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Structure characterization of a novel polysaccharide from Chinese wild fruits (*Passiflora foetida*) and its immune-enhancing activity

Ya Song, Minqian Zhu, Huili Hao, Jie Deng, Meiying Li, Yuanming Sun, Ruili Yang, Hong Wang *, Riming Huang *

Guangdong Provincial Key Laboratory of Food Quality and Safety, College of Food Science, South China Agricultural University, Guangzhou 510642, China

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ABSTRACT

A novel polysaccharide (PFP1) with an average molecular weight of 2.02×10^5 g/mol was isolated from *Passiflora foetida* fruits through hot water extraction, ethanol precipitation and column chromatography. The structure of PFP1 was determined by GPC-MALS-RI, IC, FT-IR, GC–MS and NMR. The structural analysis showed that PFP1 was a heteropolysaccharide and composed of mannose (48.83%), galactose (32.46%), glucose (6.21%), arabinose (5.88%), fructose (2.24%), galacturonic acid (2.20%), xylose (1.17%), fucose (0.17%), ribose (0.05%), and glucuronic acid (0.78%), with a backbone structure of →1)-α-D-Man*p*→1,2)-β-D-Man*p*-linked 1,2,6)-β-D-Man*p* residues and side chains consisted of →1)-β-D-Gal*p*, →1,4)-α-D-Man*p*, →1, 4)-β-D-Glc*p*, →1,3)-α-D-Gal*p*, →1,6)-β-D-Man*p*, →1,6)-β-D-Gal*p*, →1,2,3)-β-D-Man*p* and →1,3,6)-β-D-Gal*p* residues. The results of immune-enhancing assays revealed that PFP1 could obviously promote the production of NO and secretion of cytokines (TNF-α and IL-6) of macrophage RAW264.7. These findings demonstrate that *P. foetida* fruit polysaccharides can be utilized as a potential immune-enhancing functional food.

1. Introduction

In recent years, polysaccharides of fruits have attracted a great deal of attention due to their structural diversity and numerous biological functions. Previous researches have shown that polysaccharides obtained from *Citrus medica* L. var. *sarcodactylis*, *Zizyphus jujuba* cv. *Huizao*, *Ficus carica*, *Zizyphus jujuba* cv. *Junzao*, *Lycium europaeum* Linn, strawberry, mulberry, *Ribes nigrum* L., *Prunus amygdalus*, pomegranate, *Ziziphus jujuba* cv. *Muzao*, *Psidium guajava*, *Rosa roxburghii* Tratt, *yzygium jambos* and *Camptotheca acuminata* fruits possessed a wide range of bioactivities, such as immunoregulatory [1–3], anti-inflammatory [4–6], antioxidant [7,8], hepatoprotective [9], hypoglycemic [10–12] and antitumor [13,14] activities. Therefore, fruit polysaccharides have benefits to pharmaceutical agents and nutritional supplements for human health. However, many fruit polysaccharides are unexplored. In order to promote further exploitation and utilization of these fruit polysaccharides, more fruit polysaccharides should be extensively investigated. In depth investigation of these fruit polysaccharides will give us novel insights on the specific structural characteristic required for the observed function.

Passiflora foetida belong to genus *Passiflora*, yields edible fruits that have been used as food and medicine for a long history in China and India. These fruits could be used to relieve asthma, treat acute edema and treat traumatic cornea or conjunctivitis [15]. Previous chemical investigation of *P. foetida* have exhibited the presence of cyanohydrin glycosides [16], polyketides [17], alkaloids, flavonoids, tannins, steroids, gums, glycosides [18], unsaturated fatty acids and phenolics [19], some of these molecules possessed various biological functions, such as antibacterial [20], anti-osteoporotic [21], anti-inflammatory [15] and antioxidant properties [19]. Although some polysaccharides obtained from fruits of genus *Passiflora* (*P. edulis, P. liguralis and P. incarnata*) with antitumor [22], antioxidant [23], neuropharmacological [24] and anxiolytic [25] effects, have been reported, there was no investigation of polysaccharides of *P. foetida* fruits.

In order to widen the utilization of the polysaccharides from *P. foetida* fruits, an understanding of the pharmaceutical and nutritional functions of these polysaccharides, especially the immune-enhancing function related to disease epidemiology in the pharmaceutical and biomedical industries is fundamental and crucial. In our present investigation, the extraction, purification and structural features of a novel polysaccharide (PFP1) from *P. foetida* fruits were performed by a series of methods including hot-water extraction, ethanol precipitation, column chromatography, Fourier transform-infrared spectroscopy (FT-IR), gel-permeation chromatography with multi angle light scattering and refractive index detectors (GPC-MALS-RI), ion chromatograph (IC), high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), gas chromatography-mass spectrometer (GC–MS), and nuclear magnetic resonance (NMR) spectroscopy Moreover, the immune-enhancing activity of PFP1 was also evaluated using RAW264.7 macrophages to investigate the effects on

* Corresponding authors.
E-mail addresses: gzwhongd@scau.edu.cn (H. Wang), huangriming@scau.edu.cn (R. Huang).

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cell viability, and production of nitric oxide (NO) and cytokines (TNF-α and IL-6). The present results might provide a useful information for the development and application of *P. foetida* fruits as a potential immunomodulating functional food.

2. Materials and methods

2.1. Materials and reagents

P. foetida fruits were obtained from Dongfang (Hainan province, China) in May 2018. Disease and mechanical damage-free fruits with uniform shape and color were pitted and thoroughly washed with tap water. The air-dried sample was transported to our laboratory in Guangzhou, Guangdong Province, and ground in a cutting mill and then passed through a 40-mesh sieve to obtain *P. foetida* fruits powder. The powder was stored in normal sealed polyethylene packing at −20 °C before use. A voucher specimen (No. 20180501) was deposited in Guangdong Provincial Key Laboratory of Food Quality and Safety, South China Agricultural University, China.

