Food Chemistry 258 (2018) 35–42

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Immunomodulatory acidic polysaccharides from *Zizyphus jujuba* cv. *Huizao*: Insights into their chemical characteristics and modes of action

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ARTICLE INFO

Chemical compounds studied in this article:
Pullulan (PubChem CID: 92024139)
Rhamnose (PubChem CID: 5460029)
Arabinose (PubChem CID: 66308)
Mannose (PubChem CID: 18950)
Glucose (PubChem CID: 5793)
Galactose (PubChem CID: 6036)

Keywords:
Neutral and acidic monosaccharides
Metal elements
Molecular weight
Reaction groups
Mouse bio-indices interplay

ABSTRACT

Chinese jujube is commonly used in folklore medicine. This study aimed to examine the *in vivo* immunomodulatory activity of two acidic polysaccharides, HP1 and HP2, extracted and purified from *Zizyphus jujuba* cv. *Huizao* (which remains extensively unexplored). HP1 and HP2 had the same monosaccharide species and manganese contents, but differed in their molar rhamnose, arabinose, mannose, glucose, galactose and uronic acid contents (7.32 and 35.9%, as galacturonic acid), Mw (68.7 and 111 kDa, respectively), and contents of K, Cr, Cu, Zn, Pb and Ca. Both HP1 and HP2 could significantly ($P < 0.05$) increase spleen and thymus indices, promote serum hemolysin formation, enhance the phagocytic activity of macrophages and inhibit footpad edema of mice, with HP2 likely being a more consistent and potent immunomodulator. This study clearly demonstrates the potential of *Z. jujuba* cv. *Huizao* polysaccharides as immunomodulators and their associated chemical characteristics and working mechanisms.

1. Introduction

Chinese jujube (*Zizyphus jujuba* Mill. *Rhamnaceae*), also known as "chinese date" or "tsao (zao)", is a native fruit of China with over 4000 years of history. It has long been used for human consumption as a fruit or a traditional Chinese herb for palliative, analeptic and antibechic purposes. The fruit is rich in nutrients, such as proteins (3–7% dry weight), carbohydrates (including fibre polysaccharides at 2–9% dry weight), vitamins (especially vitamin B and C) and important minerals (e.g. manganese, iron, phosphorus, potassium, magnesium, calcium and zinc) (Gao, Wu, & Wang, 2013; Li, Fan, Ding, & Ding, 2007), while containing significant amounts of bioactive secondary metabolites, such as triterpenoids (Fujiwara et al., 2011), flavonoids (Choi, Ahn, Kozukue, Levin, & Friedman, 2011) and phenolic acids (Gao, Wu, Wang, Xu, & Du, 2012).

China is the main producer of the *Z. jujuba* fruits and accounts for up to 90% of the global yield. Increasing demand for *Z. jujuba* fruits, as a result of mounting evidence of their health benefits, has stimulated the production of Chinese jujube in the past decades. The annual yield has reached about 400,000 tons in 2014 (Qiao, Wang, Xiang, Zhang, & He, 2014), and approximately 700 cultivars of Chinese jujube have now been established in China (Gao et al., 2013). Among them, *Zizyphus jujuba* cv. *Huizao*, a major cultivar in Akasu, Xinjiang Province, is attracting increasing attention (Gao et al., 2013). Research has covered a wide range of topics related to *Z. jujuba* fruits, from fruit postharvest handling, preservation and processing, to analysis of nutrient and bioactive contents and evaluation of *in vitro* antioxidant capacities of fruit-derived products.

