JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Structural characterization and immunostimulatory activity of polysaccharides from Brassica rapa L.

Zhuo-Er CHEN, Reziyamu WUFUER, Jin-Hu JI, Jin-Fang LI, Yu-Feng CHENG, Caixia Dong, and Hailiqian TAOERDAHONG

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.7b03902 • Publication Date (Web): 10 Oct 2017

Just Accepted

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Structural characterization and immunostimulatory activity of polysaccharides from *Brassica rapa* L.

Zhuo-Er CHEN[1‖], Reziyamu WUFUER[1‖], Jin-Hu JI[2], Jin-Fang LI[3], Yu-Feng CHENG[1], Cai-Xia DONG[4*], Hailiqian TAOERDAHONG[1*]

[1]College of Pharmacy, Xinjiang Medical University,Urumqi, Xinjiang Uygur Autonomous Region,China;

[2]Medical Engineering Technology Institute, Xinjiang Medical University, Urumqi, Xinjiang Uygur Autonomous Region, China;

[3]The Experimental Teach Center, College of HouBo, Xinjiang Medical University, Karamay, Xinjiang Uygur Autonomous Region, China;

[4]Tianjin Key Laboratory on Technologies Enabling Development of Clinical Therapeutics and Diagnosis, School of Pharmacy, Tianjin Medical University, Tianjin 300070, China.

Corresponding Authors

*(C.-X.D.) Tel: +86-183-2247-3708; E-mail: dongcaixia@tmu.edu.cn

*(H.T.) Tel: +86-139-9987-9870; E-mail: hailiqian2471@sina.com

Author Contributions

‖Z.-R.C. and R.W. contributed equally.

ABSTRACT

Two neutral polysaccharides (BRNP-1 6.9 kD, BRNP-2 4.8 kD) were purified from the common edible plant *Brassica rapa* L. via the combined techniques of ion-exchange chromatography and high-performance gel permeation chromatography. Monosaccharide composition analysis showed that BRNP-1 and BRNP-2 were composed of glucosyl residues. Methylation and 1D- and 2D-NMR analyses revealed that both BRNP-1 and BRNP-2 contained a backbone chain that was composed of *α*-D-(1→4)-linked Glc*p* residues and side chains that were composed of terminally linked Glc*p* residues attached at the *O*-6 position of backbone-glycosyl residues. BRNP-1 and BRNP-2, however, differed in branch degree and molecular weight. Bioassay results showed that treatment with the higher dosage (400 μg/mL) of BRNP-1 and BRNP-2 stimulated the proliferation, NO release, and cytokine secretion (IL-6 and TNF-α) of RAW264.7 macrophages. These results suggested that BRNP-1 and BRNP-2 may enhance macrophage-mediated immune responses.

KEYWORDS: *Brassica rapa* L., polysaccharides, structural characterization, immunostimulatory activity

1. INTRODUCTION

The innate immune system comprises macrophages, monocytes, granulocytes, and humoral elements[1]. Macrophages, the first line of immune defense, perform a variety of complex biological activities, including phagocytosis, surveillance, chemotaxis, and destruction of targeted organisms[2]. These activities suggest that regulating macrophage activity is a potential strategy against disease[3].In recent years, many kinds of plant-derived polysaccharides have been widely investigated by researchers because of their immunostimulatory activity on macrophages[4], such as water-soluble polysaccharide (SNP) from *Sipunculus nudus* L.[5] , a *Laminaria japonica* polysaccharide[6], and Ginseng fruits polysaccharides[7], as well as Astragalus polysaccharides [8-10], and polysaccharides from *Tinospora cordifolia*[11].And studies demonstrated that polysaccharides may mainly exert immunomodulatory effects on macrophage RAW264.7 via TLR4-mediated MyD88-dependent signaling pathways[8-11].

*Brassica rapa*L., a common edible plant that belongs to the family of Cruciferae, is widely distributed at high-altitude regions in Xinjiang Uygur Autonomous Region, China. The root of *B. rapa* is a traditional Uyghur folk medicine for moistening lungs and relieving cough and asthma[12].Studies have shown that the pharmacological activities of *B. rapa* result from its nutritional benefits, including vitamins[13], glucosinolates[14], flavonoids[15], ferredoxin-sulfite reductase[16], chalcone glycosides[17], and polysaccharides[18,19]. Among them, glucosinolates (GLSs) have attracted the more attention in view of their chemopreventive activities against numerous chronic degener-ative diseases, together with cancer, cardiovascular diseases, neurodegeneration and diabetes.[14,18] Compared with GLSs, however, polysaccharides as the main water-soluble components in *B. rapa* were few investigated, especially in structural characterization[18,19]. It has been reported that only three polysaccharides with relatively low molecular weight[18] and three polysaccharides

with large molecular weight[19] were isolated from *B. rapa*. However, there is no more information reported on the structural characterization except the monosaccharide composition. In addition, increasing evidence over recent years has demonstrated that polysaccharides exhibit immunomodulatory activity. Experimental evidencefor the immunodulatory activities of other plant-derived polysaccharides and the use of *B. rapa* as traditional folk medicine imply that polysaccharides from *B. rapa*may also have immunomodulatory activity. To the best of our knowledge, however, no other studies have investigated polysaccharides from *B. rapa*and their immunological activity. In this paper, we report the isolation, structural characterization, and in *vitro* immunostimulatory activity of polysaccharides from *B. rapa* L.

2. MATERIALS AND METHODS

2.1 Materials. *B. rapa*roots were collected from a commercial market in Urumqi, Xinjiang Uygur, Autonomous Region of China. Toyopearl DEAE 650 M was purchased from Tosoh (Tokyo, Japan). PL aquagel-OH MIXED-H (7.5 mm × 300 mm, 8 μm) and Sugar-pack TM (6.5 mm × 300 mm, 10 μm) columns were purchased from Agilent (USA) and Waters Co., respectively. Sepharose 6B and Sephacryl S-300 HR were obtained from GE Healthcare (Amersham Biosciences AB, Uppsala, Sweden). Other chemicals were obtained from Sigma-Aldrich Co. LLC. Fetal bovine serum (FBS), trypsin, Dulbecco Modified Eagle Medium (DMEM), phosphate buffered solution (PBS), streptomycin, penicillin, and dimethyl sulfoxide (DMSO) were obtained from Hyclone Co. (UT, USA). Lipopolysaccharide (LPS) was purchased from Sigma Chemical Co. (MO, USA). Cell Counting Kit-8 (CCK-8 kit), and IL-6 and TNF-α ELISA kits were purchased from Wuhan Boster Co. (Wuhan, China). Nitric oxide (NO) kit was supplied by Promega Co. (Wisconsin, USA).

HPLC analyses were conducted on an Agilent 1200 system that was coupled with a

refractive index detector (RID). GC–MS analysis was performed on an Agilent GC-MS 7890A-5975 instrument with helium as the carrier gas. Fourier transform infrared (FT–IR) spectra were recorded on a Nicolet 380 FT–IR spectrophotometer.

2.2 Extraction, Isolation, and Purification of Polysaccharides. Extraction was performed in accordance with a previously described method[20]. Dried *B. rapa* powder was defatted by soaking in 95% ethanol (EtOH) at room temperature. After removing the solvent, residues were extracted with hot water thrice for 2 h per extraction. The residues were then filtered through defatted cotton and concentrated in vacuo. The supernatant was precipitated with 80% EtOH. The precipitates were redissolved and dialyzed against distilled water (cut off, 7000 Da). Crude *B. rapa* polysaccharides (BRP) were obtained after freeze-drying.

