Structural characterization and immunomodulating activities of a novel polysaccharide from *Nervilia fordii*

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ABSTRACT

Nervilia fordii (Hance) Schltr. has been widely used as a medicinal and edible herb in Southwest China and Southeast Asia. In this study, NFP-1, a new water-soluble polysaccharidewith a purity of 97.8%, was purified from water extract of *Nervilia fordii* by DEAE-cellulose and Sephadex G-100 chromatography. NFP-1 has a relative molecular weight of 950 kDa determined by high performance gel-permeation chromatography (HPGPC). Its monosaccharide compositions were analyzed by high performance liquid chromatography (HPLC) after pre-column derivatizing its hydrolysate with 1-phenyl-3-methyl-5-pyrazolone (PMP). NFP-1 mainly consists of galactose, arabinose, rhamnose, and galacturonic acid. Based on FT-IR, methylation and GC–MS analysis, and NMR, the structure unit of NFP-1 was established as →4)-α-Rha*p*-(2→4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→2)-α-Rha*p*-(4→1)-β-Gal*p*-T containing two branch chains of →2,4)-α-Rha*p*-(1→5)-α-Ara*f*-(1→3)-α-Ara*f*-(1→, and →2,4)-α-Rha*p*-(1→4)-β-Gal*p*-(1→. The immunomodulatory assays revealed the dual-functionalities of NFP-1. NFP-1 could significantly induce the secretion of nitric oxide (NO), and promote the secretions of TNF-α, IL-6, and IL-1β in RAW264.7 macrophages. NFP-1 could also significantly inhibit the production of NO, depress the secretions of TNF-α, IL-6 and IL-1β in RAW264.7 macrophages activated by lipopolysaccharide (LPS), and promote the production of IL-10 meanwhile. Our study suggested that *Nervilia fordii* could be an ideal medicinal or functional food due to its dual immunomodulatory activities.

1. Introduction

Nervilia fordii (Hance) Schltr. (family *Orchidaceae*), is distributed mainly in Southwest China and Southeast Asia, and has been cultivated in recent years [1]. The stems and leaves of *Nervilia fordii* have been used as medicinal and edible herb, and its water extract is often used to treat lung diseases, such as cough, pharyngitis, tuberculosis and pneumonia in folk medicine for a long history [2]. Local residents in Southwest China often drink herbal tea containing *Nervilia fordii* as main component, and they also use the leaves of *Nervilia fordii* to make Chinese herbal pork soup.

Nervilia fordii became a well-knownherb for being used as the main ingredient to make decoctions together with *Scutellaria baicalensis*, *Taraxacum mongolicum*, *Pinellia ternata*, etc. to treat severe acute respiratory syndrome (SARS) in 2003 [3–5]. During SARS epidemic period, it showed that the decoctions of traditional Chinese herbs could significantly alleviate SARS symptoms in clinic [3,4,6–8]. Thus, we have been studying the phytochemicals and pharmacological activities of *Nervilia fordii* since 2009 [9–13]. Modern pharmacological studies demonstrated that *Nervilia fordii* has antitumor, antiviral, analgesic, and anti-inflammatory activities [14–17]. In our previous work, we found that the flavonoids and polysaccharides are two main constituents in *Nervilia fordii* which display compelling anti- inflammatory activities [10,11,13].

Macrophage is the main innate immune cell which plays important roles in the immune response [18,19]. Macrophages can kill invading pathogens through releasing of cytokines including tumor necrosis factor α (TNF-α) and interleukins (IL-1β, IL-2, IL-6, IL-8, IL-10, and IL-12), and NO [20–22]. It has been known that immune response would be activated when the specific membrane receptors of macrophage were binding with polysaccharides [23,24]. Thus, macrophages are usually used as cell models to evaluate the immunomodulatory activities of polysaccharides.

It was found that the bioactivities of polysaccharide are closely related to its structure factors, such as molecular weight, the composition of monosaccharide residues, the connection position and sequences of monosaccharide residues, types of substituents, and conformations of glycosidic bonds [25]. When the factor changes, the activity of the

polysaccharide might change accordingly [26]. It is meaningful to elucidate the structural characterization of the polysaccharide. It could be helpful to disclose the mechanisms involved in the activation of macrophages by polysaccharide.