The macrophage RAW264.7 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was grown in DMEM medium that contained 10% FBS and 100 Units/mL of penicillin and 100 μg/mL of streptomycin at 37 °C with 5% CO_2. Standard monosaccharides (glucose, galactose, arabinose, rhamnose, fucose, xylose, mannose, ribose, galacturonic acid, and glucuronic acid) were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). DEAE-cellulose DE-52 and Sephadex G-100 were from Yuanye Bio-Chem Technology Co. Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin (Pen), streptomycin (Strep) and trypsin were the products of Gibco BRL (Gaithersburg, MD, USA). Lipopolysaccharide (LPS) and polymyxin B were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). NO detection kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). ELISA kits for quantitation mice TNF-α and IL-6 were purchased from NEOBIOSCIENCE Institute of Biotechnology (Shenzhen, China). Acetonitrile and trifluoroacetic acid (TFA) were of chromatographic grade from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). All other chemicals used were of analytical grade and purchased from Yuanye Bio-Chem Technology Co. Ltd. (Shanghai, China).

2.2. Extraction, isolation and purification of the polysaccharide

The milled *P. foetida* (2 kg) was dipped into 10 vols of distilled water and extracted three times at 65 °C for 4 h each time and then centrifuged at 12000 r/min for 10 min. The supernatants were combined and concentrated in a rotary concentrator and precipitated by adding to a final concentration of 80% ethanol, then maintained overnight at 4 °C. After centrifugation at 12000 r/min for 10 min, the polysaccharides were collected and lyophilized.

The proteins were removed by Sevag reagent (chloro-form/butanol = 4:1, *v*/v) for 3 times. After all the organic solvents were removed by drying (XMTD-8222, JINGHONG, Shanghai, China), the residue was dissolved in distilled water (50 mg/mL) and dialyzed in a cellulose membrane (5 kDa) with distilled water at room temperature for 48 h, followed by lyophilization to obtain the crude *P. foetida* polysaccharides (PFP). PFP was (20 mg/mL distilled water) subjected to ion-exchange chromatography with DEAE-cellulose Fast Flow (1.6 × 40 cm, Buchi, Flawil, Switzerland), eluted with distilled H_2O (4 column bed volume for each gradient) at a flow rate of 1 mL/min. The eluent was collected in 10 mL fractions per tube. All the fractions were measured by phenol-sulphuric acid method [26] and results were expressed as a histogram. The peak with high polysaccharide content was collected, concentrated at 60 °C, and followed by lyophilization to obtain the polysaccharide. The crude polysaccharide (30 mg/4 mL distilled water) was fractionated on a Sephadex G-100 flow column (60 cm × 2.5 cm, Buchi, Flawil, Switzerland) and eluted with 0.1 mol/L NaCl at a flow rate of 1 mL/min.

2.3. Spectra and scanning electron micrographs analysis

The UV spectrum of PFP1 was performed on a UV–visible spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, MMAS, USA) by scanning from 190 to 500 nm. The Fourier transformed infrared (FT-IR) spectrum of PFP1 was measured on a FT-IR instrument (VERTEX 33, Bruker, Rheinstetten, Germany) in the 4000–400 cm^{-1} wave number region.

Scanning electron micrographs (LEO1530VP, Zeiss, Oberkochen, Germany) of the samples were conducted. Sample of PFP1 was placed on a specimen holder with the help of double-sided adhesive tapes and coated with gold powder by vacuum coating apparatus. The sample was observed at an accelerating potential of 15 kV during micrography.

2.4. Molecular weight determination

PFP1 (5 ± 0.05 mg) was performed by gel permeation chromatography with a Waters Series 1500 Pump and multi angle light scattering detector (DAWN HELEOS II, Wyatt technology, CA, USA) and a refractive index detector (Optilab Trex, Wyatt technology, CA, USA) (GPC-MALS-RI), equipped with a TSKgel G3000PWXL column (300 × 7.8 mm, Tosoh, Japan) for low molecular weight polysaccharides and a TSKgel G6000PWXL column (300 × 7.8 mm, Tosoh, Japan) for the high molecular weight polysaccharides. The eluent was 0.02 mol/L NaCl added in 0.005 mol/L sodium phosphate buffer (pH 7.5) at a flow rate of 0.5 mL/min; the column oven was kept at 30 °C. The 0.1% polysaccharide solution (*w*/*v*) was dissolved using eluent.

2.5. Monosaccharide composition determination

PFP1 (5 ± 0.05 mg) was hydrolyzed with 2 mol/L trifluoroacetic acid at 121 °C for 2 h in a sealed tube. Excessive acid was removed by a vacuum rotary evaporator (EYELA N-1100VW, Tokyo, Japan). Hydrolysates were dissolved in 2 mL ultrapure water. After being filtered through 0.22 μm syringe filters, the samples were injected into the ion chromatograph system (ICS5000, Thermo Fisher Scientific, MMAS, USA) for analysis, performed on a Dionex ICS5000 chromatographic system with an efficient anion exchange column of Dionex™ Carbopac™ PA20 column (150 mm × 3 mm) and a Dionex pulsed amperometric detector equipped with an Au electrode (Thermo Fisher Scientific, MMAS, USA).

2.6. Methylation analysis

The reduction of uronic acids was performed according to the method of literature with minor modification [27]. Briefly, PFP1 (5 ± 0.05 mg) was diluted in 1.0 mL distilled water, then mixed with 0.2 mL 2-(4-Morpholino) ethanesulfonic acid (0.2 mol/L) and 0.4 mL carbodiimide (2.6 mol/L), and incubated at room temperature for 3 h. After incubation, 1.0 mL imidazole (4 mol/L) was added to the reaction product and incubated on the ice for 30 min. The reaction mixture was divided into two parts on average, then mixed with 1 mL sodium borohydride ($NaBH_4$, 70 mg/mL) and 1 mL boron sodium deuteride ($NaBD_4$, 70 mg/mL), and incubated at room temperature for 3 h. The reaction terminated with 0.5 mL chromatographic glacial acetic acid and dialyzed in a cellulose membrane (5 kDa) against distilled water at room temperature for 24 h, following by lyophilization to obtain the reduzates.