BRP (3.0 g) was suspended in H_2O. The suspension was stirred at room temperature and then centrifuged at 4000 rpm for 15 min to remove insoluble portions (17.6%). The supernatant was then subjected to an ion-exchange chromatography on a Toyopearl DEAE 650 M column (5.0 i.d. × 20 cm). The supernatant was successively eluted with distilled 0.8 Lof H_2O, 0.5 M, 1.0 M NaCl, 2.0 M NaCl, and 0.2 M NaOH to yield BRN (*B. rapa* L. neutral polysaccharide fraction) (11.2%), BRA1 (32.9%), BRA2(0.7%), BRA3 (0.1%), and BRA4 (1.4%), respectively.Fractions of 15 mL were collected and monitored at 490 nm via phenol–H_2SO_4 method[21].

The water-eluted polysaccharide fraction BRN (500 mg) was loaded on a Sepharose 6B (5.0 i.d. × 90 cm) column and eluted with 0.1 M NaCl (1.2 L) to yield two major fractions, BRN-1 and BRN-2. BRN-1 was further purified by gel filtration on Sephacryl S-300 (2.2 i.d. × 90 cm) and eluted with 0.1 M NaCl (0.4 L) to yield BRNP-1 (240 mg). BRN-2 was purified to yield BRNP-2 (250 mg). Fractions of 10 mL were collected and monitored at 490 nm via phenol–H_2SO_4 method and at 280 nm via UV absorbance

spectroscopy.

2.3 Estimation of Homogeneity and Apparent Molecular Weight. The apparent molecular weights of BRNP-1 and BRNP-2 were estimated with high-performance gel permeation chromatography (HPGPC) analysis on an Agilent 1200 system equipped with an RID detector. Samples (5 mg/mL, 10 μL) were applied to a PL Aquagel-OH MIXED-H column (7.5 mm × 300 mm, 8 μm) and eluted with 0.1M $NaNO_3$ at 0.6 mL/min with the column temperature maintained at 35 °C. Commercially available T-series dextrans (MW 2000, 670, 410, 270, 150, 80, 50, 12, 5, and 1 kD) were used as standard molecular markers.

2.4 Colorimetric Analyses. Total carbohydrate and uronic acid contents were determined by phenol–sulfuric acid[21] and m-hydroxybiphenyl[22] methods with galactose as the standard. Protein content was analyzed using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard.

2.5 Sugar Composition Analysis. The sugar components of BRNP-1 and BRNP-2 were directly hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h in accordance with the routine method of complete hydrolysis for neutral polysaccharides. After the removal of TFA under nitrogen gas, the hydrolysates were converted into alditol acetates for GC–MS analysis, which was conducted with a fused silica capillary column (HP-5 MS, 30 m × 0.25 mm, 0.25 μm, Agilent, USA). Injection and detector temperatures were maintained at 280 °C. The oven temperature was programmed to increase from 160 °C to 190 °C at 2 °C/min, then to 240 °C at 5 °C/min, and maintained at 240 °C for 5 min. Helium was used as the carrier gas.

2.6 Methylation Analysis. The methylation analyses of BRNP-1 and BRNP-2 were performed in accordance with a previously described method[23]. After posttreatment, the resultant products were hydrolyzed with 2 M TFA at 120 °C for 2 h, followed by

reduction with $NaBD_4$ and acetylation with acetic anhydride to yield partially methylated alditol acetates. These acetates were analyzed with GC–MS using a HP-5 MS fused silica capillary column (30 m × 0.25 mm, 0.25 μm, Agilent). The column temperature was set to 120 °C during injection, then increased by 4 °C/min to 280 °C, and maintained at 280 °C for 5 min. Helium was used as the carrier gas. Mass spectra were interpreted to identify the compounds that corresponded to each peak. The molar ratio of each residue was calculated on the basis of peak areas.

2.7 Infrared Spectral Analysis. The infrared spectra of the polysaccharide samples were obtained with a FT–IR spectrophotometer. The purified polysaccharides were ground with KBr powder and then pressed into a polymer film for FT–IR measurement in the frequency range of 4000–400 cm^{-1}.

2.8 NMR Analysis. Approximately 20 mg BRNP-1 and BRNP-2 were dissolved in 0.6 mL D_2O and then analyzed via NMR at 30 °C. The maps of 1D- and 2D-NMR were then obtained.

2.9 Cell Line and Culture Medium. The macrophage RAW 264.7 cell line was purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai. The cell line was grown in DMEM medium that contained 10% FBS and 100 U/mL of penicillin and 100 μg/mL of streptomycin.

2.10 Macrophage Viability Test by CCK-8 Assay. RAW 264.7 cells were seeded at a density of 1.0×10^4 cells/mL in 96-well plates and cultured for 24 h at 37 °C in a humidified incubator with 5% CO_2. Polysaccharide samples were added at the final concentrations of 0, 1.6, 3.2, 8, 40, 80, 200, 400, 1000, and 2000 μg/mL. After 24 h of incubation at 37 °C in 5% CO_2 humidified atmosphere, 10 μL of CCK-8 solution was added to each well. After 1 h of incubation, absorbance was recorded at 450 nm using an ELISA plate reader and then converted into macrophage viability ratio for comparison.

2.11 Nitric Oxide Production and Cytokine Production after BRNP-1 and BRNP-2 Treatment. After 24 h of incubation in the above conditions, RAW 264.7 cells (2×10^4 cells/mL) were treated with BRNP-1 and BRNP-2(0, 80, or 400 μg/mL) or LPS(1 μg/mL) in DMEM medium. The treated cells were then incubated for an additional 24 h. The conditioned culture medium was collected to analyze nitric oxide (NO) and cytokine release by RAW 264.7 cells. NO, TNF-α, and IL-6 contents were determined using commercial kits in accordance with the manufacturer's instructions. Cells that were treated with 1 μg/mL LPS were used as the positive control. Cells that were cultured in DMEM medium without polysaccharides and LPS were used as the normal control.

2.12 Statistical Analysis. All experiments were repeated thrice. 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 Structural Characterization of BRNP-1 and BRNP-2. The purified polysaccharides BRNP-1 and BRNP-2 were colorless, highly water-soluble powders. The two polysaccharides were eluted as a single and symmetrical sharp peak on the HPGPC chromatogram. As shown in Fig.1, these results indicated that BRNP-1 and BRNP-2 were homogeneous. The apparent molecular weight of these polysaccharides were estimated as 6.9 kD and 4.8 kD from a dextran standard curve. The total carbohydrate, uronic acid, and protein contents of BRNP-1 and BRNP-2 were determined through colorimetric analysis. Results showed that carbohydrates dominated the chemical composition of BRNP-1 (99.8%) and BRNP-2 (99.9%), whereas protein was present only in trace amounts (BRNP-1: 0.11%; BRNP: 0.07%).Uronic acid was not detected, thus suggesting that the two polysaccharides are neutral polysaccharides.

The monosaccharide compositions of BRNP-1 and BRNP-2 were hydrolyzed and acetylated intoalditol acetates for GC–MC analysis. As shown in Fig.2, only one peak appeared at 21.1 min. Comparing the retention time of this peak with those of authenticated standards revealed its identity as glucose (Glc). Therefore, BRNP-1 and BRNP-2are composed of glucosyl residues.

