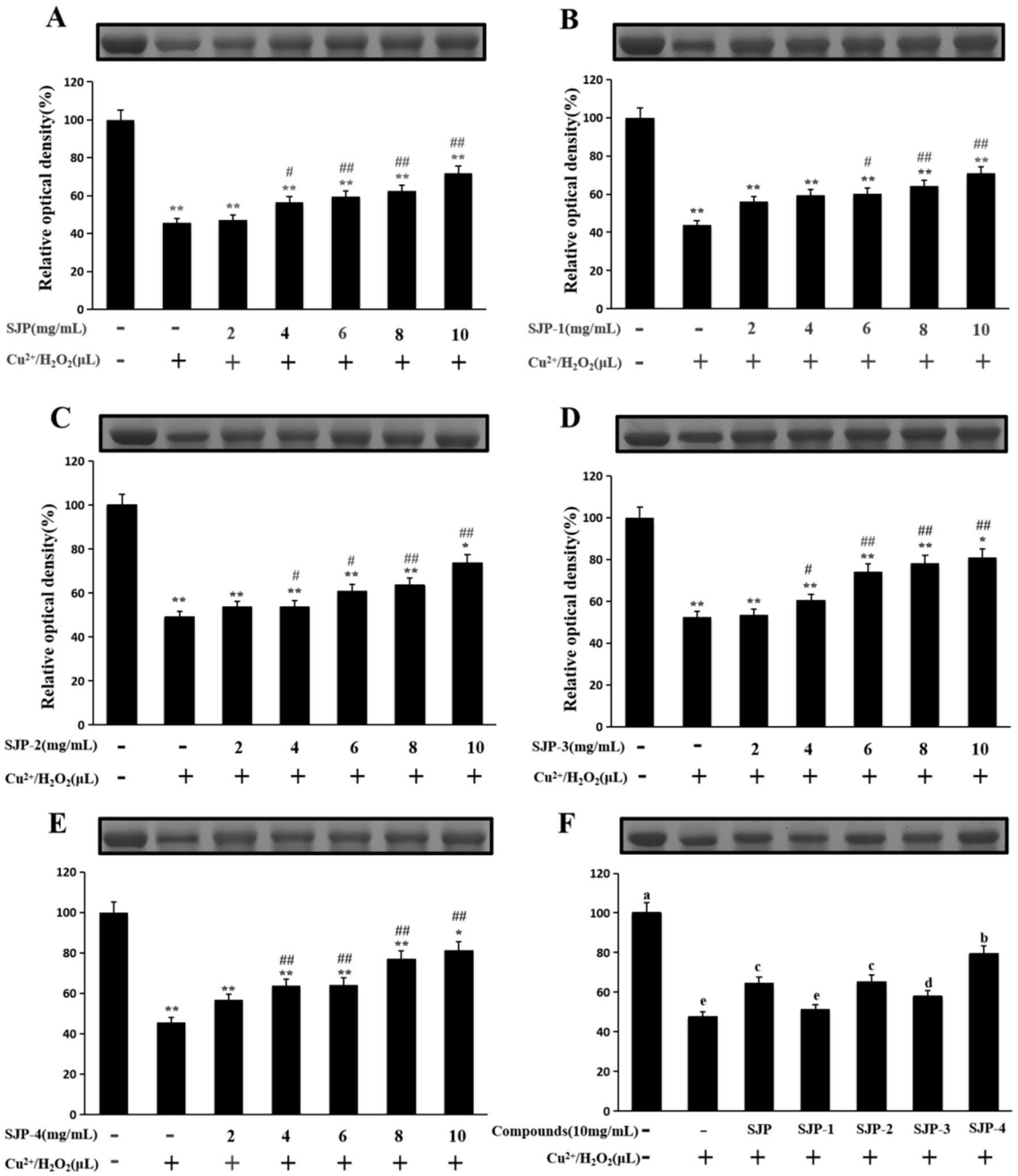

Fig. 4 Effects of the crude polysaccharides and its fractional precipitated components on Cu^{2+}/H_2O_2-induced BSA oxidative damage. **A** SJP; **B** SJP-1; **C** SJP-2; **D** SJP-3; **E** SJP-4; and **F** SJP and fractional precipitated components at 10 mg/mL. The date presented are means ± SD (n = 3). *p < 0.05, **p < 0.01 compared with control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with Cu^{2+}/H_2O_2-induced group. $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$ compared with SJP-4. The same letter indicates no significant difference (P > 0.05), while different letters indicate significant difference (P < 0.05)

Effect on acid phosphatase activity of macrophages

As shown in Fig. 6B, the polysaccharide treatment groups increased the acid phosphatase activity to different degrees as compared to the blank group; the acid phosphatase activity increase was the most obvious in SJP-4 treated cells. At 100 μg/mL, the activity index of SJP, SJP-1, SJP-2, SJP-3 was 1.03, 1.02, 1.04, 1.08, respectively. SJP-4 significantly enhanced the acid phosphatase activity (P < 0.001) with an activity index of 1.17, which was close to that of the positive control (1.21) and significantly higher than that of other polysaccahride samples.

