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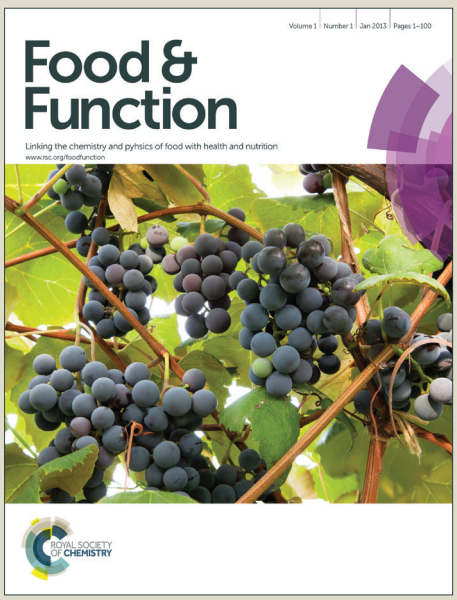

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Structural Characterization and Immunomodulatory Activity of a New Heteropolysaccharide from *Prunella vulgaris*

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Abstract

A new heterpolysaccharide, here called P1, was isolated from the fruit clusters of *Prunella vulgaris* using hot water extraction method. Chemical and physical analyses indicated that P1 had a spherical conformation with an average molecular weight of 1,750 kDa, and consisted of arabinose (28.37%), xylose (54.67%), mannose (5.61%), glucose (5.46%), and galactose (5.89%). The main linkage types of P1 were proved to be (1→5)-linked α-L-Ara, (1→)-linked α-L-Ara, (1→3)-linked α-D-xyl, (1→3)-linked β-D-Gal, (1→3,6)-linked β-D-Gal, (1→3,6)-linked α-D-Man and (1→6)-linked α-D-Glc according to periodate oxidation-Smith degradation and NMR analyses. P1 could significantly enhance the secretion of NO, TNF-α, and IL-6 in murine RAW 264.7 cells involving the toll-like receptor 2 (TLR2), TLR4 and complement receptor 3 (CR3). Further studies showed that P1 exhibited stable immune activities in the pH range of 4.0-10.0 and below 121 ℃. The results suggested that P1 could be used as a potent immunomodulatory agent in functional foods and pharmacological fields.

Key words: polysaccharide, *Prunella vulgaris*, structural characterization, immunomodulatory, toll-like receptor

1. Introduction

Macrophages, a kind of innate immune cells, are involved in many processes, such as tissue remodeling during embryogenesis, wound repair, clearance of apoptotic cells, hematopoiesis, and homeostasis. They also play pivotal roles in the defense against microbial invasion and tumorigenesis.[1, 2] Therefore, macrophages are usually chosen as ideal cell models to examine the immune-modulating effects of bioactive compounds. The role of activated macrophages in the immune system involves the release of various inflammatory mediators and cytokines, such as nitric oxide (NO), tumor necrosis factor (TNF), interleukin (IL), and reactive oxygen species (ROS).[3] Activation of macrophages required activation signals to trigger cytokine synthesis and release.[4] Development of polysaccharides to augment innate immune responses has attracted much attention during the past several decades.

Polysaccharides, considered as biological response modifiers (BRMs), show a number of beneficial therapeutic properties. They are widely used as potent immunotherapeutic agents with no serious side effects.[5, 6] The primary effect of polysaccharides is to enhance and/or activate macrophages immune responses through membrane receptors, resulting in immunomodulation, anti-tumor activity, wound-healing and other therapeutic effects.[3] Glucans are an important type of polysaccharides and their bioactivities are highly dependent on their structural characteristics, including molecular weight, monosaccharide composition, backbone linkage, degrees of branching, and branch linkage. The β-glucan with a 1→3 and 1→6 linked main chain shows antitumor and immunomodulatory activities, while α-glucan with 1→4 linked main chain usually serves as food nutrients. For example, polysaccharides isolated from mushrooms are typical β-glucans, showing strong antitumor and immunomodulatory activities.[7, 8] Therefore, it is important to search new polysaccharides with unique structure and high

immunity activity as novel BRMs.

Prunella vulgaris L., which belongs to the family of Lamiaceae, is a medicine food homology plant widely cultivated in China and Europe. The fruit clusters of *P. vulgaris* have long been used as a traditional medicine to alleviate sore throat, reduce fever and accelerate wound healing in folk.[9] In China, it has been extensively used as a health-promoting food and traditional Chinese medicine (TCM) for treatment of hypertension, jaundice, hepatitis, tuberculosis, mammitis, diabetes mellitus, *etc*.[10] To date, some polysaccharides isolated from the fruit clusters of *P. vulgaris* have been demonstrated to have antioxidant, antiproliferative and immune-stimulatory effects.[11-13] However, little work has been reported on the structural characterization, biological function and mechanisms of the immunomodulatory activities of the polysaccharides from *P. vulgaris*.

In the present study, a new heterpolysaccharide, named P1, was extracted from *P. vulgaris* using hot water extraction. The primary structures and conformation of P1 were characterized and its immunomodulatory activities and immune receptors using murine macrophage RAW 264.7 cells were investigated. In addition, the stability of P1 bioactivity under different pH values and thermal treatments were investigated. The results will provide significant information for the application of this plant in functional foods and pharmacological fields in the future.

2. Materials and Methods

2.1. Materials and Chemicals

The fruit clusters of *P. vulgaris,* derived from Hubei province of China, were purchased from Qingping medicinal material market (Guangzhou, China). The murine macrophage RAW 246.7

cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin and phosphate-buffered saline (PBS, pH 7.4) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biological Technology Co. (Zhejiang, China). Anti-TLR2 antibody, anti-TLR4 antibody and Anti-CR3 antibody were purchased from Abcam Inc. (Cambridge, MA). Laminarin (Lam), lipopolysaccharide (LPS, isolated from *E. coli* strain 055: B5) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Standards of dextran and monosaccharides, uronic acid, glycol, glycerol, and erythritol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DEAE (diethylaminoethyl)-Sepharose and Sephadex G-200 were purchased from GE Healthcare Life Science (Piscataway, NJ, USA). Nitric oxide (NO)-detecting kit was purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). The mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit and the mouse IL-6 ELISA kit were purchased from Neobioscience Technology Co., Ltd. (Shenzhen, China). Other chemicals and reagents used in this study were of analytical grade.

2.2. Extraction, Isolation, and Purification of Polysaccharides

The crude polysaccharides were prepared and then fractionated according to the method described previously.[13] Three fractions, PV-P1, PV-P2, and PV-P3 were obtained by freeze-drying. The PV-P1 showed the most pronounced immune ability (data not shown). For this reason, PV-P1 was further purified by Sephadex G-100 column chromatography. Ten milligrams of PV-P1 were re-dissolved in 1 mL of distilled water and loaded onto a Sephadex G-100 column (10 mm × 400 mm). The sample was eluted with distilled water at a speed of 0.2

mL/min. The eluent was collected with an automatic collector and then examined. The elution curve was drawn using the tube number and absorbance (490 nm). The elution peaks were evaluated and the P1 fraction (Fig.1A) was collected and then freeze-dried. The carbohydrate content of P1 was measured by the phenol-sulfuric acid method.[14]

2.3. Physicochemical Properties

Physicochemical properties of P1 were determined by color observation, solubility test, and iodination reaction. The absorption spectrum of P1 solution was recorded using a TU-1901 UV-vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) in the wavelength range of 190-800 nm.