Among the functional components of the Chinese *Z. jujuba* fruit, polysaccharides may be one of the most important species, given their relative abundance. Diverse polysaccharides with distinct structures associated with sequences of sugar units, compositions of backbones or monosaccharide residues, glycosidic linkages, degrees of polymerization and branching, have been isolated from Chinese jujube in past decades. Many of them exhibit antioxidant capacities (Kozarski et al., 2011), antiviral effects (Saha, Navid, Bandyopadhyay, Schnitzler, & Ray, 2012), anticoagulant functions (Ye, Xu, & Li, 2012), anticancer benefits (Huang, Zhang, Jiang, Kang, & Zhao, 2012), immune-modulating properties (Patra, Das, Behera, Maiti, & Islam, 2012) and hepatoprotective activities (Ma et al., 2012). Growing interests in the use of immunomodulators, to enhance the host defence responses, as promising alternatives to traditional drug treatment (Tzianabos, 2000),

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https://doi.org/10.1016/j.foodchem.2018.03.052
Received 1 October 2017; Received in revised form 10 February 2018; Accepted 12 March 2018
Available online 14 March 2018
0308-8146/

have led to an increasing number of investigations on the immunomodulatory activities of jujube polysaccharides (Li, Shan, Liu, Fan, & Ai, 2011; Zhao, Liu, & Tu, 2007). Results reveal that the immunomodulatory effects of jujube polysaccharides are affected by fruit cultivar, growing and handling conditions, as well as structural characteristics of derived polysaccharides (such as molecular weight, monosaccharide composition, glycosidic bonds and degree of branching) (Chang, Hsu, & Chen, 2010). Accordingly, this research was set up as a preliminary study on structural characteristics and immunomodulatory effects of the polysaccharides from *Zizyphus jujuba* cv. *Huizao* (which remain largely unexplored). Two acidic polysaccharides were extracted, purified using a DEAE-52 cellulose anion-exchange column and Sephadex G-200 column chromatography, and subjected to chemical analyses and *in vivo* assessment of immunomodulatory activity in mice.

2. Materials and methods

2.1. Materials and chemicals

Zizyphus jujuba cv *Huizao* fruits were obtained from Akasu (Xinjiang, China). The fruits were harvested at optimum stage of maturity, transported to our laboratory in Taian, Shandong Province, and stored in normal sealed polyethylene packing at −2°C and 90% relative humidity before use. Monosaccharide standard chemicals (rhamnose, ribose, arabinose, xylose, mannose, glucose and galactose) were purchased from Shanghai Yuanye Bio-Technology Co. Ltd (Shanghai, China). Pullulan standards (Polyanalytik) were purchased from Shanghai Zzbio Co. Ltd (Shanghai, China). Anion-exchange DEAE-52 cellulose and Sephadex G-200 were obtained from Pharmacia & Upjohn Company (Bridgewater, NJ, USA). All other chemicals used were of analytical reagent grade and purchased from local chemical suppliers.

2.2. Extraction of crude polysaccharides

The jujube fruits were sliced into 5 mm pieces, dried at 60 ± 2 °C in an oven to a constant weight, comminuted and sieved through a 40 mesh screen. The dried and sieved jujube particles (150 g) were mixed with distilled water (3 l) at 70 °C for 6 h under continuous and gentle stirring, before centrifugation at 4000 rpm for 15 min. The resultant supernatant was concentrated in a rotary evaporator under reduced pressure, from which the crude polysaccharides were precipitated by adding a four-fold volume of absolute ethanol at 4 °C overnight and recovered via centrifugation at 4000 rpm for 15 min. The obtained precipitate was then deproteinated, following the Sevage method (Staub, 1965), dialyzed for 48 h against deionized water to remove impurities of low molecular weight (M_w < 8000 Da), and lyophilized using a freeze-dryer (Alpha-2, Christ, Germany). The content of polysaccharides was determined by the phenol-sulfuric acid method, using glucose as a standard (Chaplin & Kennedy, 1994).