The reduzate was subjected to methylation analysis with NaOH/DMSO-MeI as described by Anumula and Taylor [28]. The methylated products were extracted five times with dichloromethane followed by vacuum concentration at 40 °C. The methylation procedure was repeated until exhaustive methylation was confirmed by IR detection.

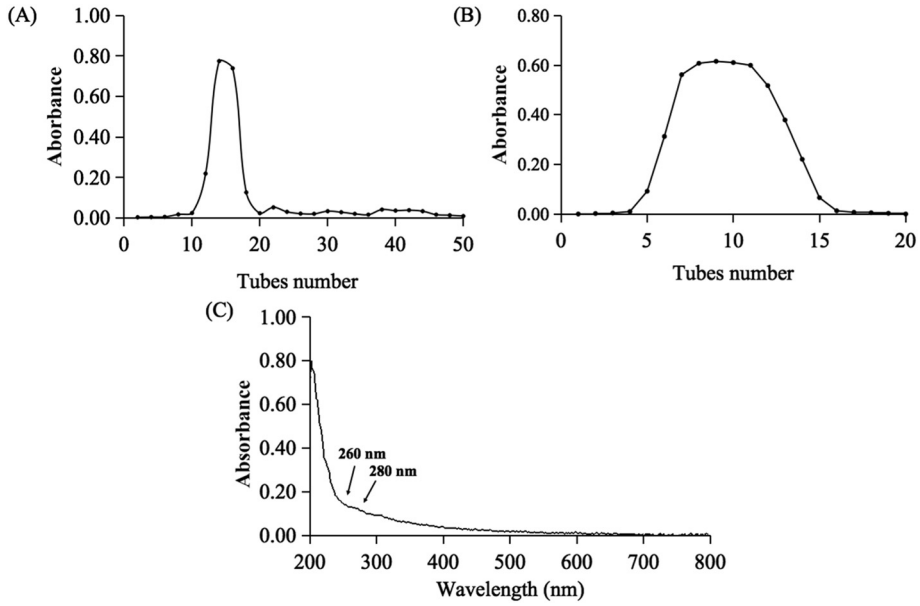

Fig. 1. Separation of PFP1. DEAE-cellulose elution curve (A); Sephadex G-100 elution curve (B). General properties of PFP1. UV–Vis spectrum (C).

The methylated products were hydrolyzed with trifluoroacetic acid (TFA, 2 mol/L, 1 mL) at 121 °C for 90 min. The hydrolysates were evaporated to dryness under reduced pressure at 40 °C, followed by 50 μL ammonium hydroxide (2 mol/L) and 50 μL $NaBD_4$ (1 mol/L) were added, then vortexed and incubated at room temperature for 2.5 h. The reaction terminated with 0.5 mL chromatographic glacial acetic acid and was evaporated to dryness under nitrogen, followed by washing in ethanol several times. After ethanol was removed by nitrogen, 0.25 mL acetic anhydride was added and incubated at 100 °C for 2.5 h. 0.25 mL of distilled water were added to terminate the acetylation reaction. The acetylated derivatives were extracted by 1 mL dichloromethane for 3 times. The dichloromethane extracts were combined and re-extracted with 3 mL water. The chloroform fraction was collected and concentrated at 40 °C. The ultimate derivatives were loaded into a gas chromatography (6890A-5975C, Agilent, California, USA) equipped with a HP-5 capillary column (19091S-433, Agilent, California, USA) and a flame ionization detector. The injection temperature and detector temperature were 240 °C. The column temperature was started with

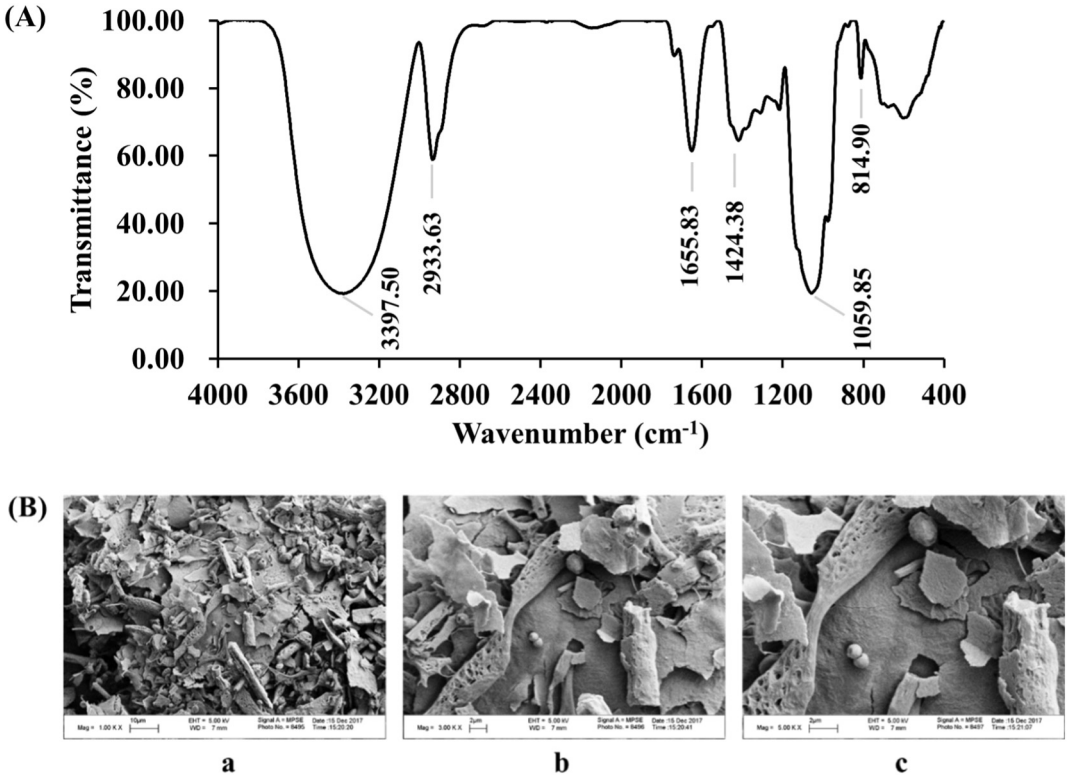

Fig. 2. FT-IR spectrum of PFP1 (A). SEM images of PFP1 (a: ×1 k; b: ×3 k; c: ×5 k) (B).