The FT–IR spectra of BRNP-1 and BRNP-2 were similar (Fig. 3). The absorbance band at 3409.3 cm^{-1} represented the stretching vibration of O–H bonds in constituent sugar residues. Bands at approximately 2929.4 cm^{-1} were associated with the stretching vibration of C–H in sugar rings. The band at approximately 1649.1 cm^{-1} indicated bound water[24]. Absorbance bands at 1154.1 cm^{-1}, 1078.0 cm^{-1}, and 1025.7 cm^{-1}indicated the bending vibrational modes of C–O stretching in pyranose[25]. In addition, the absorption peak at 842.2 cm^{-1}suggestedthat glucosyl residues are mainly present inα-configuration[26], whereas that at approximately 928.4 cm^{-1} isa characteristic of α-glucans[27]. These wave numbers indicated that the major glucosyl residues in these two polysaccharides are of α-configuration and are α-glucans.

To identify the linkage type between glucosyl residues, BRNP-1 and BRNP-2 were subjected to methylation analyses. Results are shown in Fig. 4 and are summarized in Table 1.The total ionization chromatogram (TIC) showed that derivatized BRNP-1 and BRNP-2 exhibited the same peak-signal pattern, which was composed of three peak signals (1–3). These signals were identified based on the combination of fragment analysis and monosaccharide composition results. Peak 1 was identified as1,5-*di*-*O*-acetyl-(1-deuterio)-2,3,4,6-*tetra*-*O*-methyl glucitol as deduced from the major primary ion fragments at *m/z* 161 and 162 that appeared in nearly equal amounts and the diagnostic ion fragments at *m/z* 205 and 118.These ion fragments confirmed the presence of *t*-linked Glc*p*[28]. Peak 2 corresponded to the signal of

1,4,5-*tri*-*O*-acetyl-(1-deuterio)-2,3,6-*tri*-*O*-methyl glucitol, as deduced from the major primary ion fragments at *m/z* 233 and 118 and the diagnostic fragment at *m/z* 162. These fragments indicated the presence of (1→4)-linked Glc*p* units. As deduced from the diagnostic pair *m/z* 118 and 261, peak 3 was assigned to 1,4,5,6-*tetra*-*O*-acetyl-(1-deuterio)-2,3-*di*-*O*-methyl glucitol, suggesting the presence of (1→4,6)-linked Glc*p*[28]. Taken together, both BRNP-1 and BRNP-2 were predominantly composed of (1→4)-linked Glc*p* residues, suggesting the presence of a (1→4)-linked glucan backbone. In addition, (1→4,6)- and *t*-linked Glc*p* residues were also present in small amounts, indicating that the glucan backbone, which is attached by *t*-linked Glc*p* residues, branches at the *O*-6 position. As summarized in Table 1, BRNP-1 and BRNP-2 exhibited similar glycosyl linkage composition but different apparent molecular weight and branch degree (DB). DB was calculated according to the formula[29](DB) = (NT+NB)/(NT+NB+NL)，where NT, NB, and NL are the total numbers of the terminally linked residues, branched residues, and linear residues, respectively. On the basis of this formula, the DB values of BRNP-1 and BRNP-2 were calculated as 0.243 and 0.238, respectively.

To further interpret the structures of BRNP-1 and BRNP-2, the two polysaccharides were analyzed via 1D- (^{1}H- and^{13}C-NMR)and 2D-NMR (HSQC and HMBC). The obtained spectra were data from the literature. As shown in Fig.5A-a, the ^{1}H- and^{13}C-NMR spectra of these two polysaccharides have similar signal patterns. In the low-field region, three anomeric hydrogen signals appeared at δ 4.5-5.5 ppm in ^{1}H-NMR spectra. These signals corresponded to three anomeric carbon signals at δ 90–110 ppm in ^{13}C-NMR spectra. Taking the results of methylation analysis into consideration, the presence of these anomeric signals suggested three glucopyranosyl residues in the repeating units of BRNP-1 and BRNP-2. In the case of BRNP-1, signals at δ4.88 (98.63),

5.30 (99.83), and 5.27 (100.02) ppm were assigned to the H1 (C1) of *t*-linked Glc*p* residue (residue A), (1→4)-linked Glc*p* residue (residue B), and (1→4, 6)-linked Glc*p* residue (residue C), respectively. Consistent with the results of IR analysis, the signals in the lower field indicated that all glucosyl residues haveα-configuration. In high field, the major signals at δ 3.76, 3.87, 3.57, 3.55, and 3.77/3.76 in ^{1}H-NMR spectrum were assigned to H-2, H-3, H-4, H-5, and H-6a/H-6b of residue B in accordance with the literature[30,31].Corresponding carbons were easily determined from correlations in HSQC, as summarized in Table 2. Other signals were assigned by the combination of 2D-NMR and evidence from the literature[30,31].

The linkage sequence was deduced from HMBC spectra (Fig.5B-b). A strong cross-peak signal at δ 3.57/99.83 ppm was assigned to the correlation between H-4 and C-1 of inter-residue B, suggesting a(1→4)-*α*-linked glucan backbone. This deduction was also supported by the weak cross-peak signal at δ 5.30/77.12 ppm; on the basis of data from the literature, this signal was ascribed to the correlation between the H-1 and C-4 of inter-residue B[30]. In addition, a very weak cross-peak signal at 4.88/69.44 ppm was attributed to the correlation between the H-1(δ 4.88) of residue A and the C-6(δ 69.44) of residue C, indicating that the H-1 of residue A is linked to the *O*-6 of residue C. In accordance with the results of methylation analysis, deductions from the HMBC spectra suggested that BRNP-1 contains a (1→4)-linked glucan backbone that branches at the *O*-6 position by *t*-linked Glc*p* residues.

BRNP-2 showed similar ^{1}H- and ^{13}C-NMR and HMBC spectra (not shown here) as BRNP-1, suggesting that BRNP-1 and BRNP-2 have similar structural characteristics. Considering all of the information obtained from NMR spectra and methylation analyses, both BRNP-1 and BRNP-2 contain a (1→4)-*α*-linked glucan backbone chain that branches at the *O*-6 position of the backbone units with *t*-*α*-linked Glc*p* residues as side chains. The

two polysaccharides, however, differ in apparent molecular weight and DB. The possible structures of BRNP-1 and BRNP-2 are shown in Fig. 6.

3.2 Immunomodulatory Activity of BRNP-1 and BRNP-2. The cytotoxicity of BRNP-1 and BRNP-2 against RAW 264.7 macrophages was evaluated by CCK-8 assay. The viability of control wells (no sample) was considered to be 100%. The viability ratios of macrophages that were activated by samples were determined at the indicated concentrations. As shown in Fig.7A, BRNP-1 showed low cytotoxicity at the low concentration of 1.6–80 μg/mL, exerted no cytotoxicity at concentrations higher than 80 μg/mL, and exhibited the best stimulatory activity on macrophages at 400 μg/mL. BRNP-2 exhibited significant proliferation activity in a dose-dependent manner and exerted no cytotoxic effects at concentrations of 1.6 to 2000 μg/mL. Thus, our results revealed that higher concentrations of BRNP-1 and BRNP-2 stimulate macrophage proliferation. Therefore, the concentrations of 80, 400 μg/mL of BRNP-1 and BRNP-2 were used as treatments in the following experiments.