2.4. Determination of Molecular Weight

The molecular weight of the purified polysaccharide was measured using gel-permeation chromatography (GPC) performed on a Waters instrument equipped with the TSK-GEL columns (Tosoh Co., Ltd, Tokyo, Japan) in series of G5000 PW_{XL} (7.8 × 300 mm i.d., 10 μm) and G3000PW_{XL} (7.8 × 300 mm i.d., 5 μm). The mobile phase was 0.02 M KH_2PO_4 (pH 6.0) at a flow rate of 0.6 mL/min. The column temperature was kept at 35.0 ± 0.1 ℃. The injection volume was 20 μL in each run. The calibration curve of dextran standards obtained was $\mathrm{Log}Mw=36.803-5.5527V+0.3325V^2-0.007V^3$ (where Mw represents the molecular weight, while V represents elution volume) with a correlation coefficient of 0.9999. The elution volume of P1 was plotted in the same calibration curve, and the molecular weight was determined.

2.5. Infrared (IR) Spectrum

The infrared spectrum was collected using a Vector 33 FT-IR spectrophotometer (Bruker, Ettilingen, Germany). The P1 was ground with KBr power and then pressed into pellets for

transmission IR measurement in the wavenumber range of 500-4000 cm^{-1}.[15]

2.6. Uronic Acid Analysis

Galacturonic acid and glucuronic acid contents were measured according to the method described previously.[8] Briefly, P1 (5 mg) was hydrolyzed in a sealed tube with 2 M trifluoroacetic acid (4 mL) at 105 ℃ for 6 h. The residue was re-dissolved in distilled water (10 mL) and filtered through a 0.22 μm microporous filtering film. The operation was done using a Dionex ion chromatography ICS 5000 (Sunnyvale, CA, USA) with a CarboPac PA1 analytic column (4 × 250 mm). Galacturonic acid and glucuronic acid were used as standards.

2.7. Monosaccharide Composition Analysis

The monosaccharide composition was determined as previously described.[16] P1 (5 mg) was hydrolyzed in a sealed tube with 2 M trifluoroacetic acid (4 mL) at 105 ℃ for 6 h. Excess trifluoroacetic acid was removed by evaporation under reduced pressure. The residue was dissolved in methanol and evaporated to dryness for three times. The residue was re-dissolved in methanol (2 mL), and transferred to a glass tube. The solution was blow-dried with nitrogen. Hydroxylamine hydrochloride (10 mg), inositol hexacetate (1 mg), and pyridine (2.0 mL) were added in the tube. The sealed tube was immersed in a thermostatic water bath at 90 ± 1 ℃ for 30 min. Then, acetic anhydride (2 mL) was added and kept at 90 ℃ for 30 min. Distilled water (2 mL) was added to terminate the reaction. The acetylated derivatives were extracted with methylene chloride (4 mL). The methylene chloride layer was treated with anhydrous sodium sulfate and filtered through a 0.22 μm microporous filtering film. GC was performed on a gas chromatography/mass spectrometer (Trace DSQ II, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a TR-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The temperature

program was set as follows: the initial temperature of the column was 100 ℃ and held for 2 min, then increased to 280 ℃ at 5 ℃/min and held for 5 min. The flow rate was 1 mL/min. The injection temperature was 250 ℃. The ion source of the mass spectrometer was set at 280 ℃. The injection volume was 1μL and the split ratio was 10:1.

2.8. Periodate Oxidation-Smith Degradation

The periodate oxidation-Smith degradation assay was carried out according to the method described previously.[8] Briefly, P1 (20 mg) was dissolved in distilled water (12.5 mL), and 30 mM $NaIO_4$ (12.5 mL) was then added. The solution was kept in the dark at room temperature until the optical density value at 223 nm became stable. The oxidation solution (2 mL) was titrated with NaOH standard solution (0.01 M) to calculate the production of formic acid. The rest was dialyzed against distilled water, and the residue was reduced with $NaBH_4$ in the dark for 24 h, neutralized to pH 6.0-7.0 with 50% acetic acid, dialyzed, and concentrated to dryness. The residue was hydrolyzed with 2 M trifluoroacetic acid in a sealed glass tube. The residue was dissolved in methanol and evaporated to dryness for three times. Acetylation was carried out with hydroxylamine hydrochloride and pyridine in the sealed tube at 90 ℃ for 30 min. Then, acetic anhydride (1 mL) was added for another 30 min. The acetate derivate was analyzed using a gas chromatography (GC) spectrometer. Glycol, glycerol, erythritol, rhamnose, arabinose, xylose, mannose, glucose, and galactose were used as standards.

2.9. NMR Spectroscopy

NMR analysis was carried out on a Bruker 600 MHz (Bruker Corp., Fallanden, Switzerland). P1 (30 mg) was dissolved in D_2O (0.6 mL). ^1H NMR and ^{13}C NMR spectra were obtained at 25 ℃. All chemical shifts were expressed in ppm.

2.10. Atomic Force Microscopy (AFM)

The morphological characteristics of the polysaccharide P1 was observed by Tapping Mode (TM)-AFM (Nanoscope 3A Multimode, Veeco Co., USA) in air at room temperature with a relative humidity of 65% according to the method described previously.[17] P1 (1 mg) was dissolved in distilled water (1.0 mL) with continuous stirring for 2 h in a water bath at 60 ℃. After cooling to room temperature, the solution was diluted to a final concentration of 2.5 μg/mL, gently stirred overnight and filtered through a 0.45 μm microporous filtering film. Approximately 5 μL of aliquot (2.5 μg/mL) was pipetted onto freshly cleaved surface of mica sheet. After drying fully in air, the mica sheet was pasted to a round metal plate and then placed on the magnetic observing platform. The image was examined using TM-AFM with a silicon probe (Tap 150-G-10, Ted Pella, INC., USA) and captured at a scan rate of 1.0 Hz and tip velocity of 600 μm/S. Software *WSxM* (Nanotec Electronica, Spain) was used to process AFM image.

2.11. Preparation of P1 Solutions for Cell Culture

(1) P1 solutions were prepared in culture medium at concentrations of 62.5, 125, 250, and 500 μg/mL. (2) P1 in phosphate buffers (PBS) (6 mL) at a concentration of 2 mg/mL was divided into three parts and thermal treated for 30 min at 100, 121, and 145 ℃, respectively. Then each part was adjusted to a concentration of 400 μg/mL using culture medium. (3) P1 (2 mg/mL) was dissolved in PBS to adjust final pH 2.0, 4.0, 6.0, 7.4, 8.0, and 10.0, respectively. All samples were placed overnight at room temperature and then adjusted to the concentration of 400 μg/mL with culture medium. All solutions were sterilized using a 0.22 μm microporous filtering film and then incubated with RAW 264.7 cells.

2.12. Cell Culture and Sample Treatment

RAW 264.7 cells were cultured in DMEM containing 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin in a humidified incubator with 5% CO_2 at 37 ℃. Cells in the logarithmic growth phase were adjusted to the concentration of 1×10^6 cells/mL. The cell solutions (100 μL) and sterilized PBS (100 μL) were added to each well. Cells were cultured at 37 ℃ in a 5% CO_2 humidified atmosphere incubator for 24 h. The culture medium was then refreshed, and cells were incubated with P1 sample. The immune receptors of P1 were determined by antibody inhibition experiments. The cells were incubated with the monoclonal antibodies (5 μg/mL) and Lam (500 μg/mL) for 1 h before stimulation with P1. LPS (50 μg/mL) was used as the positive control. The supernatants were collected after 24 h incubation. The concentrations of NO, TNF-α and IL-6 were determined using the NO-detecting kit, mouse TNF-α kit and mouse IL-6 ELISA kit, respectively, according to the manufacturer's instructions.