2.3. Purification of the polysaccharides

The lyophilized crude polysaccharide extract (300 mg) was dispersed in 6 ml of distilled water and centrifuged at 4000 rpm for 15 min, using a TDL-40B High-speed centrifuge (Shanghai Anting Scientific Equipment Inc., Shanghai, China). The supernatant was applied onto a DEAE-52 cellulose anion-exchange column (2.6 × 50 cm) pre-equilibrated with distilled water. Elution was then conducted with a stepwise gradient of NaCl solutions, from low concentration (0.1 M to high concentration 1.0 M), at a flow rate of 1 ml/min. Three fractions were produced (profiles Fig. 1A): the first fraction (Peak 1), the second fraction (Peak 2, HP1, obtained from the 53rd tube to 100th tube) and the third fraction (Peak 3, HP2, obtained from the 110th tube to 142th tube) with mass yields of 22.6% and 26.7%, respectively. The polysaccharide fractions were collected using a BSZ-100 fraction collector (Shanghai Precision Scientific Instrument Co. Ltd., Shanghai, China), based on the absorbance at 490 nm monitored by a UNICO UV-2000 spectrophotometer (Shanghai Instruments Co. Ltd., Shanghai, China), concentrated using a rotary evaporator under reduced pressure, dialyzed using dialysis tubes (M_w cutoff = 3000 Da, Thermo Fisher Scientific, Waltham, MA) against distilled water for 48 h, and lyophilized using an Alpha 1–2 freeze-dryer (Martin Christ Corporation, Osterode am Harz, Germany).

The lyophilized powder (25 mg) was re-dissolved in 5 ml of distilled water, and loaded onto a Sephadex G-200 gel column (1.6 × 60 cm). The column was eluted with distilled water at a flow rate of 0.2 ml/min (fraction profiles as shown in Fig. 1B). Two major purified polysaccharide fractions, HP1 and HP2, were collected, based on the absorbance at 490 nm and lyophilized for further analysis.

2.4. Determination of monosaccharide composition

Monosaccharide composition of the HP1 and HP2 polysaccharide fractions was determined by gas chromatography (GC) according to reported procedures (Li, Li, You, Fu, & Liu, 2017) with slight modifications, using a GC-2010 (Shimadzu Corporation, Kyoto, Japan) equipped with Agilent DB-17 capillary column (30 m × 0.25 mm × 0.25 μm). The injector and detector temperatures were set 260 °C and 280 °C, respectively. The split ratio was 2.0:1. The GC oven temperature was maintained initially at 190 °C for 20 min, then ramped to 200 °C at a rate of 10 °C/min and held for 25 min. The helium carrier gas flow was set at 0.74 ml/min and the injection volume was 0.1 μl. The identification and preliminary quantification of the peaks in the GC chromatograms were achieved by comparing the retention times with those of monosaccharide standards (rhamnose, ribose, arabinose, xylose, mannose, glucose and galactose). The quantification of the monosaccharides in the HP1 and HP2 polysaccharide fractions was achieved by using inositol as the internal standard (to monitor the recovery of individual monosaccharide, based on its corresponding correction factor). The uronic acid contents (as galacturonic acid) were determined according to the procedures described by Sun-Waterhouse, Smith, O'Connor and Melton (2008).

2.5. Fourier transform infrared (FTIR) spectroscopy analysis of crude polysaccharide extract and its purified fractions

The FT-IR spectra of crude polysaccharide fractions, HP1 and HP2, were obtained with a FT-IR spectrometer (Vertex 70 V, Bruker Company, Ettlingen, Germany) in the range of 650–4000 cm^{-1}. The samples were diluted in KBr powder and pellets were made to perform the measurement.

2.6. Determination of molecular weight distribution and metal element contents

The molecular weight (Mw) distributions of HP1 and HP2 were determined by high-performance gel permeation chromatography (HPGPC) following the published method (Wang et al., 2015), using a LC-20AT high-performance liquid chromatography (HPLC) apparatus (Shimadzu, Tokyo, Japan) equipped with a TSK-GEL G4000 PWXL column (7.8 × 300 mm) and a refractive index detector. The column temperature was maintained at 30 °C. An aliquot (20 μl) of each test sample or control sample was injected and eluted with 50 mM $NaNO_3$ solution (containing 0.05% NaN_3, m/v) at a flow rate of 0.4 ml/min. The M_W distribution of each polysaccharide fraction was determined, based on the calibration curve of the pullulan standards (Mw: 745,558, 371,868, 202,898, 101,237, 43,714, 19,626, 9411 and 6056. Each had a concentration of 1 mg/ml). A regression equation was established as the elution time (T) against the logarithm of their respective Mw (Eq. (1)):