In this study, a new polysaccharide named NFP-1 was purified from *Nervilia fordii* with a purity of 97.8%. The primary chemical structure and conformation of NFP-1 were characterized. Moreover, its immunmodulatory activities were tested using murine RAW264.7 macrophage with or without induced by LPS. Our work might provide useful information of *Nervilia fordii* polysaccharides, and help to better understand the structure and immunmodulatory activities of functional botanical polysaccharides.

2. Methods and materials

2.1. Materials and reagents

Stems and leaves of *Nervilia fordii* (900 g) were purchased from Yixin Pharmaceutical Group Ltd. Co. of Guangxi, and were authenticated by TCM-Pharmacist Jia-fu Wei from Guangxi Zhuang Autonomous Region Food and Drug Administration. A voucher specimen was coded as NF-02, and deposited in the laboratory of natural products of the School of Pharmaceutical Science, Guangxi Medical University.

The murine RAW264.7 macrophage cell line was obtained from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). DMEM was purchased from Hyclone Laboratories Inc. (Logan, USA). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Griess reagent were purchased from Beyotime Biotechnology Research Institute (Shanghai, China). Lipopolysaccharide (LPS; *Escherichia coli* 055: B5 endotoxin) and Dextrans were ordered from Sigma-Aldrich (St. Louis, MO, USA). Enzyme linked immunosorbent assay (ELISA) kits for cytokines were purchased from R&D systems (Minneapolis, MN, USA). The ultrapure water was obtained from a Milli-Q® water purification system of Millipore (Bedford, MA). All other chemicals and reagents used in this paper were analytical grade purchased in China.

2.2. Extraction and purification of polysaccharide from Nervilia fordii

The dried stems and leaves of *Nervilia fordii* (800 g) were extracted 3 times with 10-fold of distilled water under reflux for 2 h. Filtrates were pooled and evaporated in vacuo to afford a crude water extract. Then the crude water extract was precipitated by adding ethanol to a final concentration of 80%, and kept at 4 °C for 24 h. Then, the precipitate was collected by using a centrifuge at 4000 rpm for 10 min, and was redissolved in distilled water to give an aqueous solution. Subsequently,

the aqueous solution was deproteinated by using the Sevage method repeatedly [27]. Afterwards, the aqueous solution was dialyzed against distilled water for 48 h by a dialysis bag of 3500 Da molecular weight. Finally, the retentate was freeze-dried to obtain crude polysaccharides from *Nervilia fordii* (CPNF).

The CPNF was dissolved in deionized water, filtered through 0.45 μm of membrane and subjected onto a DEAE-52 cellulose column (6.5 × 95 cm). The column was then stepwise eluted with 0, 0.2, 0.4, 0.6, 0.8, 1.2 and 1.6 M NaCl solutions at a flow rate of 1.0 mL/min, and the eluent was fractionated by 250 mL per receiving conical flask. Seven fractions, CPNF-1-CPNF-7, were collected by checking the absorbance of each conical flask at 490 nm using the phenol sulfuric acid method [28]. The CPNF-2 was dialyzed against deionized water for 2 days, and then the retentate was filtered through 0.45 μm of membrane and subjected onto a Sephadex G-100 column (1.5 × 100 cm). Here, deionized water was employed as elution solvent at a flow rate of 1.0 mL/min, and the eluent was subfractionated by 20 mL per receiving tube.

After detected by checking the absorbance of each receiving tube at 490 nm using the phenol sulfuric acid method, the collection of receiving tube No.76–81 was pooled, concentrated in vacuo and freeze-dried stepwise, to yield a homogenous polysaccharide, named as NFP-1.