140 °C for 2 min, increasing to 230 °C at 3 °C/min, holding for 3 min. Nitrogen was used as the carrier gas and maintained at 40 mL/min.

2.7. NMR analysis

PFP1 (50 ± 0.5 mg) was dissolved in 0.5 mL D_2O and 1 μL acetone [29]. 1D and 2D NMR spectra were recorded by a Bruker AVANCE III HD 600 spectrometer (Bruker, Rheinstetten, Germany). The measured spectra included ^1H, ^{13}C, heteronuclear single quantum coherence spectroscopy (HSQC), heteronuclear multiple bond correlation spectroscopy (HMBC) and H/H correlation spectroscopy (COSY).

2.8. Immune-enhancing activity assay

RAW264.7 cells were planted onto a 96 well plate at a density of 2×10^4 cells/mL and cultured for 24 h at 37 °C in 5% CO_2 humidified atmosphere for cell adhesion. Then, the mediums containing different concentrations of PFP1 (0, 0.032, 0.16, 0.8, 4 or 20 μg/mL) or LPS (1 μg/mL) were added, then followed by another 24 h culturing. To eliminate the possibility of LPS pollution during the polysaccharide isolation, the polysaccharides were mixed with different concentrations of polymyxin B for 30 min before added to the medium.

The conditioned culture medium was collected to analyze the level of NO and cytokines released by RAW264.7 cells. NO, TNF-α and IL-6 contents were determined through using commercial kits in accordance with the manufacturer's instructions. Cells treated with 1 μg/mL LPS were used as the positive control. Cells cultured in DMEM medium without polysaccharides and LPS were used as the normal control.

2.9. Statistical analysis

All experiments were repeated in triplicate. Results were expressed as the mean ± SD of triplicate analyses. Statistical significance was analyzed by one-way ANOVA using SPSS 16.0 software. Comparisons with *P* values less than 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Isolation, purification and general properties of PFP1

The crude polysaccharide, termed PFP, was extracted from *P. foetida* fruits with hot-water, followed by ethanol precipitation, deproteinization, dialysis and lyophilization. PFP was purified by a DEAE-cellulose 52 anion exchange chromatography, eluting with distilled H_2O (Fig. 1A). The fractions with the highest yield were collected and concentrated. After lyophilization, one fraction was obtained and designated PFP1.

PFP1 was further purified by a Sephadex G-100 column. As a result, a single and symmetrical elution peak was apparent from the 0.1 mol/L NaCl eluent (Fig. 1B). For the purified PFP1, no absorbance was observed at 260 nm and 280 nm (Fig. 1C), indicating that PFP1 contained no nucleic acids and proteins. The average molecular weight of PFP1 was determined to be 2.02×10^5 g/mol by GPC-MALS-RI, which was more than that of *P. edulis* (6.0×10^4 g/mol) [22], but less than that of *P. liguralis* (greater than 1×10^6 g/mol) [23]. Moreover, the number-average molecular weight (Mn) of PFP1 was determined to be 2.83×10^4 g/mol, and the value of Mw/Mn, known as the polydispersity index (PDI) was 7.14, which indicated the high polydispersity in molecular sizes of PFP1.

3.2. FT-IR spectra and scanning electron micrographs

The FT-IR spectrum shown in Fig. 2A indicated PFP1 had characteristic absorbance of the chemical bonds and functional groups of a polysaccharide. The broad band of PFP1 at 3397.50 cm^{-1} was assigned to the stretching vibration of O—H, which is common to carbohydrates [10]. The signal at 2933.63 cm^{-1} was attributed to the stretching vibration of C—H in the sugar ring [10]. A series of absorbance peaks at 1655.83 cm^{-1} and 1424.38 cm^{-1} were assigned to asymmetrical COO– stretching vibrations and symmetrical COO– stretching vibrations, respectively. These results suggested the existence of uronic acid in PFP1 [11]. The weak absorption band at approximately 814.90 cm^{-1} might be associated with pyranose [30]. The characterization of PFP1 with FT-IR identified absorption peaks typical of polysaccharides.

The SEM morphological results of PFP1 were shown in Fig. 2B at magnifications of 1000, 3000 and 5000. PFP1 was mainly composed of irregular flake, with many different sized pores. The structure features of irregular flakes might account for the highly branched structure and many pores account for excellent water-solubility of PFP1, despite its high molecular weight [11].