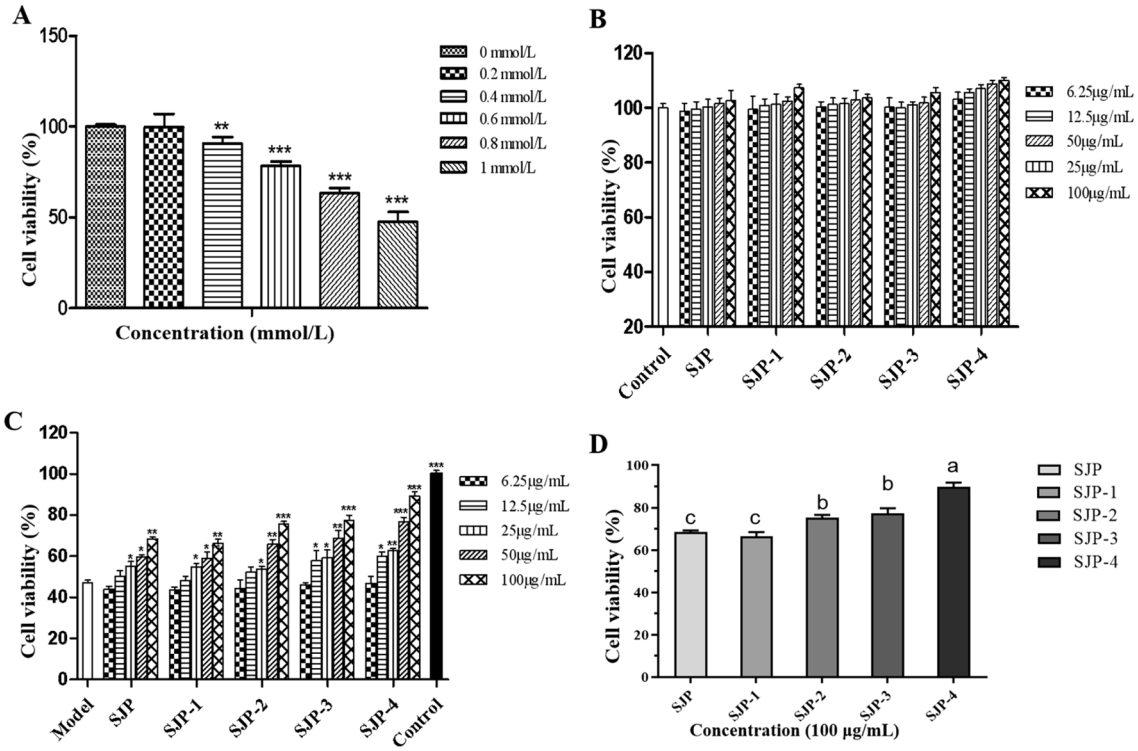

Fig. 5 Antioxidant activity of the crude polysaccharides (SJP) and its fractional precipitated components in HL-7702 cells. **A** Effects of H_2O_2 in a cell damage model; **B** Vitality of HL-7702 cells exposed to the crude polysaccharides and its fractional precipitated components; **C** Protective effects of the crude polysaccharides and its fractional precipitated components on H_2O_2 damaged HL-7702 cells. The data presented are means ± SD (n = 5); **D** SJP and fractional precipitated components at 100 μg/mL. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with H_2O_2 damaged group. The same letter indicates no significant difference (P > 0.05), while different letters indicate significant difference (P < 0.05)

Effect on phagocytic ability of macrophages

The ability of cells to phagocytose neutral red can directly reflect macrophage activity. As shown in Fig. 6C, each polysaccharide component significantly improved the macrophage phagocytic ability as compared to that of the blank group. Macrophage phagocytic ability increased with increasing polysaccharide concentration. At 100 μg/mL, the phagocytic index of SJP, SJP-1, SJP-2, SJP-3 was 1.14, 1.08, 1.10, 1.20, respectively. SJP-4 had the maximum effect on macrophage phagocytic ability; the phagocytic index was 1.24, which was close to that of the positive control (1.27) and significantly higher than that of other polysaccahride samples.