2.13. Statistical Analysis

Data were presented as mean ± standard deviation (SD) with triplicates. Significance was determined at $p < 0.05$ by one-way analysis of variance followed by Duncan's least significant using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Physicochemical Properties

Polysaccharide P1 exhibited white color and was soluble in water, especially in the hot-water. It cannot be dissolved in organic solvents such as methanol, ethanol, acetone, ethyl acetate, and butyl alcohol. The result of the iodination test indicated that P1 did not contain starch. No ultraviolet absorption peaks at 260 and 280 nm also suggested the absence of nucleic acid and

protein in P1. The total carbohydrate content of P1 was determined to be 97.5% (w/w).

3.2. Chemical Structures

The molecular weight distribution of P1 is shown in Fig. 1B. P1 comprised of a polysaccharide with an average molecular weight of 1,750 kDa. Results of gas chromatography suggested that P1 was composed of arabinose, xylose, mannose, glucose, and galactose with molar percentages of 28.37, 54.67, 5.61, 5.46, and 5.89%, respectively (Fig. 3).

The IR spectrum of P1 showed the absorption peaks at 3374, 2928, and 1422 cm^{-1}, corresponding to the stretching of the O-H, C-H, and carboxyl C-O groups (Fig. 2A). The absorption peak at 1605 cm^{-1} was due to the bound water. The peak in the region of 1249 cm^{-1} was assigned to O-H deformation vibrations. A characteristic absorption at 901 cm^{-1} indicated the presence of β anomeric configurations, while the absorption at 1046 cm^{-1} was typical for the pyranose form.[18] A weak characteristic peak at 1745 cm^{-1} indicated the existence of uronic acid in the polysaccharide structure.[19] The equation of the standard curve made by different concentrations of galacturonic acid and the peak area is shown in Fig. 2B. The result of ion chromatography analysis indicated that the content of galacturonic acid was 2.9% (w/w) (based on dry weight of P1) (Fig. 2C).

Using periodate oxidation, Smith degradation, and gas chromatography (GC), the position of glycosidic linkages in P1 was measured (Fig. 4). The standard curve of $NaIO_4$ is shown in Fig. 4C. According to the equation of standard curve, results of periodate oxidation showed that 0.794 mol of HIO_4 consumed and 0.350 mol of formic acid produced per hexose residue, indicating that the (1→)-linked or (1→6)-linked glycosyl linkages accounted for 35.0% and the (1→2)-linked or (1→4)-linked glycosyl linkages accounted for 9.4% of all linkages in the

molecule.[19] The oxidized P1 was converted into the corresponding alditol acetate for GC analysis. The presence of five monosaccharides revealed that some of the linkages were (1→3)-linked, (1→2,4)-linked, (1→2,3)-linked, (1→3,4)-linked, or (1→2,3,4)-linked glycosyl linkages, accounting for about 55.6% of all linkages in the molecule.[20] And these linkages could not be oxidized. The presence of glycerol and erythritol indicated the presence of the (1→2)-linked, (1→6)-linked, and/or (1→2,6)-linked, as well as (1→4)-linked and/or (1→4,6)-linked glycosyl bonds.[21, 22]

The spectra of ^{1}H NMR and ^{13}C NMR of P1 are shown in Fig. 5. Signals of P1 in ^{1}H NMR and ^{13}C NMR spectra were assigned based on the monosaccharide composition, linkage analysis, and chemical shifts.[20, 23-26] The ^{1}H NMR spectrum contained five signals at δ 5.48, 5.28, 5.26, 5.16, and 4.94 ppm for the anomeric protons, indicating five residues designated as (1→5)-linked α-L-Ara, (1→)-linked α-L-Ara, (1→3,6)-linked α-D-Man, (1→3)-linked α-D-xyl, and (1→6)-linked α-D-Glc, respectively (Fig. 5A). Signals at δ 4.61, 4.55, 4.53, 4.50 ppm were assigned to (1→6)-linked β-D-Gal, β-galacturonic acid, (1→3)-linked β-D-Gal, and (1→3,6)-linked β-D-Gal, respectively. The chemical shifts from δ 3.4 to 4.0 ppm were assigned to proton signals of carbons C-2 to C-6 of sugar rings.[21] The ^{13}C NMR spectrum showed seven signals in the anomeric region (δ 95-110 ppm), which were assigned to α (δ 95-102 ppm) and β (δ 103-110 ppm) anomeric configuration (Fig. 5B). Signals at δ 95.98, 97.24, 100.19, 101.33, 109.16 ppm were attributed to the anomeric carbon atoms of (1→3,6)-linked α-D-Man, (1→6)-linked α-D-Glc, (1→3)-linked α-D-xyl, (1→5)-linked α-L-Ara, and (1→)-linked α-L-Ara units, respectively. The signal at δ 105.99 ppm was due to C-1 of β-galacturonic acid, (1→3)-linked β-D-Gal, and (1→6)-linked β-D-Gal units. The signals from δ 55.17 to 86.26

ppm were assigned to carbons C-2 to C-6 of the residues.[23]

Previous chemical analysis results indicated that P1 had different chemical compositions and structure characteristics.[27, 28] The structural differences might be due to the differences in extraction methods, the specific *P. vulgaris* strain, and growing conditions. Different analytical methods may also generate different results.[16, 29]

3.3. Molecular Morphology

AFM can intuitively provide three-dimensional structural information of macromolecules and is widely used for observing the surface morphology or structure of polymers.[30] AFM image showed that the morphological chain conformation of the P1 was spherical in distilled water (Fig. 6). The spherical polysaccharides have the potential of being used in the drug delivery and controlled release fields.[31, 32]

3.4. Immunomodulatory activity

3.4.1. Effects of Different Concentrations of P1 on Cytokine Production

Cytokines, such as NO, TNF-α, and IL-6, secreted from activated macrophages play a pivotal role in fighting microbial invasion and tumorigenesis. NO, TNF-α, and IL-6 are widely used as immune response parameters.[33] Our previous study demonstrated that *P. vulgaris* polysaccharide had no cytotoxicity to RAW 264.7 cells within the tested concentrations in this study (data not shown). Effects of different concentrations of P1 on NO, TNF-α, and IL-6 production are shown in Fig. 7. NO production is regulated by the expression of inducible oxide synthase (iNOS) in activated macrophages.[34] The activation of macrophages required activation signals. Thus, the level of NO in the control was very low. P1 remarkably increased the secretion levels of NO and IL-6 in RAW 264.7 cells in a dose-dependent manner within the