NO is released from various mammalian cells, including vascular endothelial cells, macrophages, mesangial cells, spleen lymphocytes, glial cells, and fibroblasts. As a messenger molecule, NO exerts cytotoxic effects on bacteria, fungi, and tumor cells, and is implicated in inflammatory responses in response to tissue injury[32]. We determined NO production from RAW 264.7 cells treated with BRNP-1 and BRNP-2 to evaluate their activation effects on macrophages. As shown in Fig. 7B, treatment with higher dosage (400 μg/mL) of BRNP-1 or BRNP-2 on RAW264.7 cells significantly enhanced the release of NO in the culture medium compared with that in the normal control group, especially for BRNP-1 which showed even a comparative level compared to LPS group. As compared with the normal control group, however, treatment with the lower dosage (80 μg/mL) of BRNP-1 or BRNP-2 showed no significant effect on the release of NO. It is

well known that NO is a major mediator of macrophages and essential for the resistance of immune system to pathogens invasion. Glucans that are composed of 1,4-*α*-D-glucosidic linkages have been reported to have a significant effect on NO production from macrophages[33]. In our study, both BRNP-1 and BRNP-2 stimulated the NO release of RAW 264.7 cells at higher dosage (400 μg/mL). BRNP-1 showed more significant effects on NO production than BRNP-2. Given the similar backbones and side chains of the two polysaccharides, the different effects of BRNP-1 and BRNP-2 on NO production may be closely related to molecular weight and DB.

We examined the ability of BRNP-1 and BRNP-2 to stimulate cytokine secretion in macrophages. As illustrated in Fig. 7C, BRNP-1 or BRNP-2 exhibited a marked enhancement in TNF-α secretion when treated RAW 264.7 cells at higher dosage 400 μg/mL compared with the control group. The TNF-α secretion was observed to increase to 4.5 folds of that in control group with a level of 81 pg/mL, and 6.1 folds with a level of 110 pg/mL. BRNP-2 showed a stronger effect on TNF-α secretion than BRNP-1 at the higher dosage. Consistent with the effect of two polysaccharides on the NO release, the lower dosage of BRNP-1 and BRNP-2 showed little even suppressed effect on the TNF-α secretion. Unlike the case in TNF-α, both BRNP-1 and BRNP-2 exhibited a significant effect on the secretion of IL-6 (Fig. 7D) compared with that both in control group and in LPS group, but no difference were observed between the higher and lower dosage treatments.

The activation of macrophages results in the production of a large amount of NO. NO contributes to the killing of pathogens and mediates a variety of biological functions as an intracellular messenger molecule[34]. In addition, macrophage activation produces various cytokines, including TNF-α and IL-6. In turn, TNF-α and IL-6 induce the proliferation of other immune cells, including B-cells and T-cells[35]. In the present study, we found that

polysaccharides from *B. rapa* L. induced the activation of macrophages *in vitro*, as manifested by NO production and cytokine (TNF-α and IL-6) secretion. Therefore, these two polysaccharides may potentially enhance macrophage-mediated innate immune response.

In summary, two neutral polysaccharides, BRNP-1 and BRNP-2, with apparent molecular weights of 6.9 kD and 4.8 kD, respectively, were isolated from the medicinal plant *B. rapa* L. via the combination of ion-exchange chromatography and high-performance gel permeation chromatography. The structures of BRNP-1 and BRNP-2 were characterized by chemical derivatization, HPGPC, GC–MS, and 1D- and 2D-NMR technologies. BRNP-1 contains a glucan backbone chain that is composed of *α*-D-(1→4)-linked Glc*p* residues. Moreover, BRNP-1contains side chains that are composed of*t*-linked-*α*-D-Glc*p* residues that are attached at the *O*-6 position of *α*-D-(1→4)-linked Glc*p* at every eight backbone-glycosyl residues. Furthermore, the DB of BRNP-1 is 0.243. BRNP-2 has the same backbone chain and side chains as BRNP-1 but differs in DB (0.238), which indicates that its side chains are attached at the *O*-6 position of *α*-D-(1→4)-linked Glc*p* at every nine backbone-glycosyl residues. Bioassay results showed that both BRNP-1 and BRNP-2 stimulate macrophage proliferation and significantly enhance NO production and cytokine (TNF-α and IL-6) secretion when treated RAW 264.7 cells with higher dosage of 400 μg/mL. Therefore, BRNP-1 and BRNP-2 may potentially enhance macrophage-mediated immune response. This study provides preliminary information for further investigations on the antitumor effects of *B. rapa* polysaccharides.

FUNDINGS

This work was financially supported by the National Natural Science Foundation of China

(No. 81460615), the Foundation of Key Laboratory of Xinjiang Uygur Autonomous Region of China (XJDX0208-2012-1), and Tianjin Municipal Science and Technology Commission (No. 15JCYBJC50700).

REFERENCES

(1) Chávez-Sánchez, L.; Espinosa-Luna, J. E.; Chávez-Rueda, K.; Legorreta-Haquet, M. V.; Montoya-Díaz, E.; Blanco-Favela, F., Innate Immune System Cells in Atherosclerosis. *Arch. Med.Res.* **2014**, *45*, 1-14.

(2) Sica, A.; Allavena, P.; Mantovani, A., Cancer related inflammation: the macrophage connection. *Cancer Lett.* **2008**, *267*, 204-215.

(3) Grivennikov, S. I.; Greten, F. R.; Karin, M., Immunity, inflammation, and cancer. *Cell.* **2010**, *140*, 883-899.

(4) Schepetkin, I. A.; Quinn, M. T., Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. *Int. Immunopharmacol.* **2006**, *6*, 317-333.

(5) Zhang, C. X.; Dai, Z. R., Immunomodulatory activities on macrophage of a polysaccharide from *Sipunculus nudus* L. *Food Chem. Toxicol.* **2011**, *49*, 2961-2967.

(6) Zha, X.-Q.; Lu, C.-Q.; Cui, S.-H.; Pan, L.-H.; Zhang, H.-L.; Wang, J.-H.; Luo, J.-P., Structural identification and immunostimulating activity of a *Laminaria japonica* polysaccharide. *Int. J. Biol. Macromol.* **2015**, *78*, 429-438.

(7) Wang, Y.; Huang, M.; Sun, R.; Pan, L., Extraction, characterization of a Ginseng fruits polysaccharide and its immune modulating activities in rats with Lewis lung carcinoma. *Carbohydr. Polym.* **2015**, *127*, 215-221.

(8) Wei, W.;Xiao, H.; Bao, W.; Ma, D.; Leung, C.; Han, X.; Ko, C.; Lau, C.; Wong, C.; Fung, K.; Leung, P.; Bian, Z.; Han, Q.TLR-4 may mediate signaling pathways of

Astragalus polysaccharide RAP induced cytokine expression of RAW264.7 cells.*J. Ethnopharmacol.* **2016**, *179*, 243-252.

(9) Zhou, L.; Wang, Z.; Yu, S.; Long, T.; Zhou, X.; Bao, Y., Astragalus polysaccharides exerts immunomodulatory effects via TLR4-mediated MyD88-dependent signaling pathway in vitro and in vivo., *Sci. Rep.* **2017**, *7*, 1-13.

(10) Zhang, W.; Ma, W.; Zhang, J.; Song, X.; Sun, W.; Fan, Y., The immunoregulatory activities of Astragalus polysaccharide liposome on macrophages and dendritic cells. *Int. J. Biol. Macromol.*, *2017*, in press.

(11) Kumar, G.P.; Rajan, M.G.R.; Kulkami,S. Activation of murine macrophages by G1-4A, a polysaccharide from *Tinospora cordifolia*, in TLR4/MyD88 dependent manner. *Int. Immunopharmacol.* **2017**, *50*, 168-177.

(12) Liu, Y.M. *Brassica rapa* L. In *Uygur medicine tzu*, edition 1.; Liu, Y.M., Eds.; Health science and technology publishing house in Xinjiang, China; 1999; Vol. 9.; pp. 334-335.

(13) Dominguez-Perles, R.; Mena, P.; Garcia-Viguera, C.; Moreno, D. A., Brassica foods as a dietary source of vitamin C: a review. *Crit Rev Food Sci Nutr.* **2014**, *54*, 1076-1091.