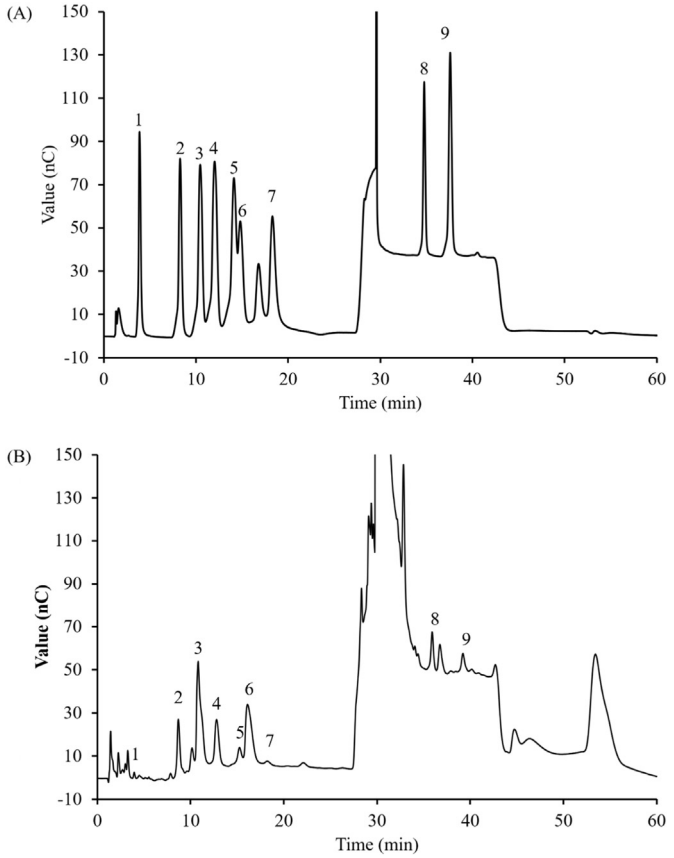

Fig. 3. The IC chromatograms of standard monosaccharides. 1, fucose; 2, arabinose; 3, galactose; 4, glucose, 5, xylose; 6, mannose; 7, fructose; 8, ribose; 9, galacturonic acid; 10, glucuronic acid(A); The IC chromatograms of PFP1(B).

Table 1
Monosaccharide compositions (%) of PFP1.

Monosaccharide compositions	Ratio (%)
Fucose	0.17
Arabinose	5.88
Galactose	32.46
Glucose	6.21
Xylose	1.17
Mannose	48.83
Fructose	2.24
Ribose	0.05
Galacturonic acid	2.20
Glucuronic acid	0.78

Table 2
Methylate analysis data of PFP1.

Retention time (min)	Methylated sugars	Type of linkage	Molar ratios (%)	Mass fragments
13.075	2, 3, 4, 6-Me_4-Man*p*	T-Man*p*	11.35	43, 71, 87, 102, 118, 129, 145, 162, 205
13.686	2, 3, 4, 6-Me_4-Gal*p*	T-Gal*p*	7.32	43, 71, 87, 102, 118, 129, 145, 161, 205
15.911	3, 4, 6-Me_3-Man*p*	1,2-Man*p*	25.51	43, 71, 88, 102, 129, 145, 161, 190
16.095	2, 3, 6-Me_3-Man*p*	1,4-Man*p*	9.29	43, 71, 87, 102, 118, 173, 233
16.23	2, 3, 6-Me_3-Glc*p*	1,4-Glc*p*	3.15	43, 71, 87, 102, 118, 129, 161, 233
16.495	2, 4, 6-Me_3-Gal*p*	1,3-Gal*p*	6.09	43, 71, 87, 101, 118, 129, 161, 234
16.802	2, 3, 4-Me_3-Man*p*	1,6-Man*p*	9.21	43, 59, 87, 102, 118, 129, 162, 190
17.634	2, 3, 4-Me_3-Gal*p*	1,6-Gal*p*	4.13	43, 85, 101, 118, 159, 201, 233, 278
18.158	4, 6-Me_2-Man*p*	1,2,3-Man*p*	5.45	43, 71, 87, 101, 118, 129, 161, 262
19.644	3, 4-Me_2-Man*p*	1,2,6-Man*p*	11.76	43, 87, 101, 129, 130, 190
20.487	2, 4-Me_2-Glc*p*	1,3,6-Glc*p*	6.73	43, 59, 87, 101, 118, 129, 160, 189, 234