tested concentrations (Fig. 7A, C). P1 also increased the TNF-α production in a dose-dependent manner below the concentration of 125 μg/mL (Fig. 7B). When the concentration increased from 125 to 250 μg/mL, the amount of TNF-α remained stable. The result indicated the existence of maximum dosage for the secretion of TNF-α. Overall, these results confirmed that P1 had significant immunomodulatory activity. Previous studies demonstrated that the anti-cancer activity and immunomodulatory activity of polysaccharide were mainly related to the (1→3)-D-glucans in its molecular structure.[7, 35, 36] P1 with 55.6% (molar percentage) of (1→3)-linked glycosyl linkages may account for its significant immunomodulatory activity. Furthermore, the triple-helix conformation of (1→3)-D-glucans played an important role in influencing cytokine stimulating activity and antitumor activity.[37, 38] Previous studies demonstrated that some polysaccharides had immunomodulatory activities in the triple-helix conformation but not as a single flexible chain.[17, 38, 39] However, Xu et al. (2012) found a polysaccharide lacking triple-helix conformation from *Lentinula edodes* also had immuno-modulating activities.[40] Our study demonstrated that polysaccharides from *P. vulgaris* had no triple-helix conformation by Congo red assay (data not shown). AFM showed that P1 had spherical conformation when dispersed in water. Previous studies demonstrated that polysaccharides with sphere-like conformation had antitumor activity *in vitro*.[41] Thus, the spherical conformation of polysaccharide would affect its immunomodulatory activity. In-depth investigations on the relationship between spherical conformation and biological activities of P1 are underway. These results indicated that P1, a new heteropolysaccharide isolated from the fruit clusters of *P. vulgaris* with spherical conformation, exhibited significant immunomodulatory activities *in vitro*. Further research is required to demonstrate that P1can

impact macrophages *in vivo*.

3.4.2. Effects of Anti-TLR2, Anti-TLR4, Anti-CR3 and Dectin-1 Treatments on P1-induced Cytokine production

Activated macrophage can kill tumor cells and pathogens by either direct killing or releasing diffusible cytokines. It is thought to be mediated through the recognition of polysaccharide by specific immune receptors.[4] Studies suggested that polysaccharides could mediate immunomodulatory effects through the TLR2, TLR4, CR3, or Dectin-1 membrane receptors.[18, 19, 42] To further determine the membrane receptor of P1, RAW 246.7 cells were treated with anti-TLR2, anti-TLR4, anti-CR3 antibodies or the Dectin-1 inhibitor Lam for 1 h before stimulation of P1. Lam, a soluble β-glucan, was reported as the inhibitor of Dectin-1 activity through binding to Dectin-1.[43] Then Lam was employed to further study the potential role of Dectin-1.[18] The production of NO, TNF-α, and IL-6 in RAW 246.7 cells was measured. As shown in Fig. 8a, treatment of RAW 246.7 cells with anti-TLR2 reduced the P1-induced NO production by 64%. However, the anti-TLR4, anti-CR3, and Lam were unable to inhibit the secretion of NO. The results suggested that P1 induced NO production through the activation of TLR2. As shown in Fig. 8c, after anti-TLR2, anti-TLR4, and anti-CR3 incubation, the production of IL-6 was decreased by 42, 37, and 38%, respectively, but still significantly higher than that in the absence of P1 stimulation. The Lam did not inhibit the increase in IL-6 production. The results suggested that P1 induced IL-6 production through the activation of TLR2, TLR4, and CR3. However, no reduced TNF-a production was observed after the treatment with four antibodies, indicating different signaling pathways for NO, TNF-α, and IL-6 production in RAW 246.7 cells (Fig. 8b). Other immune membrane receptors may also be

possible.

The TLR signaling pathways generally include two distinct pathways depending on the adaptor molecules, myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor inducing interferon-β (TRIF). TLR2 and TLR4 trigger MyD88 signaling, which activates mitogen-activated protein (MAP) kinase and nuclear factor (NF)-κB to induce expression of proinflammatory cytokins genes. TLR4 also activates TRIF-dependent signaling, which activates interferon regulatory factor 3 (IRF3) to induce the expression of proinflammatory cytokins genes and type 1 IRF3.[2] CR3 activates spleen tyrosine kinase (syk) signaling, which activates NF-κB to induce expression of proinflammatory cytokins genes.[44] Han et al. (2009a) demonstrated that an aqueous extract of *P. vulgaris* could stimulate macrophage activation via NF-κB transactivation and MAP kinase activation.[9] Therefore, we deduced that P1 might activate macrophage to release the above mediators partly involving the NF-κB signaling pathway. The results might provide references to the signal transduction and molecular mechanisms of P1-induced macrophage activation.

3.4.3. Effects of P1 on Cytokine Production under Different pH and Thermal Treatments

Thermal and different environmental condition treatments can change the structure of polysaccharide and cause the loss of activity.[19, 45] Effects of thermal and different pH treatments on the activity of P1 were investigated. As shown in Fig. 9a, there were no obvious changes in NO, TNF-α, and IL-6 production in RAW 246.7 cells induced by P1 treated with temperatures below 121 ℃. However, after high-temperature treatment at 145 ℃ for 20 min, P1 almost lost all of its secretion-inducing ability of NO, TNF-α, and IL-6. Previous studies showed that the ordered conformation of polysaccharide could be disrupted by thermal treatment at about

137-145 ℃.[45] However, the chemical bonds could not be changed.[19] The GPC analysis also demonstrated that there was no difference in the molecular weight between thermal treated and untreated P1, indicating the chemical bonds of P1 were not broken. The result indicated that the ordered conformation of P1 was changed by thermal treatment at 145 ℃, resulting in the loss of immune activity. As shown in Fig. 9b, after treatment for 12 h at solutions ranging from pH 2.0 to 10.0, P1 maintained active conformation and was also able to induce the secretion of NO, TNF-α, and IL-6. However, after treatment with pH 2.0 solvent, P1 almost lose all of its activity, with the NO, TNF-α, and IL-6 production equal to the control groups (without P1). These results showed that P1 had high stability in the pH range of 4.0 to 10.0. Therefore, P1 has the potential of being used as a immunomodulatory agent in the drug and food industries.

4. Conclusion

In this study, a new heteropolysaccharide P1 was isolated from *P. vulgaris* using boiling water. P1 had an average molecular weight of 1,750 kDa and was composed of Ara, Xyl, Man, Glu, and Gal with molar percentages of 28.37, 54.67, 5.61, 5.46, and 5.89%, respectively. AFM analysis showed that P1 had a spherical conformation when dispersed in water. P1 showed significant immuno-stimulating activities involving TLR2, TLR4 and CR3. P1 exhibited highly stable immuno-stimulating activities in the pH range of 4.0-10.0 and below 121 ℃. Our results demonstrated that P1 could be used as a potential complementary medicine or functional food. In-depth studies on the immunomodulatory activity *in vivo* and molecular mechanisms of action of P1 are in progress.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Figure Captions

Fig. 1 Chromatogram of P1 by Sephadex G-100 (A) and GPC (B)

Fig. 2 IR spectrum of P1 (A), standard curve of galacturonic acid (B) and ion chromatography of galacturonic acid in P1 (C)

Fig. 3 Gas chromatography (GC) results of acetyl derivatives of monosaccharide standards (A) and hydrolyzate of P1 (B).

Fig. 4 Gas chromatography (GC) results of acetyl derivatives of standards (A), Smith degradation product of P1 (B) and the standard curve of $NaIO_4$ (C).

Fig. 5 NMR spectra of the polysaccharide P1 in D_2O: (A) ^1H NMR and (B) ^{13}C NMR.

Fig. 6 The AFM (scale: 3.0 μm ×3.0 μm) image height of P1 in distilled water.

Fig. 7 Effects of the different concentrations of P1 on production of NO (A), TNF-α (B), and IL-6 (C) in RAW 264.7 cells. Lipopolysaccharides (LPS, 50 μg/mL) were used as the positive control. Bars with no letter in common are significantly different ($p < 0.05$).