(14) Jeong, J.; Park, H.; Hyun, H.; Kim, J.; Kim, H.; Oh, H. I.; Hwang, H. S.; Kim, D. K.; Kim, H. H., Effects of glucosinolates from Turnip (*Brassica rapa* L.) root on bone formation by human osteoblast-like MG-63 cells and in normal young rats. *Phytother Res.* **2015**, *29*, 902-909.

(15) Annalisa R., P. V., Laura I., Francesca I., Daniela H., HPLC-DAD/MS characterization of flavonoids and hydroxycinnamic derivatives in Turnip Tops (*Brassica rapa* L. Subsp. sylvestris L.). *J.Agric. Food Chem.* **2006**, *54，1342-1346*.

(16) Takahashi, S.; Yip, W. C.; Tamura, G., Purification and characterization of

ferredoxin-sulfite reductase from turnip (*Brassica rapa*) leaves and comparison of properties with ferredoxin-sulfite reductase from turnip roots. *Biosci. Biotechnol. Biochem.***1997**, *61*, 1486-1490.

(17) Hara, H.; Nakamura, Y.; Ninomiya, M.; Mochizuki, R.; Kamiya, T.; Aizenman, E.; Koketsu, M.; Adachi, T., Inhibitory effects of chalcone glycosides isolated from Brassica rapa L. 'hidabeni' and their synthetic derivatives on LPS-induced NO production in microglia. *Bioorg. Med. Chem.* **2011**, *19*, 5559-5568.

(18) Xie, Y.; Jiang, S.; Su, D.; Pi, N.; Ma, C.; Gao, P., Composition analysis and anti-hypoxia activity of polysaccharide from Brassica rapa L. *Int. J. Biol. Macromol.* **2010**, *47*, 528-533.

(19) Wang, W.; Wang, X.; Ye, H.; Hu, B.; Zhou, L.; Jabbar, S.; Zeng, X.; Shen, W., Optimization of extraction, characterization and antioxidant activity of polysaccharides from Brassica rapa L. *Int. J. Biol. Macromol.* **2016**, *82*, 979-988.

(20) Bao-lin HOU, Q. W., HailiqianTaoerdahong., Research on polysaccharide extraction and the content of Brassica rapaL. *Lishizhen Medicine And Materia Medica Research* **2009**, *20*，2759-2761.

(21) Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F., Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **1956**, *28*, 350-356.

(22) Blumenkrantz, N.; Asboe-Hansen, G., New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484-489.

(23) Ciucanu, I.; Kerek, F., A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **1984**, *131*, 209-217.

(24) Parker, F.S., Application of I.R Spectroscopy in Biochemistry, In *Biology and Medicine*, edition 1.; Parker, F.S., Eds.; Plenum Press, New York, 1971, Vol. 14.;

pp.100-104.

(25) Xu, F.; Liao, K.; Wu, Y.; Pan, Q.; Wu, L.; Jiao, H.; Guo, D.; Li, B.; Liu, B., Optimization, characterization, sulfation and antitumor activity of neutral polysaccharides from the fruit of *Borojoa sorbilis* cuter. *Carbohydr. Polym.* **2016**, *151*, 364-372.

(26) Wu, Y.; Li, Y.; Liu, C.; Li, E.; Gao, Z.; Liu, C.; Gu, W.; Huang, Y.; Liu, J.; Wang, D.; Hu, Y., Structural characterization of an acidic Epimedium polysaccharide and its immune-enhancement activity. *Carbohydr. Polym.* **2016**, *138*, 134-142.

(27) Wang, Y.-Y.; Khoo, K.-H.; Chen, S.-T.; Lin,C.-C.; Wong,C.-H.; Lin, C.-H.,Studies on the immuno-modulating and antitumor activities of *Ganoderma lucidum* (Reishi) polysaccharides: Functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities.*Bioorg. Med.Chem.* **2002**, *10*, 1057-1062.

(28) Carpita, N.C., Shea, E.M. Linkage structure of carbohydrates by gas chromatography-mass spectroscopy (GC-MS) of partially methylated alditol acetates. In *Analysis of Carbohydrate by GLC and MS*, edition 1.; Biermann, C. J., McGinnis, G. D., Eds.; CRC Press, FL: Boca Raton, 1988; Vol. pp. 157-216.

(29) Tao, Y.; Zhang, L.; Fan, Y.; Wu,X., Chain conformation of water-insoluble hyperbranched polysaccharide from fungus. *Biomacromolecules.* **2007**, *8*, 2321-2328.

(30) Xu, X.; Gu, Z.; Liu, S.; Gao, N.; He, X.; Xin, X., Purification and characterization of a glucan from Bacillus Calmette Guerin and the antitumor activity of its sulfated derivative. *Carbohydr. Polym.* **2015**, *128*, 138-146.

(31) Jiang, J.; Kong, F.; Li, N.; Zhang, D.; Yan, C.; Lv, H., Purification, structural characterization and in vitro antioxidant activity of a novel polysaccharide from Boshuzhi. *Carbohydr. Polym.* **2016**, *147*, 365-371.

(32) Huang, M.; Mei, X.; Zhang,S., Mechanism of nitric oxide production in macrophages treated with medicinal mushroom extracts (Review). *Int.J.Med.Mushrooms.* **2011**, *13*, 1-6.

(33) Wu, D.-T.; Xie, J.; Wang, L.-Y.; Ju, Y.-J.; Lv, G.-P.; Leong, F.; Zhao, J.; Li, S.-P., Characterization of bioactive polysaccharides from *Cordyceps militaris* produced in China using saccharide mapping. *J.Funct. Foods.* **2014**, *9*, 315-323.

(34) Baugh, J. A.; Bucala, R., Mechanisms for modulating TNF alpha in immune and inflammatory disease. *Curr. Opin. Drug Disc.* **2001**, *4*, 635-650.

(35) Sobota, R. M.; Müller, P. J.; Khouri, C.; Ullrich, A.; Poli, V.; Noguchi, T.; Heinrich, P. C.; Schaper, F., SHPS-1/SIRP1α contributes to interleukin-6 signalling. *Cell. Signal.* **2008**, *20*, 1385-1391.

Figure captions

Fig. 1. HPGPC chromatogram of polysaccharides BRNP-1 (A) and BRNP-2 (B). Samples were applied to PL Aquagel-OH MIXED-H column (7.5mm×300mm, 8μm) and eluted with 0.1M $NaNO_3$ at 0.6mL/min with column temperature maintained at 35 °C. Commercially available T-series dextrans were used as standard molecular markers.

Fig. 2. GC-MS chromatograms of standard monosaccharides (A) and hydrolysates of BRNP-1 (B) and BRNP-2 (C). Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Glc: glucose; Gal: galactose.

Fig. 3. FT-IR spectrum of BRNP-1 and BRNP-2 between 400 and 4000cm^{-1}.

Fig. 4. GC-MS profile of partially methylated alditol acetates of BRNP-1. (A) TIC profile; (B) MS fragments; (C) deduced residues. Peaks: 1, 1,5-*di*-*O*-acetyl-(1-deuterio)-2,3,4,6-*tetra*-*O*-methyl glucitol; 2, 1,3,4-*tri*-*O*-acetyl-(1-deuterio)-2,3,6-*tri*-*O*-methyl glucitol; 3, 1,4,5,6-*tetra*-*O*-acetyl-(1-deuterio)-2,3-*di*-*O*-methyl glucitol.

Fig. 5. ^{13}C and ^{1}H NMR spectra of BRNP-1 (A-a) and BRNP-2 (A-b) in D_2O as recorded on a Varian INOVA 600 NMR spectrometer at 30 °C. HSQC (B-a) and HMBC (B-b) spectra of BRNP-1 in D_2O as recorded on a Varian INOVA 600 NMR spectrometer at 30 °C.