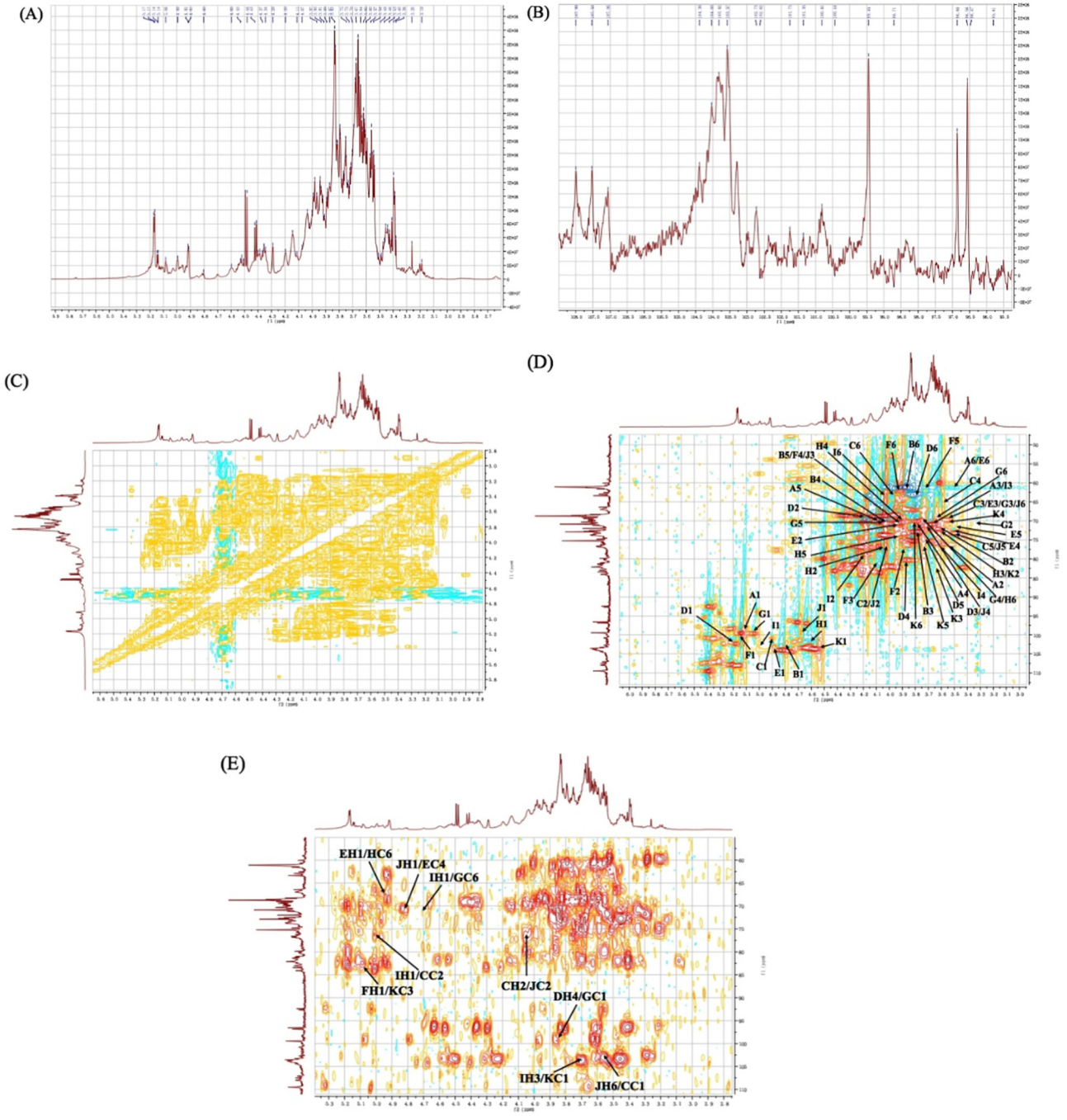

Fig. 4. NMR spectrum of PFP1. (A) ^{1}H NMR spectrum of PFP1; (B) ^{13}C NMR spectrum of PFP1; (C) ^{1}H—^{13}C COSY spectrum of PFP1; (D) ^{1}H—^{13}C HCQC spectrum of PFP1; (E) ^{1}H—^{13}C HMBC spectrum of PFP1.

3.3. Monosaccharide compositions

The ten monosaccharide compositions were determined by an ion chromatograph (Fig. 3). The results demonstrated that PFP1 was composed of fucose, arabinose, galactose, glucose, xylose, mannose, ribose, galacturonic acid and glucuronic acid. As shown in Table 1, PFP1 was mainly consisted of mannose (48.83%) and galactose (32.46%), which indicated that mannose and galactose might form the backbone structure of PFP1. However, the result was different from the polysaccharides isolated from *P. edulis* [22] and *P. liguralis* [23], which might be caused by different extraction methods and raw materials. Moreover, small amounts of glucose (6.21%), arabinose (5.88%), and xylose (1.17%) were also detected, proposing that PFP1 was a kind of heteropolysaccharide. These neutral sugars have also been found in the polysaccharide of *P. edulis* [22] and *P. liguralis* [23]. Furthermore, two uronic acids (galacturonic acid and glucuronic acid, 2.20% and 0.78%, respectively) and trace amount of fucose (0.17%) and ribose (0.05%) were found.

3.4. Methylation analysis

To further obtain the information about the structure of PFP1, the glycosidic bond types were identified by methylation and GC–MS. As summarized in Table 2, the eleven derivatives were identified as 2, 3, 4, 6-Me_4-Man*p*, 2, 3, 4, 6-Me_4-Gal*p*, 3, 4, 6-Me_3-Man*p*, 2, 3, 6-Me_3-Man*p*, 2, 3, 6-Me_3-Glc*p*, 2, 4, 6-Me_3-Gal*p*, 2, 3, 4-Me_3-Man*p*, 2, 3, 4-Me_3-Gal*p*, 4, 6-Me_2-Man*p*, 3, 4-Me_2-Man*p* and 2, 4-Me_2-Glc*p*, with a molar percentage ratios of 11.35: 7.32: 25.51: 9.29: 3.15: 6.09: 9.21: 4.13: 5.45: 11.76: 6.73, respectively. The result suggested that PFP1 contained eleven linkage forms: (1→)-linked mannose, (1→)-linked galactose, (1 → 2)-linked mannose, (1 → 4)-linked mannose, (1 → 4)-linked glucose, (1 → 3)-linked galactose, (1 → 6)-linked mannose, (1 → 6)-linked galactose, (1 → 2,3)-linked mannose, (1 → 2,6)-linked mannose and (1 → 3,6)-linked glucose. The number of terminal Man*p* and Gal*p* residues was approximately equal to the number of branched residues, suggesting that the methylation process used was effective [10]. Furthermore, the molar percentage ratios of the different types of sugar residues were consistent with the percentages calculated from the direct measurement of the monosaccharides in PFP1 described above. The results showed that PFP1 contained approximately 70.57% mannose residues and small amounts of galactose (17.54%) and glucose (9.88%) residues, which is consistent with the monosaccharide. More detailed structure deduction was confirmed by NMR spectroscopy.

3.5. NMR analysis

To further interpret the structure of PFP1 was analyzed via 1D-NMR (^{1}H- and ^{13}C-) and 2D-NMR (HSQC, HMBC and COSY spectra). The ^{1}H and ^{13}C NMR spectra of PFP1 in Fig. 4 represented typical characteristic of polysaccharides. PPF1 had eleven main anomeric proton signals at δ 5.14, 4.80, 4.91, 5.17, 4.92, 5.08, 4.99, 4.60, 5.00, 4.70 and 4.53, which were labeled residues A - K, respectively.

The chemical shifts of PFP1 in the ^{1}H and ^{13}C NMR spectra are summarized in Table 3, which are assigned on the data of COSY (Fig. 4C) and HSQC (Fig. 4D) spectra and with reference to previous studies [11,31–36]. In general, the anomeric signals in 5.1–5.8 ppm region represented the type of α-configuration and 4.4–5.0 ppm region represented the β-configuration. As shown in ^{1}H NMR (Fig. 4A), residues A, D and F, in which the characteristic signals ranging from 5.00 ppm to 5.17 ppm showed the presence of α-configuration. In the same way, residues B, C, E, G, H, I, *J* and K, in which the characteristic signals ranging from 4.53 ppm to 4.99 ppm showed the presence of β-configuration. The chemical shifts of residues A, C, D, G, I and *J* from correspond nearly to the reported values for a methyl glycoside of T-linked Man*p*, 1,2-linked Man*p*, 1,4-linked Man*p*, 1,6-linked Man*p*, 1,2,3-linked Man*p* and 1,2,6-linked Man*p*. Considering the results of FT-IR spectrum, methylation analysis, NMR results and those of literatures, it could be concluded that residues A, C, D, G, I and *J* were →1)-α-D-Man*p*, →1,2)-β-D-Man*p*,→1,4)-β-D-Man*p*, →1,6)-β-D-Man*p*, 1,2,3)-β-D-Man*p* and 1,2,6)-β-D-Man*p* [31,33,34].