2.3. Determination of homogeneity and molecular weight of NFP-1

Homogeneity and molecular weight of NFP-1 were evaluated and determined by HPGPC. A Shimadzu LC-20A instrument equipped with a refractive index detector and a TSK-GEL G4000 PWXL column (7.8 × 300 mm, 10 μm, Tosoh Co., Ltd., Tokyo, Japan) was employed. The mobile phase was ultra-pure water at a flow rate of 1.0 mL/min, and the column temperature was set at 40 °C. The molecular weight and polydispersity index (M_w/M_n) were estimated by HPGPC, and the standard curve was established by series standard dextrans (150, 270, 500, 1100 and 2000 kDa).

2.4. Analysis of monosaccharide composition

The monosaccharide composition was analyzed by HPLC [29]. 5 mg of NFP-1 was hydrolyzed with 10 mL of 2.0 M trifluoroacetic acid (TFA) at 100 °C for 4 h. After repeated evaporation with methanol to completely remove TFA, the residue was dissolved in 1 mL distilled water to prepare a sample solution. Then, derivatization was carried out by mixing 100 μL of the sample solution and 100 μL of 0.3 M NaOH, together with 120 μL of 0.5 M PMP at 70 °C for 1 h. After neutralization with 100 μL 0.3 M HCl, the mixture was extracted in triplicate by chloroform. Then, the water layer was centrifugated at 5000 rpm for 10
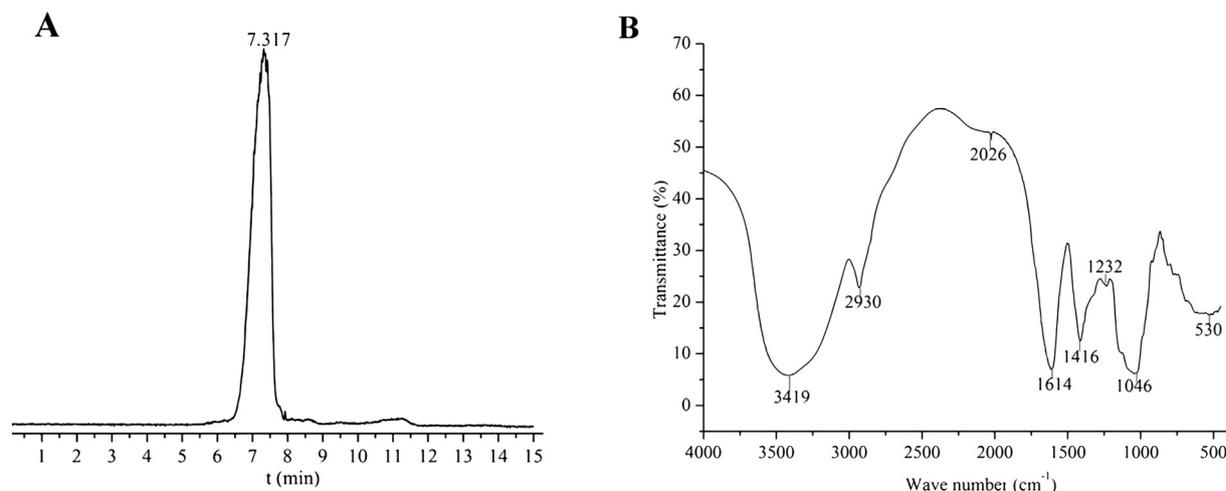
Fig. 1. Chromatography of the polysaccharides NFP-1 from *Nervilia fordii* by HPGPC (A), FTIR spectrum (B).