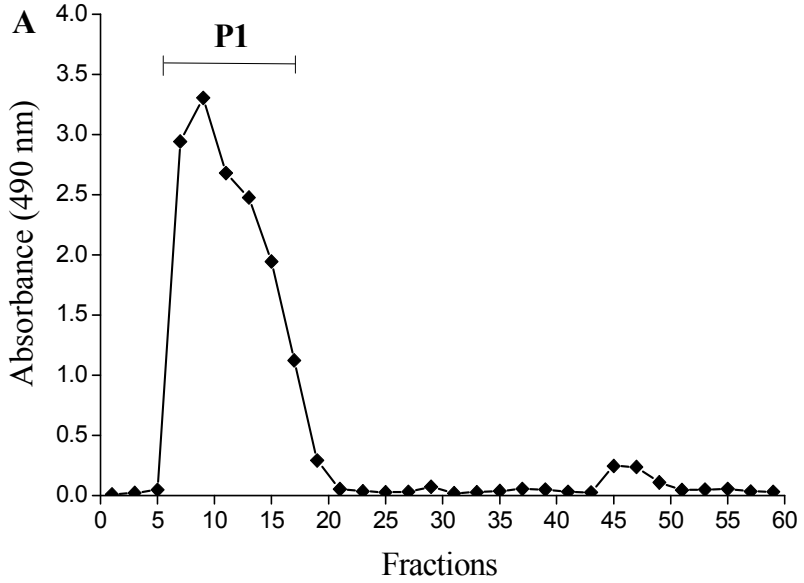
Fig. 8 Effects of anti-TLR2 (aTLR2), anti-TLR4 (aTLR4), anti-CR3 (aCR3), and Dectin-1 treatments on P1-induced NO (A), TNF-α (B), and IL-6 (C) production in RAW 264.7 cells. Laminarin (Lam) was used as the Dectin-1 inhibitor. The cells were incubated with monoclonal antibodies for 1 h and then washed extensively before stimulation with P1 (250 μg/mL). $^a p < 0.05$ versus the P1 group.

Fig. 9a Effects of different temperature on P1-induced NO (A), TNF-α (B), and IL-6 (C) production in RAW 264.7 cells. The cells were incubated with P1 (400 μg/mL) for 24 h. The P1 was treated at different temperature. $^a p < 0.05$ versus the untreated group.

Fig. 9b Effects of different pH on P1-induced NO (A), TNF-α (B), and IL-6 (C) production in

RAW 264.7 cells. The cells were incubated with P1 (400 μg/mL) for 24 h. The P1 was treated at PBS of different pH. [a]$p < 0.05$ versus the untreated group.