Table 3
Chemical shift assignment of glycosidic linkages in D_2O.

Glycosidic linkage		1	2	3	4	5	6
A → 1)-α-D-Man*p*	H	5.14	3.58	3.66	3.83	4.09	3.48
	C	99.45	76.32	71.04	70.23	70.43	61.41
B → 1)-β-D-Gal*p*	H	4.80	3.62	3.77	3.97	3.91	3.89
	C	104.39	72.22	73.15	70.92	70.55	61.17
C → 1,4)-β-D-Man*p*	H	4.91	4.06	3.56	3.58	3.48	3.94
	C	102.73	77.01	69.86	65.88	73.48	61.53
D → 1,4)-α-D-Man*p*	H	5.17	4.18	3.69	3.85	3.74	3.81
	C	101.75	68.68	72.34	80.42	75.02	63.25
E → 1,4)-β-D-Glc*p*	H	4.92	3.91	3.56	3.47	3.46	3.47
	C	102.73	70.55	69.74	71.97	70.79	61.29
F → 1,3)-α-D-Gal*p*	H	5.08	3.88	4.08	3.92	3.49	3.77
	C	101.35	76.56	81.24	70.55	73.40	61.53
G → 1,6)-β-D-Man*p*	H	4.99	3.37	3.55	3.74	4.03	3.65
	C	96.56	70.79	69.98	67.63	70.79	70.43
H → 1,6)-β-D-Gal*p*	H	4.60	3.97	3.58	3.89	3.88	3.73
	C	100.81	76.65	73.40	69.05	73.41	67.14
I → 1,2,3)-β-D-Man*p*	H	5.00	4.21	3.66	3.63	3.58	4.03
	C	103.57	78.88	71.16	68.19	76.20	62.59
J → 1,2,6)-β-D-Man*p*	H	4.70	4.09	3.91	3.67	3.50	3.56
	C	99.45	77.13	70.55	71.05	73.40	69.71
K → 1,3,6)-β-D-Gal*p*	H	4.53	3.50	3.63	3.59	3.60	3.80
	C	103.57	73.27	83.35	70.43	76.08	69.25

Taking the results of methylation analysis into consideration, the presence of anomeric signals suggested three Gal*p* residues in repeating units of PFP1. The signals at δ 104.39 (4.80), 101.35 (5.08), 100.81 (4.60) and 103.57 (4.53) ppm were assigned to the C-1(H-1) of T-linked Gal*p* residue (residue B), (1 → 3)-linked Gal*p* residue (residue F) (1 → 6)-linked Gal*p* residue (residue H) and (1 → 3,6)-linked Gal*p* residue (residue K), respectively [11,32,35,36].

The chemical shift in the anomeric proton of residue E was δ 4.92 ppm, while the corresponding chemical shift in the anomeric carbon was δ 102.73 ppm. These results are consistent with the methylation and FT-IR results, indicating that residue E was →1, 4)-β-D-Glc*p* [32,35].

The heteronuclear multiple bond correlation (HMBC) analysis has confirmed the linkage of glycoside residues in PFP1. In the HMBC spectrum (Fig. 4E) of PFP1, the cross-peak signals between H-2 (δ 4.06, residue C)/C-2 (δ 77.13, residue J), H-6 (δ 3.56, residue J)/C-1 (δ 102.73, residue C) and H-1 (δ 5.00, residue I)/C-2 (δ 77.01, residue C) could be obtained from HMBC spectrum of PFP-1. Furthermore, the cross-peak signals between H-1 (δ 4.80, residue J)/C-4 (δ 71.97, residue E), H-1 (δ 4.92, residue E)/C-6 (δ 67.14, residue H), H-1 (δ 4.70, residue I)/C-6 (δ 70.43, residue G), H-4 (δ 3.85, residue D)/C-1 (δ 96.56, residue G), H-3 (δ 3.66, residue I)/C-1 (δ 103.57, residue K) and H-1 (δ 5.08, residue F)/C-3 (δ 83.35, residue K) were also observed. Combined the methylation analysis and HMBC results, the possible linkage sequence of PFP-1 was deduced to be a heteropolysaccharide, with a backbone structure of →1)-α-D-Man*p*→1,2)-β-D-Man*p*-linked 1,2,6)-β-D-Man*p* residues and side chains were consist of →1)-β-D-Gal*p*, →1,4)-α-D-Man*p*, →1,4)-β-D-Glc*p*, →1,3)-α-D-Gal*p*, →1,6)-β-D-Man*p*, →1,6)-β-D-Gal*p*, →1,2,3)-β-D-Man*p* and →1,3,6)-β-D-Gal*p* residues.

3.6. Immune-enhancing activity of PFP1

Macrophage plays a pivotal role in multiple pathophysiological processes, such as regulate immune responses and contribute to fight against infection and inflammation. Scientists reported that macrophage activation by immunomodulators could enhance the secretion of NO, IL-6 and TNF-α [3,37]. To further evaluate the immune-

enhancing effects of PFP1, the production of TNF-α, IL-6 and NO was tested in culture medium when RAW264.7 macrophages were exposed to PFP1 at different concentrations (0.032–20 μg/mL). In the present study, the results showed that PFP1 at different concentrations significantly induced the NO release from RAW264.7 cells, with significant dosage effects (Fig. 5A). To eliminate the possibility that the induction was due to the pollution of LPS during the PFP1 extraction, a concentration gradient (0, 100 or 200 μg/mL) of polymyxin B (a specific LPS inhibitor) [38] was applied to neutralize the effect of LPS. The results (Fig. 5B) showed that PFP1 could not be inhibited by the polymyxin B, while LPS treatment was significantly inhibited, indicating that the effect of PFP1 was not due to the pollution of LPS.