Fig. 6. Predicted structure of the repeating units of BRNP-1 (A) and BRNP-2 (B).

Fig.7. The effect of BRNP-1 and BRNP-2 on the proliferation (A), NO production (B), and TNF-α (C) and IL-6 (D) secretion of RAW264.7 macrophages. $^{*}P < 0.05$, *vs* the control group; $^{\#}P < 0.05$, *vs* the LPS positive control group.

Table 1. The Deduced Glycosidic Linkage Type And Composition Of BRNP-1 And BRNP-2 Determined By Methylation And GC-MS Analyses

Sugar derivatives	mol%		Deduced residues
	BRNP-1	BRNP-2	
1,5-*di*-*O*-acetyl-(1-deuterio)-2,3,4,6-*tetra*-*O*-methyl glucitol	14.9	15.3	t-Glc*p*
1,4,5-*tri*-*O*-acetyl-(1-deuterio)-2,3,6-*tri*-*O*-methyl glucitol	75.7	76.2	(1→4)-Glc*p*
1,4,5,6-*tetra*-*O*-acetyl-(1-deuterio)-2,3-*di*-*O*-methyl glucitol	9.4	8.5	(1→4,6)-Glc*p*

Table 2. The ^{1}H-, ^{13}C-NMR Chemical Shifts of BRNP-1 Isolated From *Brassica rapa* L.

Glycosyl residues		Chemical shifts, δ (ppm)					
		1	2	3	4	5	6
t-Glc*p*	H	4.97	3.51	3.95	3.68	3.87	3.68
	C	98.49	74.58	76.71	70.30	76.44	60.47
(1→4)-Glc*p*	H	5.27	3.53	3.88	3.88	3.76	3.76
	C	99.67	71.42	73.27	76.54	71.18	60.43
(1→4,6)-Glc*p*	H	5.30	3.50	3.55	3.57	3.88	3.33
	C	99.99	71.52	73.45	77.05	71.20	69.28

Fig. 1

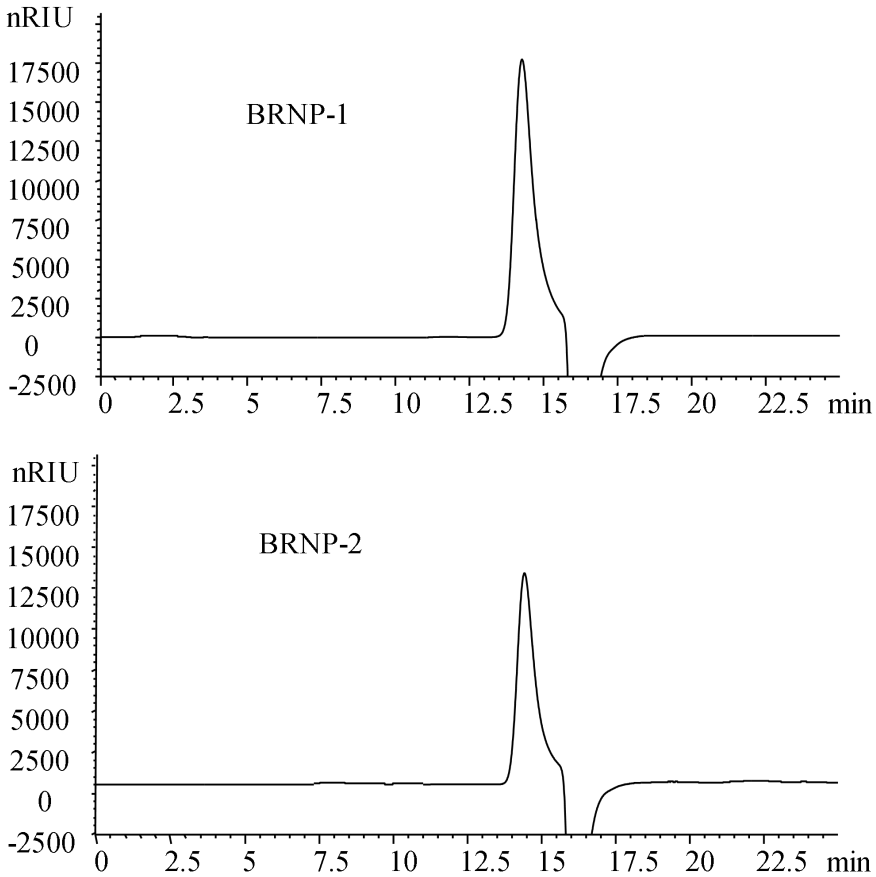

nRIU
17500
15000
12500
10000
7500
5000
2500
0
-2500
BRNP-1
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
min
nRIU
17500
15000
12500
10000
7500
5000
2500
0
-2500
BRNP-2
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
min

Fig. 2

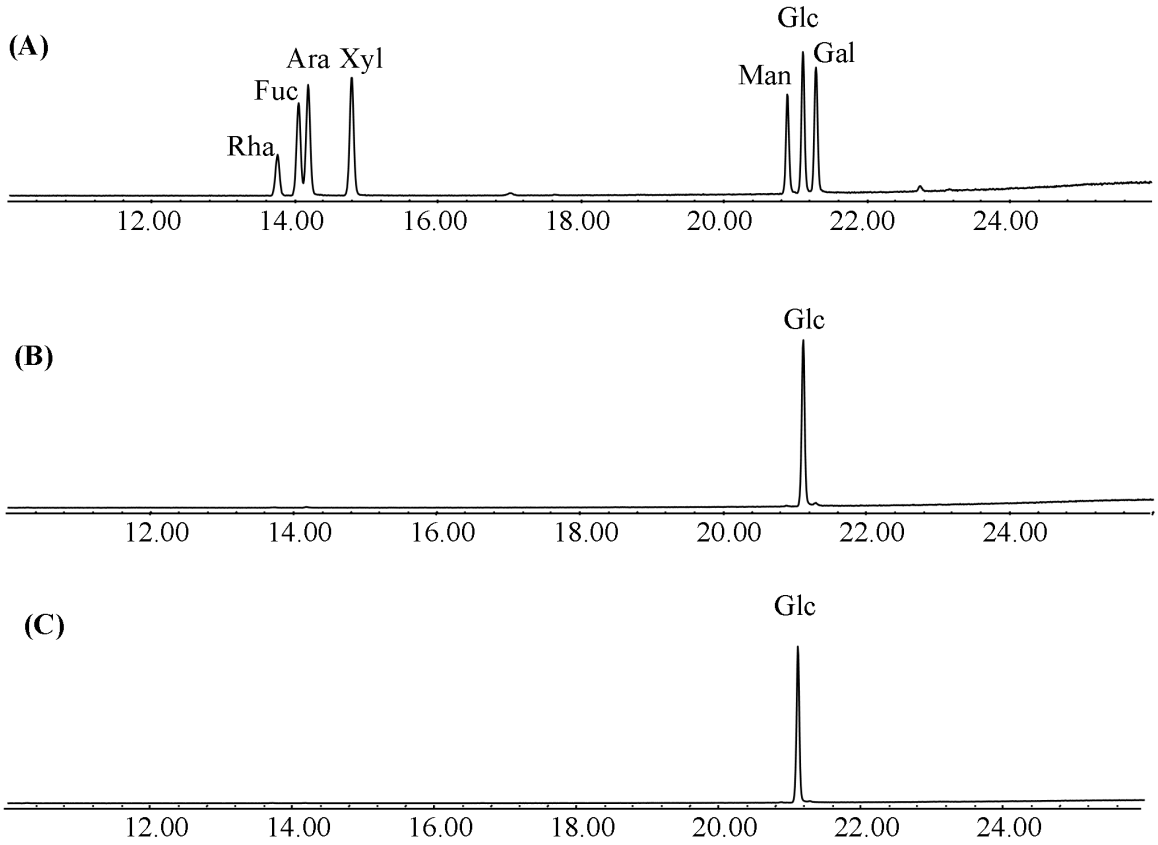

(A)
Rha
Fuc
Ara
Xyl
Man
Glc
Gal
12.00
14.00
16.00
18.00
20.00
22.00
24.00
(B)
Glc
12.00
14.00
16.00
18.00
20.00
22.00
24.00
(C)
Glc
12.00
14.00
16.00
18.00
20.00
22.00
24.00