A

B
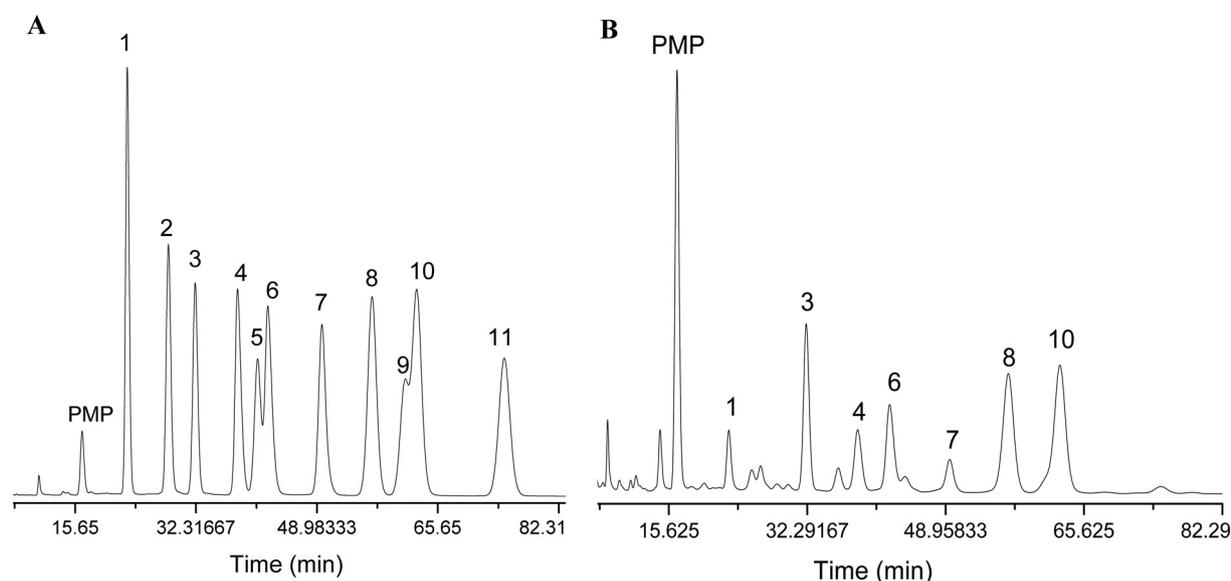
Fig. 2. HPLC chromatography of the PMP derivatives of the monosaccharide mixture (A) and NFP-1 sample (B). The peaks are identified as follows: 1, manose; 2, glucosamine; 3, rhamnose; 4, glucuronic acid; 5, galactosamine; 6, galacturonic acid; 7, glucose; 8, galactose; 9, xylose; 10, Arabinose; 11, fucose.

min. The supernatant was filtered through a 0.45 μm membrane and analyzed by a Shimadzu LC-20A HPLC using Shimadzu Inertsil C18 column (4.6 × 250 mm, 5 μm, Shimadzu, Japan) and ultraviolet (UV) detector (λ = 245 nm) at a flow rate of 1.0 mL/min at 35 °C. 82% phosphate buffer solution (pH 6.5) and 18% acetonitrile (v/v) were employed as mobile phase. Similarly, the monosaccharide standards were PMP-labeled and analyzed by HPLC accordingly.

2.5. FT-IR spectrometric analysis

The FT-IR spectrum of NFP-1 was recorded on a Spectrum 100 FT-IR spectrometer (PerkinElmer, USA) in a range of 4000–400 cm^{-1} with KBr pellets.

2.6. Methylation and GC-MS analysis

NFP-1 was methylated according to the reported method [30]. Briefly, NFP-1(dried) was dissolved in dimethyl sulfoxide (DMSO), and NaH powder was then added, the mixture was sealed in nitrogen and stirred for 1 h and then methyl iodide was added. Afterwards, distilled water was added into the solution to decompose the remaining methyl iodide. The methylated sample was dialyzed with 3500 Da semipermeable membrane, and then was frozen dried. Then, the dried methylated sample was hydrolyzed by 2 M TFA at 100 °C for 1 h. The hydrolysates were reduced with NaBH$_4$, and acetylated with pyridine and acetic anhydride at 100 °C for 1 h. Deionized water and dichloromethane were added to the acetylated derivatives, and the organic phase was dried under nitrogen, redissolved in dichloromethane, and analyzed by GC–MS (GCMS-QP 2010, Shimadzu, Japan). Temperature program was set rising from 100 °C up to 140 °C at 3 °C/min (standing for 10 min) then up to 260 °C at 20 °C/min.

2.7. Nuclear magnetic resonance (NMR) spectroscopy analysis

NFP-1 (60 mg) was kept over P$_2$O$_5$ in vacuo for five days to get rid of water. Then, 60 mg NFP-1 was dissolved and held in 0.6 mL D$_2$O for two days, and freeze-dried before being redissovled in 0.6 mL D$_2$O again. The procedure described above should be done in triplicate to insure the H/D exchange completely. Then, the ^{1}H, ^{13}C and 2D NMR (HSQC, HMBC) spectra of NFP-1 were recorded on a Bruker Advance III HD 600 spectrometer (Bruker Corp., Zurich, Switzerland).