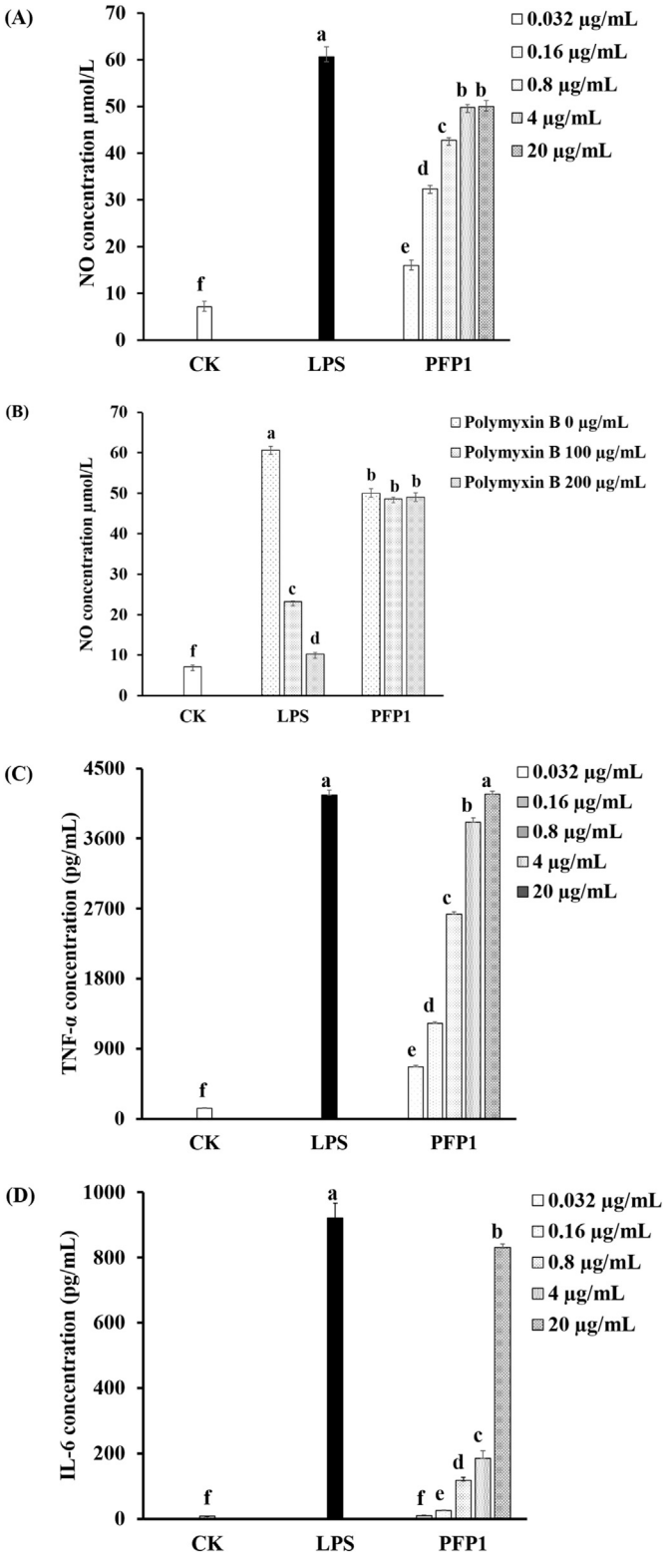

Fig. 5. NO release detection of RAW264.7 cell activation. By LPS and PFP1 (A). By LPS and PFP1 with polymyxin B (B). Elisa detection of TNF-α (C) and IL-6 (D).

Various immune factors were induced by PFP1. By ELISA analysis, cellular release of TNF-α (Fig. 5C) and IL-6 (Fig. 5D) were elevated by PFP1, with significant dosage effects (0.032–20 μg/mL). These results demonstrated that PFP1 could exert immune-enhancing activity by stimulating the release of TNF-α, IL-6 and NO in RAW264.7 macrophages, which was necessary for killing pathogens, microorganisms, and mediating a variety of biological functions as intracellular messenger molecules [3].

The immune-enhancing effect is one of the most important biological activities of natural polysaccharides [39]. The immune-enhancing activity has been related with the structural features of polysaccharides, such as monosaccharide and glycosidic-linkage composition, conformation, molecular weight, functional groups, and branching characteristics [39–41]. Several studies have indicated that mannans are important structural polysaccharides in plants, and have good immunomodulatory effects [40]. For example, the mannans from *Aloe vera* and fenugreek (*Trigonella foenum-graecum* L.) with a back-bone of Man*p* residues that were ramified with other monosaccharides [40,42]. The result of present study agreed with the studies on *Aloe vera* and fenugreek.

4. Conclusions

To sum up, the research applied ion-exchange and gel filtration chromatography to fractionate the polysaccharides of *P. foetida* fruits, and the purified polysaccharide fraction (PFP1) was obtained. According to the results of FT-IR, SEM, monosaccharides, methylation and NMR analysis, PFP1 was deduced to be a heteropolysaccharide, with a backbone structure of →1)-α-D-Man*p*→1,2)-β-D-Man*p*-linked1,2, 6)-β-D-Man*p* residues and side chains consisted of →1)-β-D-Gal*p*, →1, 4)-α-D-Man*p*, →1,4)-β-D-Glc*p*, →1,3)-α-D-Gal*p*, →1,6)-β-D-Man*p*, →1,6)-β-D-Gal*p*, →1,2,3)-β-D-Man*p* and →1,3,6)-β-D-Gal*p* residues. Besides, PFP1 possessed promoting the secretion of cytotoxic molecules (NO) and cytokines (TNF-α and IL-6) of RAW264.7 macrophages. From the above results, *P. foetida* fruit polysaccharides could be a source of a functional food supplement candidate for hypoimmunity population.

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