Fig. 1



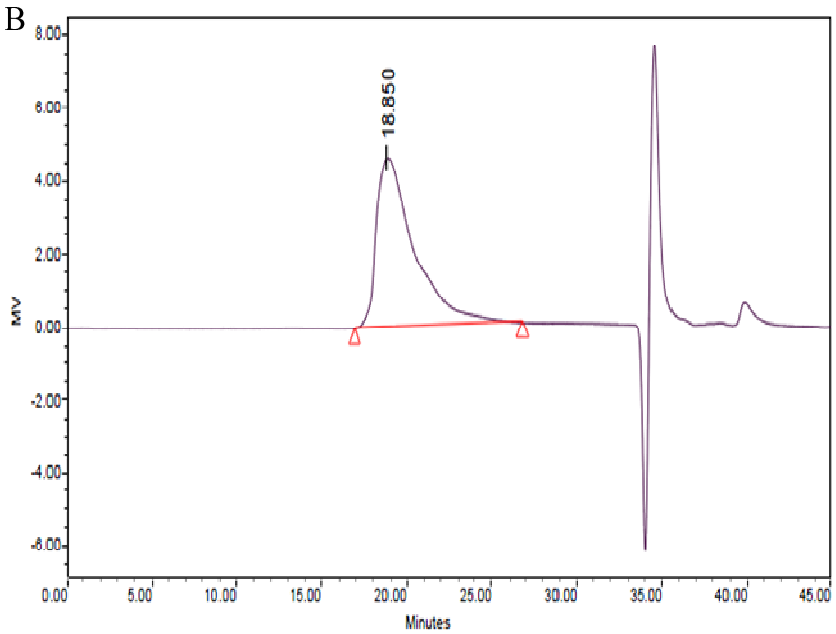


Fig. 2

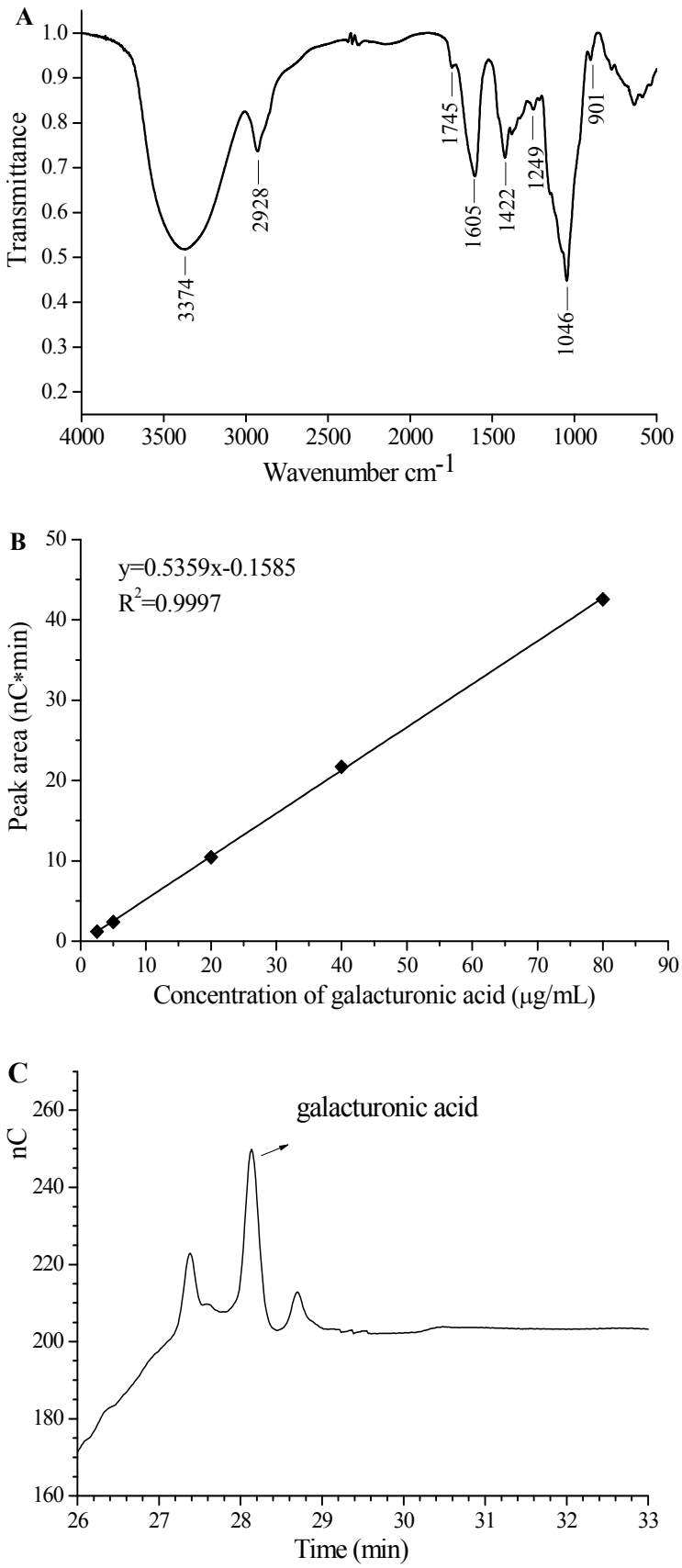
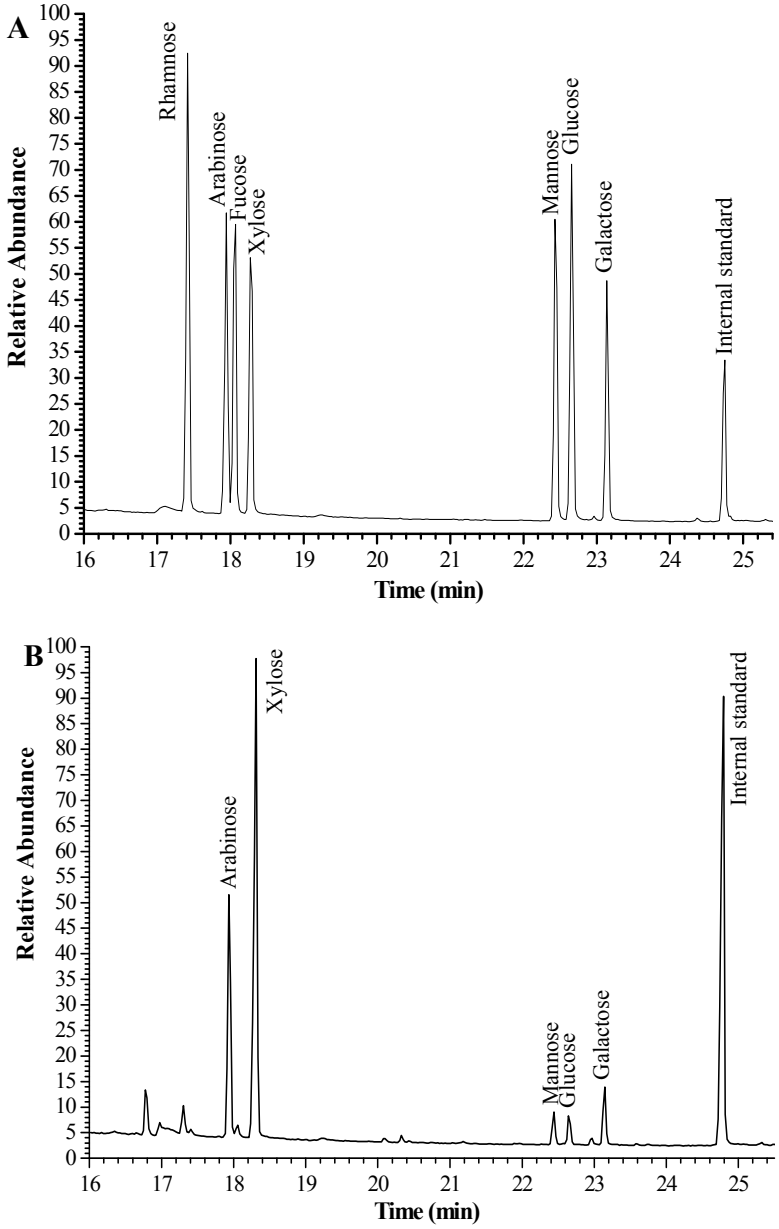


Fig. 3



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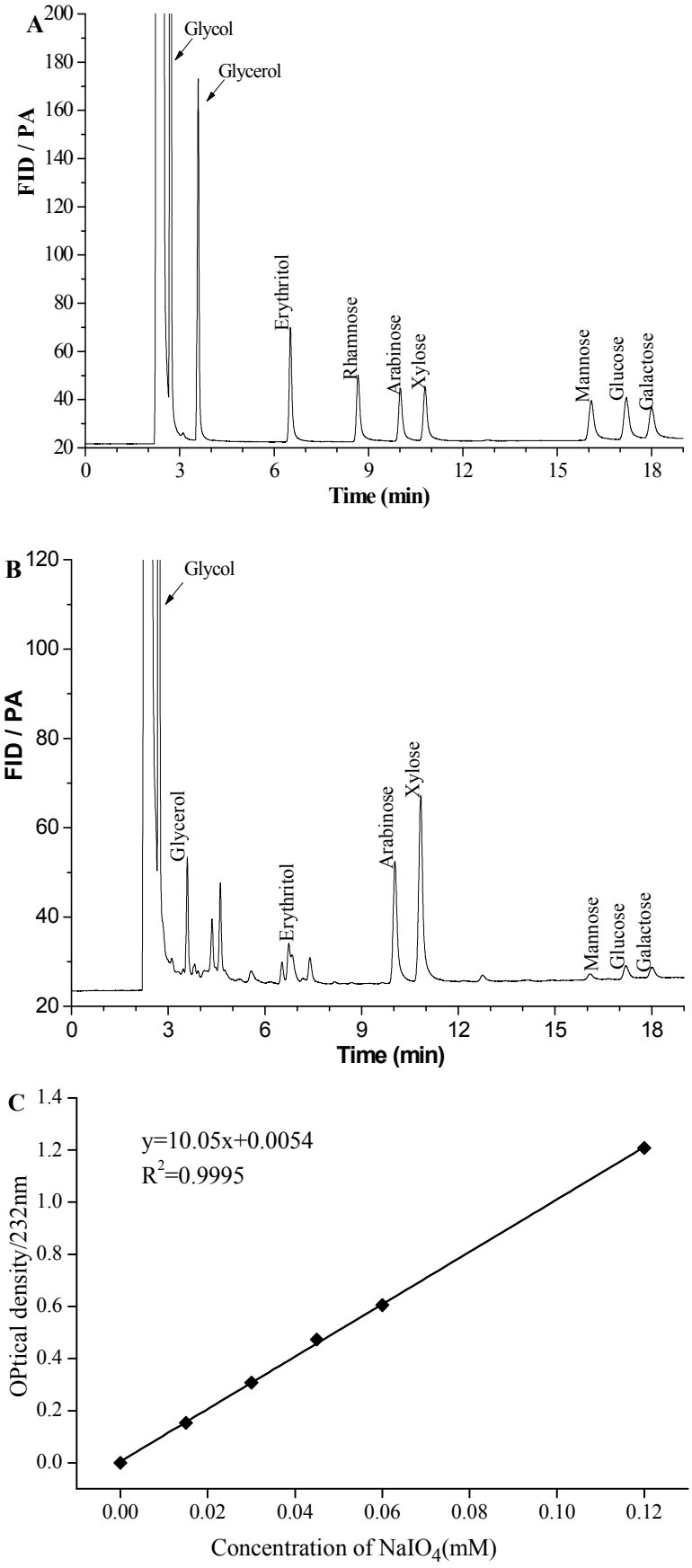