2.8. Cell culture

RAW264.7 cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO$_2$ incubator. The cells were detached by 0.05% trypsin/0.02% EDTA in Mg^{2+}/Ca^{2+}.

2.9. Cell viability assay

The effect of NFP-1 on the viability of RAW264.7 macrophages was measured using MTT assay. The 1×10^3 cells were seeded in ninety six wells plate and incubated in the DMEM medium, 5% CO$_2$ at 37 °C. After 12 h, the cells in each well were treated with or without various concentrations of NFP-1 (5–120 μg/mL). After co-incubation for 24 h, MTT solution (5 mg/mL) was added to each cell supernatant and incubated for 4 h at 37 °C. The culture medium was discarded and then 100 μL DMSO was added into the wells followed by 10 min with horizontally shaking to solubilize the formazan. The absorbance at 490 nm was measured using a spectrophotometer (Molecular Devices, CA, USA). The cell viability was calculated by the following formula:

$$\text{Cell viability }(\%) = A_{\text{experimental group}-\text{blank group}}/A_{\text{control group}-\text{blank group}} \times 100\%.$$

Table 1
Glycosidic linkage composition of methylated NFP-1.

Residues	Methylated sugars	Linkage	Molar ratio
D-Gal$p$[a]	2,3,4,6-Me$_4$-Gal	T- Gal$p$	13.18%
	2,3,6-Me$_3$-Gal	1,4- Gal$p$	22.52%
L-Ara$f$[b]	2,3-Me$_2$-Ara	1,5- Ara$f$	13.08%
	2,5-Me$_2$-Ara	1,3- Ara$f$	14.63%
L-Rha$p$[c]	3,4-Me$_2$-Rha	1,2- Rha$p$	17.73%
	3-Me-Rha	1.2.4-Rha$p$	9.04%

[a] Gal$p$, galactopyranose.
[b] Ara$f$, arabinofuranose.
[c] Rha$p$, rhamnopyranose.
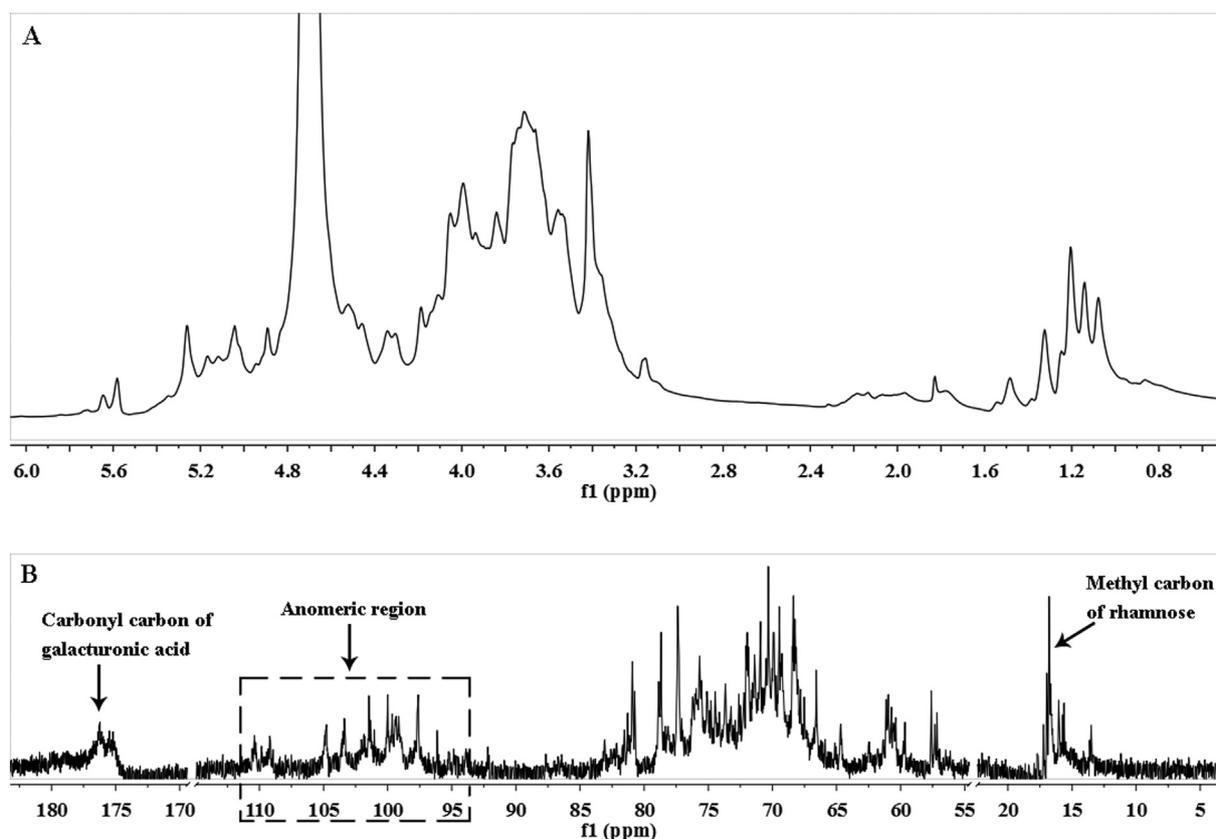
Fig. 3. ^{1}H NMR spectrum (A) and ^{13}C NMR spectrum (B) of NFP-1.