Fig. 3

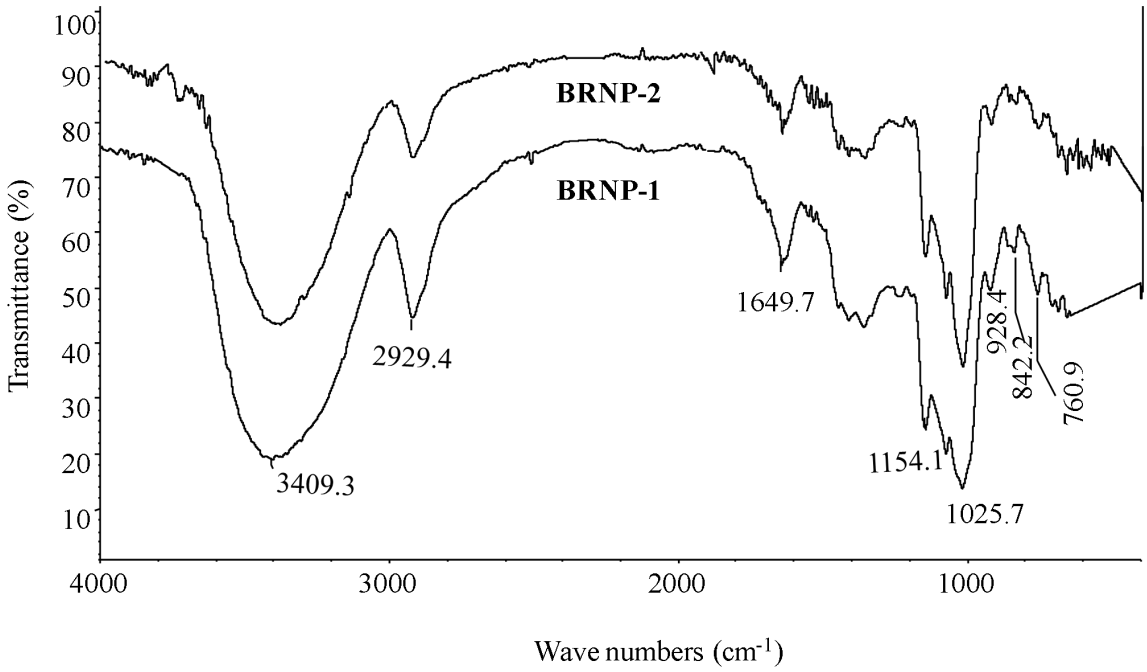

BRNP-2
BRNP-1
Transmittance (%)
100
90
80
70
60
50
40
30
20
10
3409.3
2929.4
1649.7
1154.1
1025.7
928.4
842.2
760.9
4000
3000
2000
1000
Wave numbers (cm-1)

Fig. 4

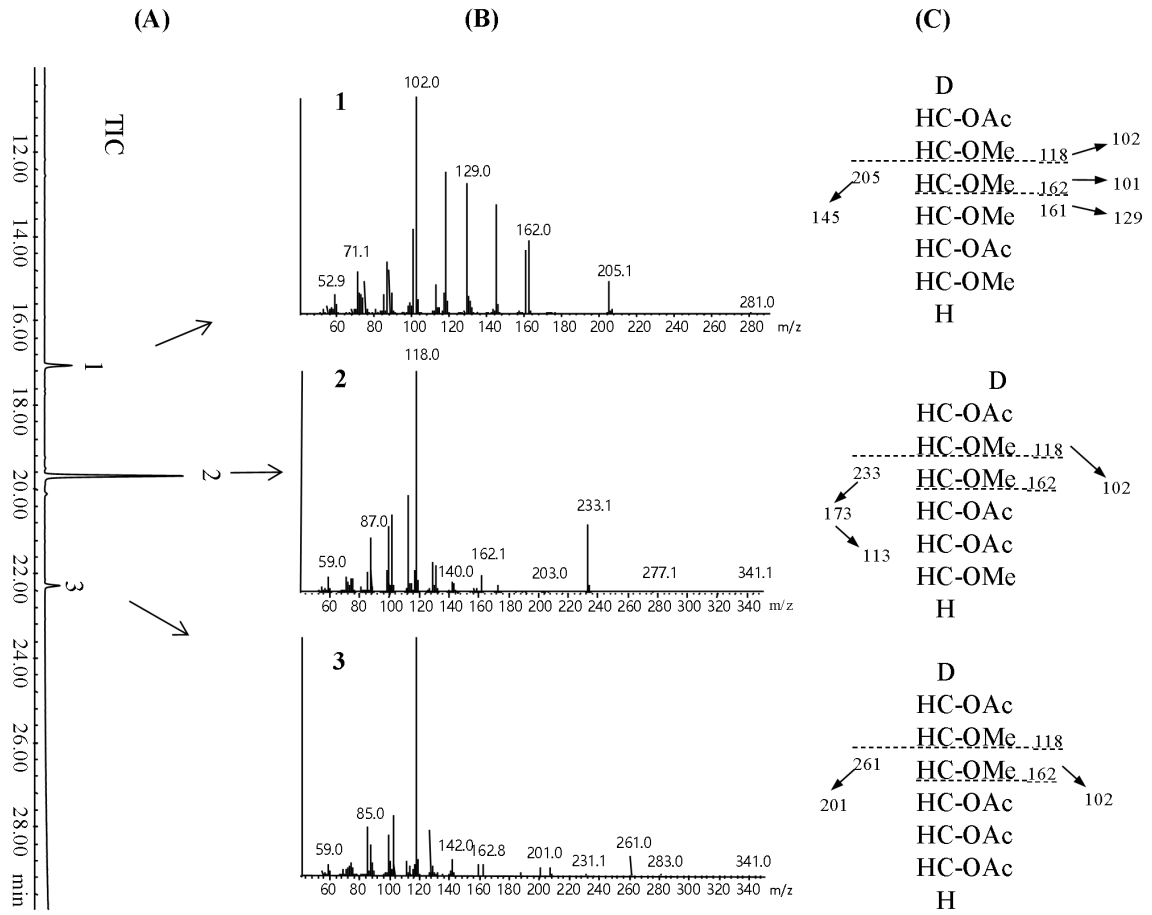

(A)
(B)
(C)
TIC
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
min
1
2
3
102.0
129.0
162.0
71.1
52.9
205.1
281.0
118.0
233.1
87.0
59.0
162.1
140.0
203.0
277.1
341.1
85.0
142.0
162.8
201.0
231.1
261.0
283.0
341.0
m/z
D
HC-OAc
HC-OMe
HC-OMe
HC-OMe
HC-OAc
HC-OMe
H
118
162
161
205
145
102
101
129
233
173
113
261
201