Effect on NO secretion by macrophages

As shown in Fig. 6D, each polysaccharide component significantly increased the NO content secreted by macrophages in a dose-dependent manner as compared that secreted by the blank group. At 100 μg/mL, the maximum NO secretion with SJP-4 was 20.20 μM, while that with SJP, SJP-1, SJP-2, and SJP-3 was 18.65, 18.47, 18.46, and 19.06 μM, respectively.

Discussion

In this study, the crude polysaccharides (SJP) derived from *Ziziphus Jujuba cv. Goutouzao* fruits was fractionally precipitated using ethanol to obtain SJP-1, SJP-2, SJP-3, and SJP-4 with a yield of 1.68%, 0.36%, 0.21%, and 0.19%, respectively. These four components showed different physicochemical properties. Zhan et al. obtained crude polysaccharides from Xinjiang red jujube and purified them using column chromatography to obtain a polysaccharide fraction mainly composed of GalA with a yield of 0.17% [27]. Li et al. obtained ZP2a polysaccharides from red jujube using hot water extraction and purification by a DEAE-Sepharose CL-6B column [26]. ZP2a was composed of Rha (30.23%), Ara (39.53%), Glc (6.98%), and Gal (23.25%). In our study, the SJP-1 was mainly composed of 82.5% galacturonic acid, and SJP-2 was composed of Rha (16.5%), Ara (35.4%), Glc

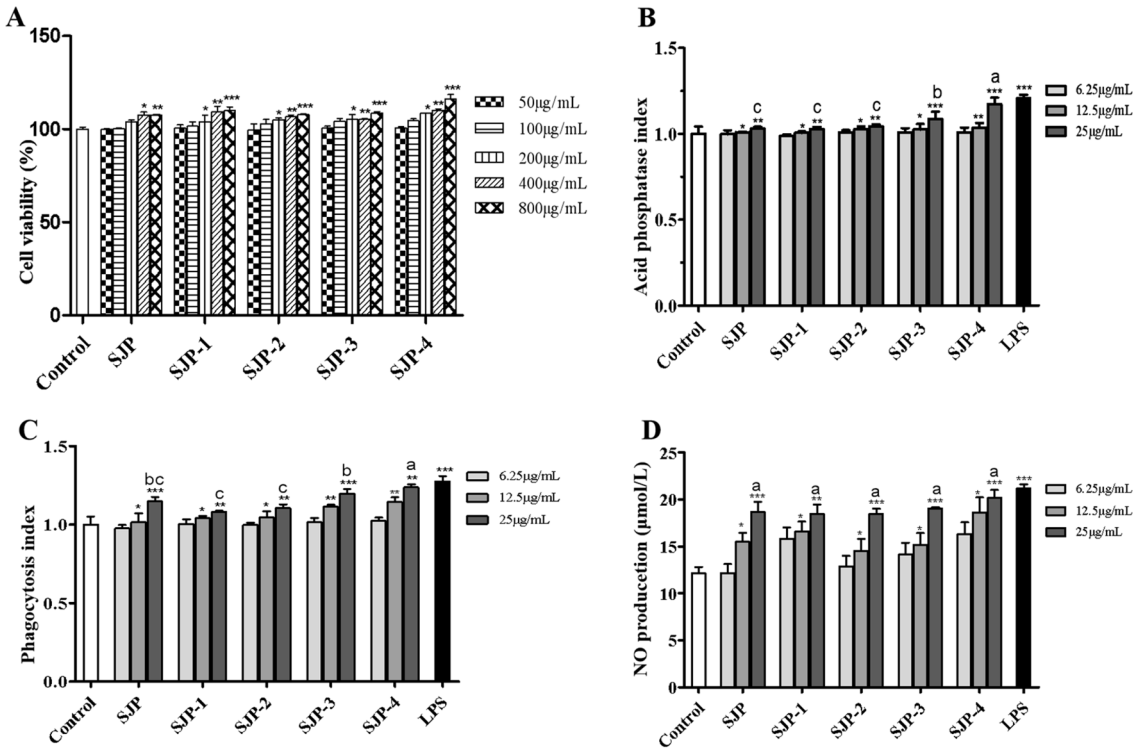

Fig. 6 Immunostimulatory activity of the crude polysaccharides and its fractional precipitated components in vitro. **A** Cell viability; **B** Acid phosphatase activity; **C** Phagocytosis; **D** NO production; LPS (10 μg/mL) was used as the positive control group. The data presented are means ± SD (n = 5). *p < 0.05, **p < 0.01, and ***p < 0.001 compared with control group. The same letter indicates no significant difference (P > 0.05), while different letters indicate significant difference (P < 0.05)

(9.4%), and Gal (38.7%). At the same time, we also obtained two additional polysaccharide components, SJP-3, and SJP-4. In addition, the four components were obtained by ethanol precipitation for 12 h, concentration, and lyophilized. The traditional ion-exchange column chromatography method need equilibration, sample loading, elution, concentration, dialysis, concentration and lyophilized, and the preparation process need about 10 days. Therefore, our study utilized a relatively simple method that result in higher yield with high convenience, saving time and lower material loss; thus, laying the foundation for subsequent biological activity evaluation.