2.10. Immunomodulatory assays

RAW264.7 cells (1×10^5 cells) were plated in 96-well plates and incubated for 12 h. Then, the cells were incubated with a series of concentrations of NFP-1 (10, 20, 50, 100 μg/mL) with or without LPS (1 μg/mL) for 24 h. After that, the culture media of cells were collected and the levels of NO, TNF-α, IL-1β, IL-6, and IL-10 were determined by using Griess regent, mouse TNF-α, IL-1β, IL-6 and IL-10 ELISA kits, respectively.

2.11. Statistical analysis

Data were presented as mean ± standard deviation (SD) for at least three replicates and analyzed by SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Statistical comparisons between multiple groups were performed by one-way ANOVA. *P* value of 0.05 or less was considered statistically significant.

3. Results

3.1. Homogeneity and molecular weight of NFP-1

96 g CPNF was isolated from 800 g dried stems and leaves of *Nervilia fordii* by water extraction and ethanol precipitation with a yield of 12%. After isolated and purified by DEAE-52 cellulose and Sephadex G-100, 400 mg dried NFP-1 was obtained with a yield of 0.42% from CPNF.

Homogeneity and molecular weight of NFP-1 were evaluated and determined by HPGPC. A single peak of NFP-1 was observed (Fig. 1A) indicating that NFP-1 was a homogeneous polysaccharide. The purity of

Table 2
Chemical shifts of resonances in the ^{1}H and ^{13}C NMR spectra of NFP-1.

Glycosyl residue	Chemical shifts, δ (ppm)					
	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
→5-α-Ara$f$ [a]-(1→(**A**)	110.2/5.04	81.8/4.31	77.6/3.94	81.8/4.06	68.6/3.73	−/−
→3-α-Ara$f$-(1 → (**B**)	109.2/5.17	81.0/3.99	81.8/3.78	81.8/4.06	60.8/3.69	−/−
T-β-Gal$p$[b] (**C**)	104.8/4.46	71.7/3.53	72.0/3.66	70.9/4.20	77.3/3.62	60.8/3.73
→4-α-Gal$p$A [c]-(1→(**D**)	101.5/5.02	68.6/3.76	69.1/3.87	78.1/4.46	75.9/4.20	175.2/−
→4-β-Gal$p$-(1→(**E**)	103.4/4.89	72.6/3.54	74.4/3.71	76.4/3.83	77.3/3.62	61.0/3.78
→2-α-Rha$p$ [d]-(1→(**F**)	100.0/5.26	78.7/4.06	66.2/3.87	71.6/3.38	68.6/3.76	17.2/1.33
→2,4)-α-Rha$p$-(1→(**G**)	97.6/5.58	75.9/4.20	69.0/4.44	83.8/3.51	68.6/3.76	16.8/1.21
→2,4)-α-Rha$p$-(1→(**H**)	99.5/4.84	77.6/4.46	68.1/3.85	87.4/4.34	71.2/4.11	16.9/1.25