Fig. 5

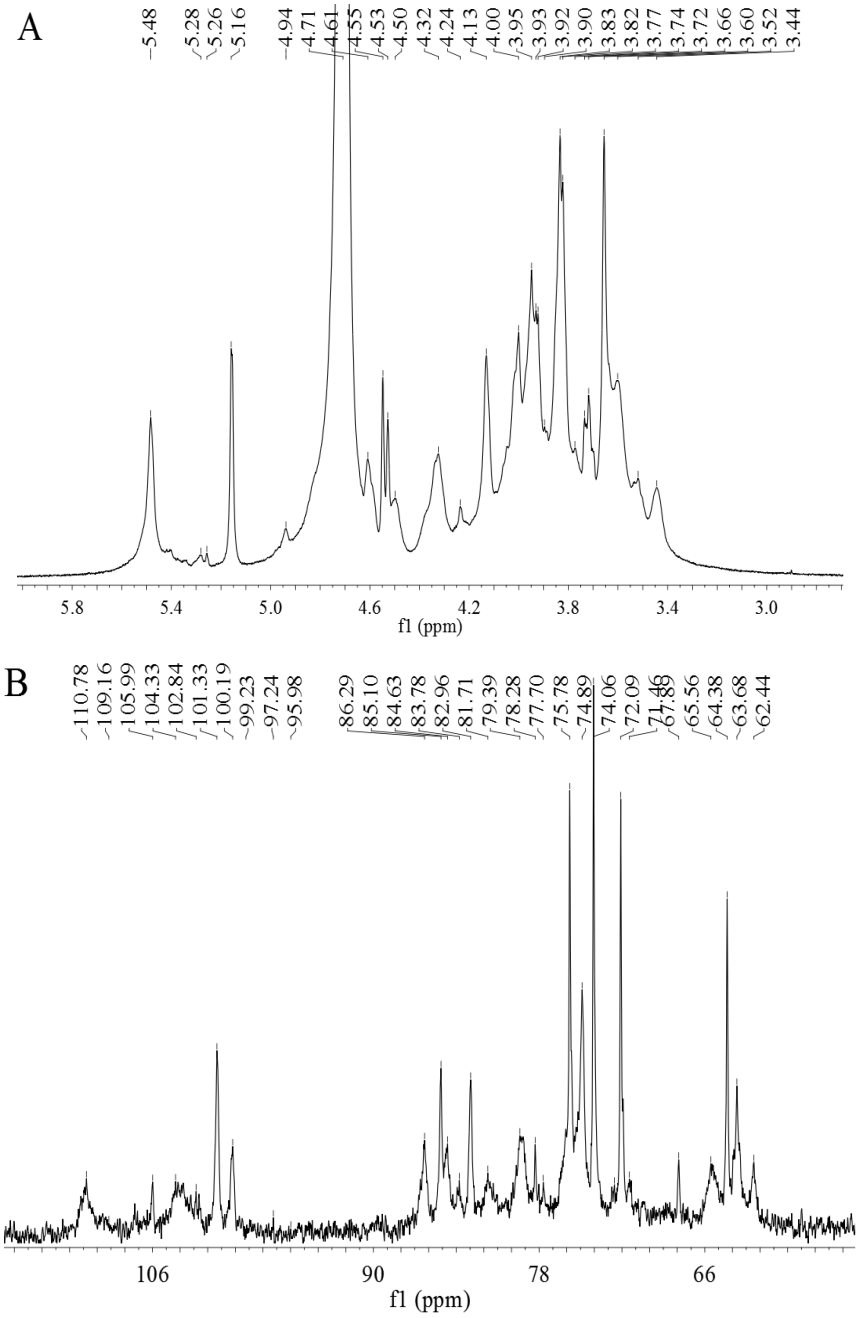
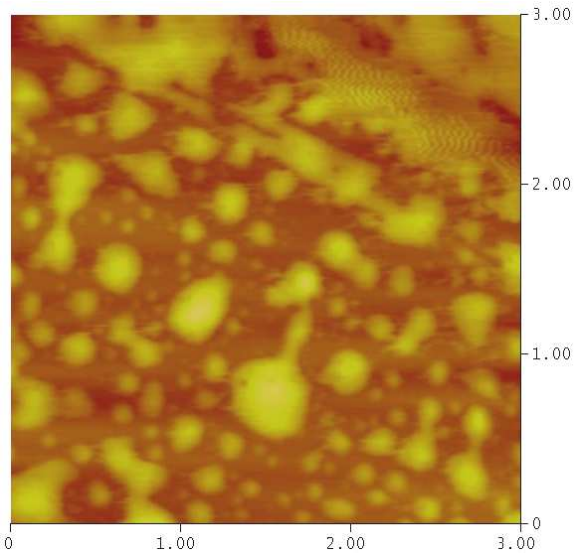


Fig. 6



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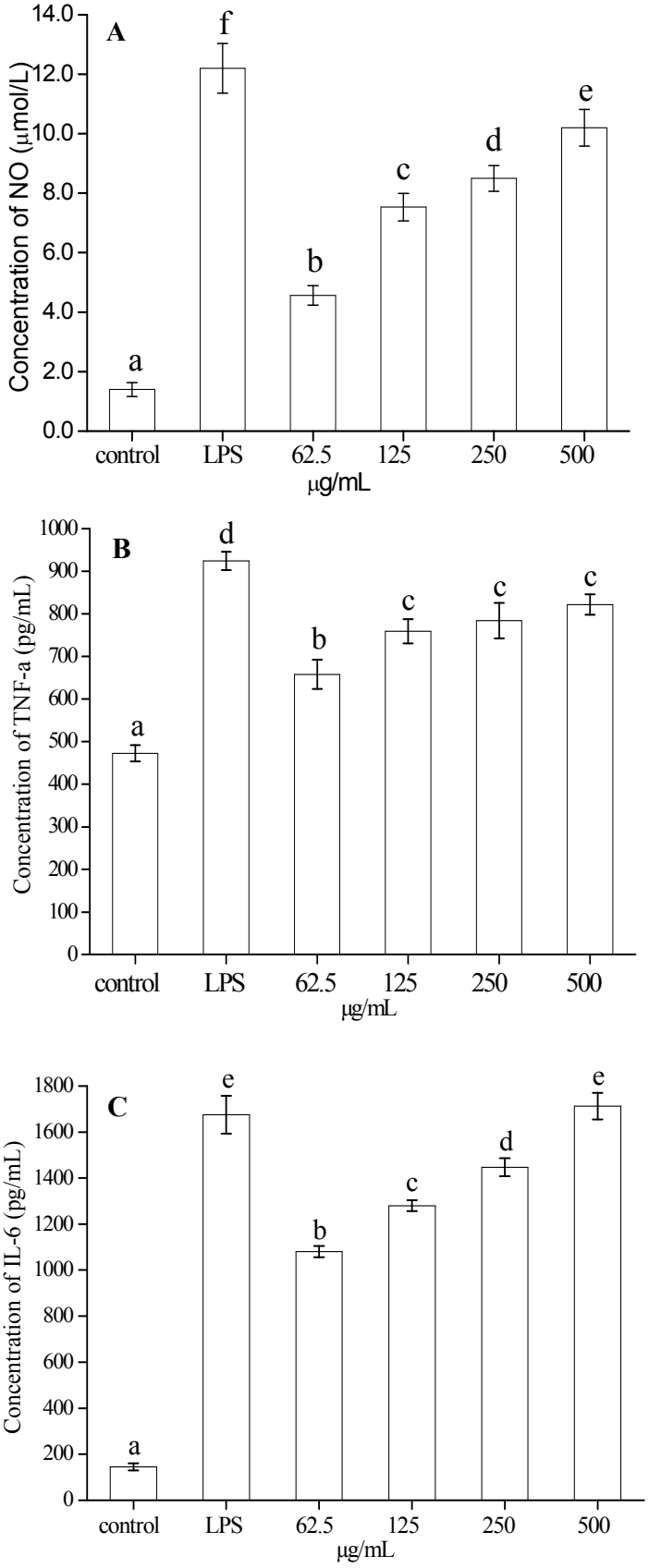