Natural antioxidants are in high demand because of their potential to treat a number of disorders, including diabetes, cardiovascular problems, anti-cancer, anti-inflammatory, and antibacterial properties. In addition, they have potential applications in the food and pharmaceutical industries [28, 29]. Therefore, the search for a natural antioxidant has become a research hotspot. These results of antioxidant activity determination showed SJP-4 had the highest antioxidant activity, which could be related to its physicochemical properties and structure. The number of active groups of polysaccharide is also related to its sugar content. The sugar content of polysaccharide is higher, and the antioxidant activity is stronger [30]. The SJP-4 and SJP-3 with higher sugar content showed the stronger antioxidant activity than that of other polysaccharide component, which was in accordance with previous research. In addition, in a certain molecular weight range, the polysaccharides with low molecular weight contain more reductive ends and its structure are more loose, which could be more likely to combine with free radicals, thereby, scavenging the free radicals at the same mass concentration [31]. Thus, the SJP-4 with the lowest the molecular weight (mainly distributed around 0.73×10^4 Da) showed the strongest the antioxidant activity.

Immune activity may be related to the monosaccharide composition and molecular weight of the polysaccharide samples [9]. Glc and Man can bind to macrophage receptors and regulate immune activity [32]. Arabinogalactans can combine with TLR4 to enhance cellular immune activity. Zhang et al. showed that *Lycium barbarum* polysaccharide LBPF4-OL can enhanced the immune activity of macrophages, wherein its arabinose to galactose ratio was 1.33:1 [33]. Gong et al. also found that arabinogalactan (LBGP-I-3) from *Lycium barbarum* significantly enhanced macrophage immune activity and its arabinose to galactose ratio was 1.08:1 [9]. Among the graded alcohol precipitation components, SJP-4 was the only component that contained

mannose (10%) and was also the component with the highest glucose content (16.8%). At the same time, the SJP-4 monosaccharide was mainly composed of Ara (29.5%) and Gal (28.2%), and the ratio of arabinose to galactose was 1.05:1, which suggested the monosaccharide composition could be the reason behind the highest immune activity of SJP-4. In addition, Chen et al. compared the immunological activity of *Schisandra sphenanthern* polysaccharide (SSP) and *Schisandra chinensis* polysaccharide (SCP), and found that low molecular weight SCP (MW 1.7×10^4 Da) showed higher immune activity [11]. The immune activity of SJP-4 may be related to its molecular weight (mainly distributed around 0.73×10^4 Da).

In addition, the sugar part and protein part of polysaccharide-protein complex exhibit a synergistic effect on its immunological activity [34]. The sugar part of polysaccharide-protein complex (LBPF4) from *Lycium barbarum* fruit showed the immune-enhancing activity by regulating the function of macrophages, while the effect of LBPF4 on the activation of T cells disappeared after removing the protein part of LBPF4, suggesting that the protein part may play an important role in the activation of T cells [33]. SJP-4 had the highest protein content, highlighting the immune-enhancing activity of SJP-4 may be synergistic between the sugar part and protein part. Thus, monosaccharide composition, molecular weight and protein content may be reason behind immune activity of SJP-4. In the follow-up experiments, we will further purify SJP-4 to determine the the main effective constituents that exert immune activity.

Conclusion

To the best of our knowledge, for the first time, all *Ziziphus Jujuba cv. Goutouzao* polysaccharides components (SJP-1-SJP-4) with different physicochemical properties were obtained via fractional precipitation. SJP-4 had the highest antioxidant and immune activity. SJP-4 was comprised of 29.5% Ara, 10% Man, 16.8% Glc, and 28.2% Gal, and its molecular weight was mainly distributed at 0.73×10^4 Da. Therefore, the study provided a preliminary experimental result, and antioxidant and immune activities of SJP-4 should be further estimated in vivo. In addition, the main effective constituents of SJP-4 should be elucidated and its detailed structure should be characterized in future studies. The results of this study will lay down the foundation for the development of *Ziziphus Jujuba cv. Goutouzao* as a natural antioxidant and a potential immunomodulator.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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