[a] Ara$f$, arabinofuranose;
[b] Gal$p$, galactopyranose;
[c] Gal$p$A, galacturonic acid;
[d] Rha$p$, rhamnopyranose.
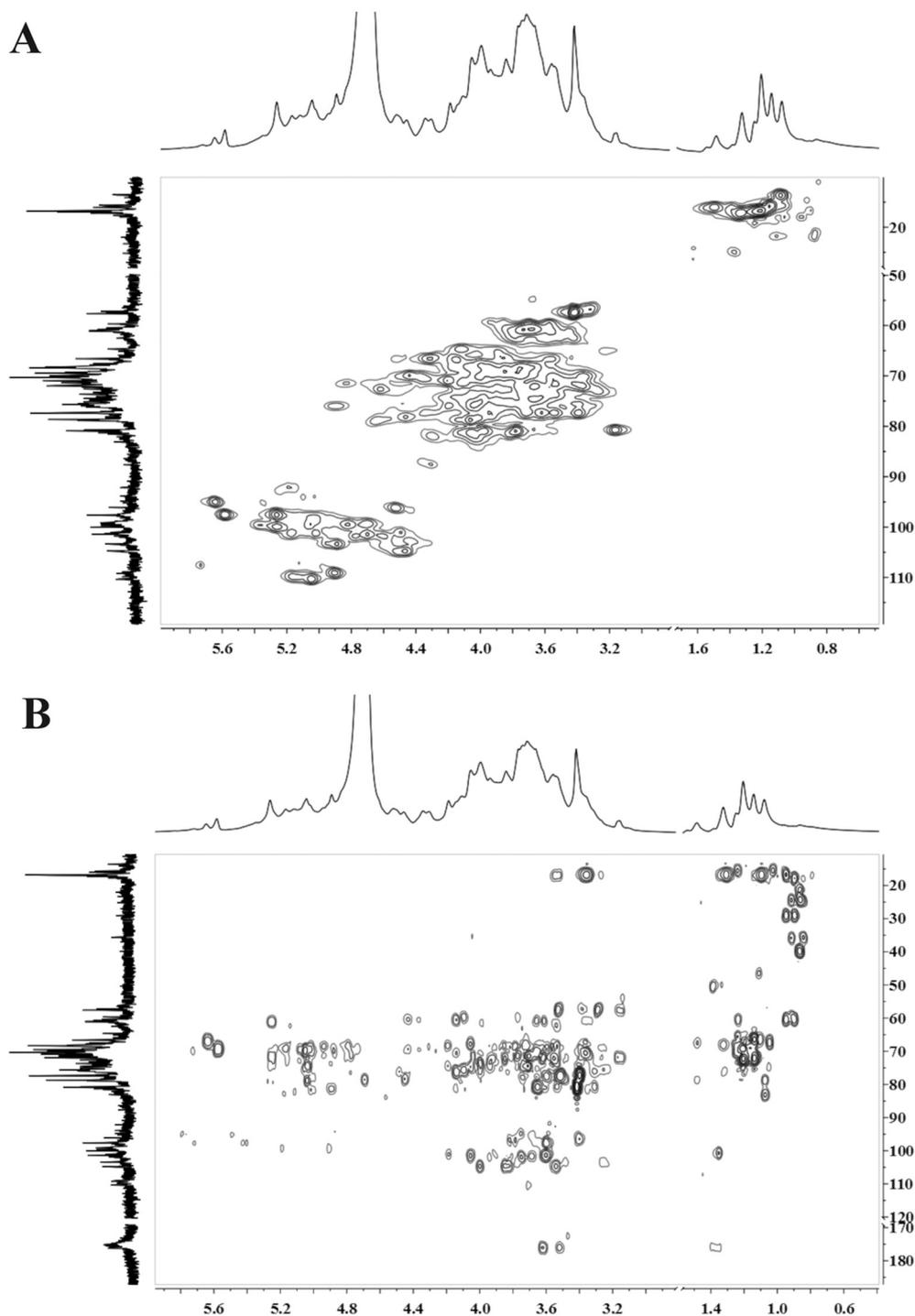
Fig. 4. Partial HSQC (A) and HMBC (B) spectra of NFP-1.