Fig. 8

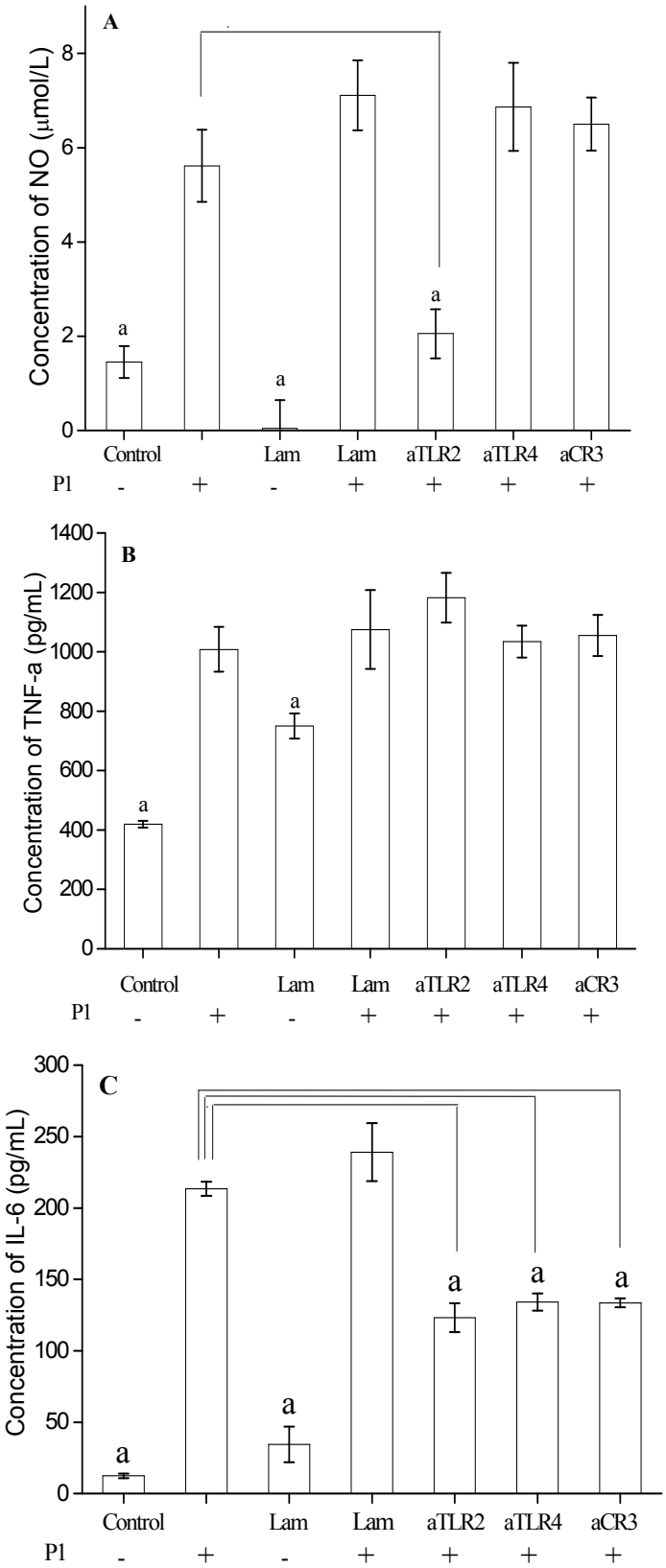

Fig. 9a

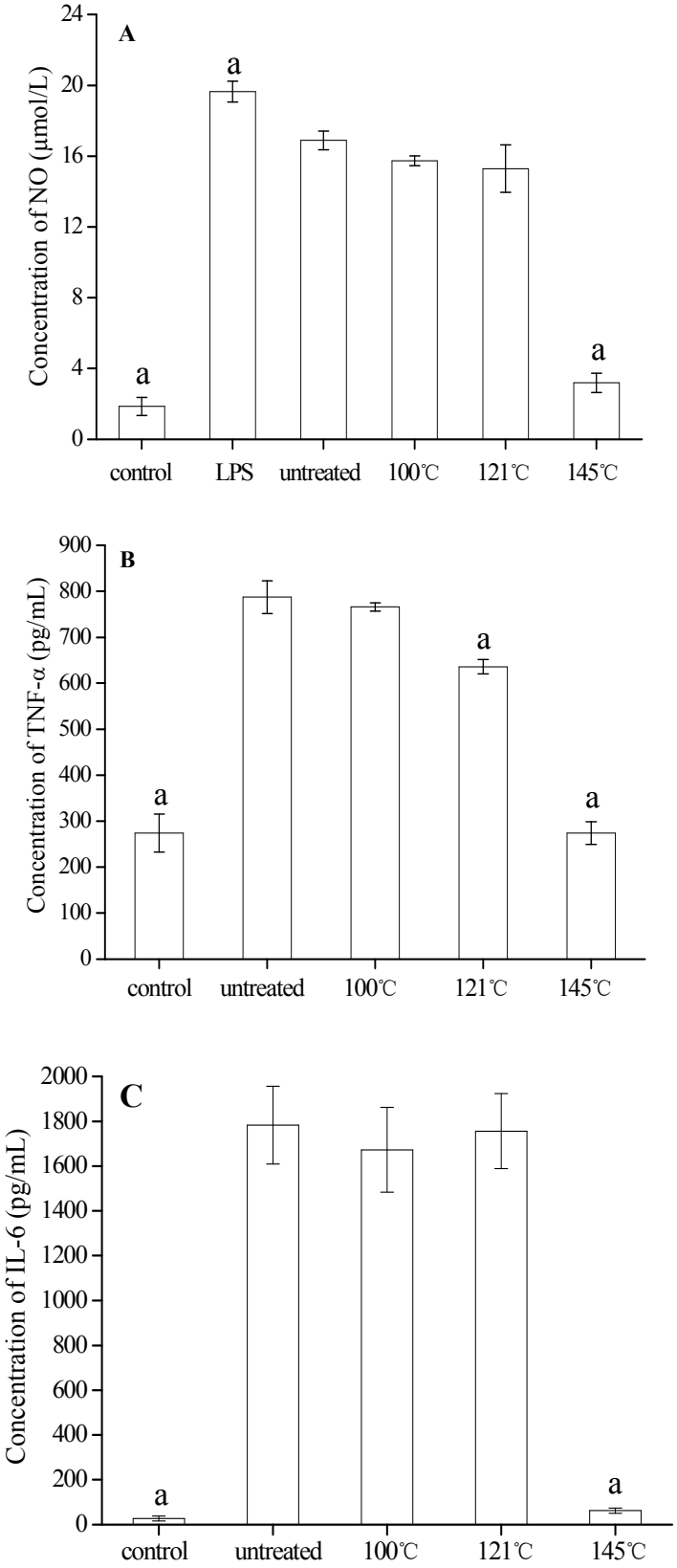

Fig. 9b