Fig. 5

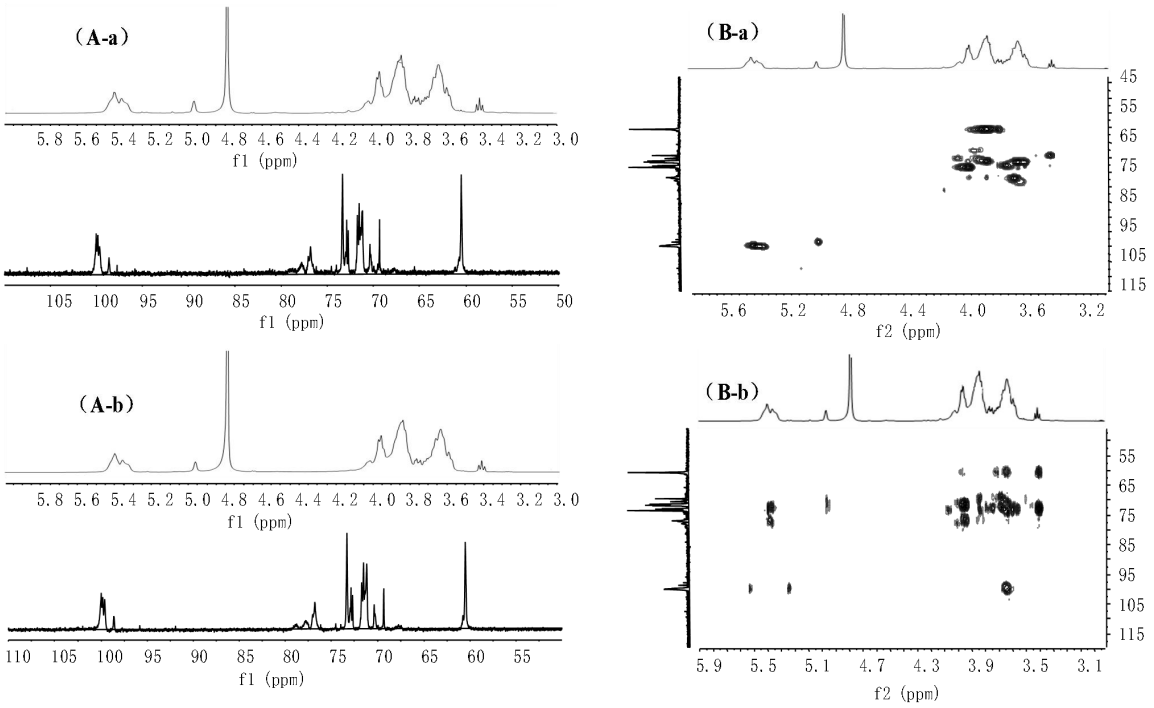

(A-a)
5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0
f1 (ppm)
105 100 95 90 85 80 75 70 65 60 55 50
f1 (ppm)
(B-a)
45 55 65 75 85 95 105 115
5.6 5.2 4.8 4.4 4.0 3.6 3.2
f2 (ppm)
(A-b)
5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0
f1 (ppm)
110 105 100 95 90 85 80 75 70 65 60 55
f1 (ppm)
(B-b)
55 65 75 85 95 105 115
5.9 5.5 5.1 4.7 4.3 3.9 3.5 3.1
f2 (ppm)

Fig. 6

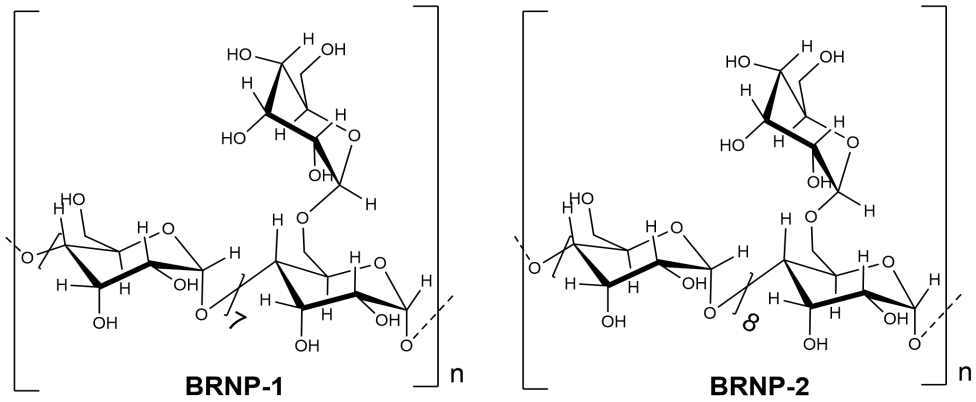
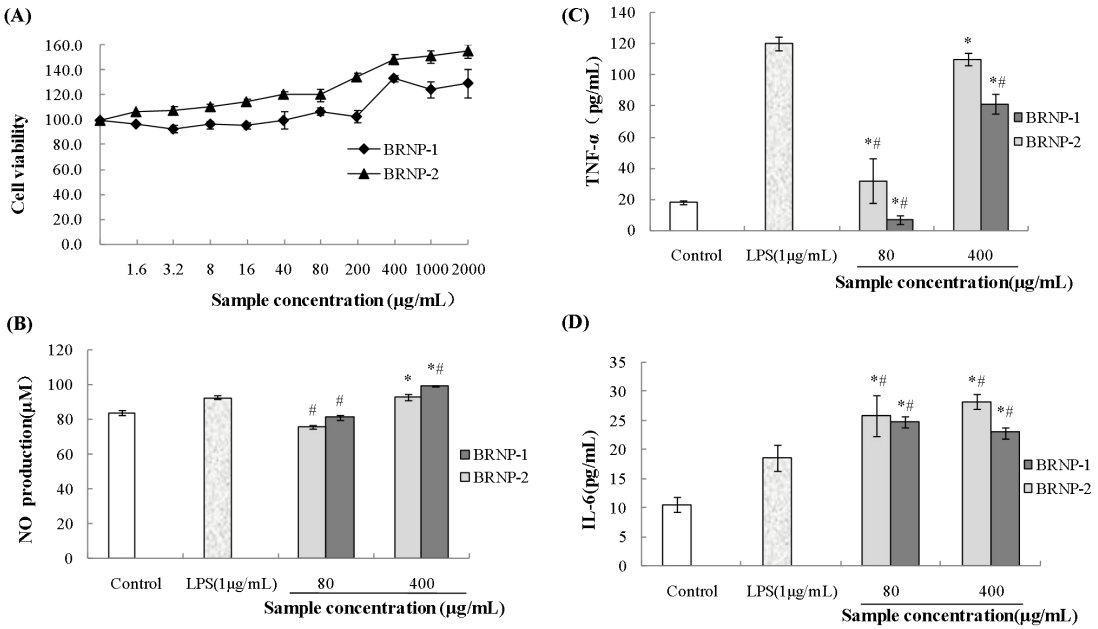


Fig. 7


(A)
Cell viability
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
BRNP-1
BRNP-2
1.6 3.2 8 16 40 80 200 400 1000 2000
Sample concentration (μg/mL)
(B)
NO production(μM)
120
100
80
60
40
20
0
BRNP-1
BRNP-2
Control
LPS(1μg/mL)
80
400
Sample concentration (μg/mL)
(C)
TNF-α（pg/mL）
140
120
100
80
60
40
20
0
BRNP-1
BRNP-2
Control
LPS(1μg/mL)
80
400
Sample concentration(μg/mL)
(D)
IL-6(pg/mL)
35
30
25
20
15
10
5
0
BRNP-1
BRNP-2
Control
LPS(1μg/mL)
80
400
Sample concentration(μg/mL)

TOC Graphic