NFP-1 was 97.8%. Based on the calibration with standard dextran, molecular weight of NFP-1 was calculated to be 950 kDa.

3.2. FT-IR spectrum of NFP-1

FT-IR spectra of NFP-1displayed a broad and intense characteristic absorption peak at 3401 cm^{-1} (Fig. 1B) which was attributed to the stretching vibration of O—H, a weak absorption peak at 2930 cm^{-1} which was due to the stretching vibration of C—H, an absorption peak at 1614 cm^{-1} indicating the presence of C=O bond, an absorption peak at 1416 cm^{-1} which was attributed to the O—H bending vibration,

and a typical absorption peak at 1046 cm^{-1} representing the characteristic vibration of C—O—H in pyranose ring [31–36].

3.3. Monosaccharide composition of NFP-1

Monosaccharide composition of NFP-1 was analyzed using HPLC after pre-column derivatizing its hydrolysate with PMP. NFP-1 mainly consists of arabinose, galactose, rhamnose, and galacturonic acid in the molar ratio of 2.1:2.0:1.0:0.4, and also contained a few mannose, glucose, and glucuronic acid in trace (Fig. 2).

3.4. Methylation and GC-MS analysis of NFP-1

Methylation and GC-MS analysis were performed to further study the glycosidic bond types of the homogenous polysaccharide [37]. The results are showed in Table 1. It is showed that the D-galactose in NFP-1 has two kinds of glycosidic linkages which are T-β-Gal$p$-(1→ and →4)-β-Gal$p$-(1→, here, T means the terminal of a sugar chain. It is also revealed that the D-arabinose in NFP-1 has two kinds of glycosidic linkages which are →5)-α-Ara$f$-(1→ and →3)-α-Ara$f$-(1→. Furthermore, the methylation and GC–MS analysis also demonstrate that the L-rhamnose in NFP-1 has two kinds of glycosidic linkages which are →2)-α-Rha$p$-(1 → and →2,4)-α-Rha$p$-(1→. Here, the linkage pattern of Gal$p$A in NFP-1 could not be identified because Gal$p$A was unmethylated.

3.5. NMR elucidation of NFP-1

The ^{1}H and ^{13}C NMR spectra of NFP-1 are shown in Fig. 3A, B. The NMR spectral data of NFP-1 are exhibited in Table 2. The proton signals between δ 3.0–5.5 and the carbon signals between δ 60–110 are in the characteristic signal regions of polysaccharide [38]. According to the ^{1}H, ^{13}C NMR and HSQC spectra (Fig. 4A) of NFP-1, there are four anomeric proton signals (δ 5.17, 5.02, 4.89, 4.46) and four corresponding anomeric carbon signals (δ 109.2, 101.5, 103.4, 104.8) which suggested that there are four anomeric resdues in NFP-1 which are →3)-α-Ara$f$-(1→ (**B**), →4)-α-Gal$p$A-(1→ (**D**), →4)-β-Gal$p$-(1→ (**E**), and terminal sugar residue of α-Gal$p$ (T-Gal$p$) (**C**), respectively [35,36,39]. There are two proton signals (δ 5.04, 3.73) and two corresponding carbon signals (δ 110.2, 68.6) which indicated that there is a residue of →5)-α-Ara$f$-(1→ (**A**) and a hydroxyl at C-5 of the Ara$f$ residue has been substituted [40–42]. Furthermore, a quaternary carbon signal at δ 175.2 is attributed to the presence of →4)-α-Gal$p$A-(1→ (**D**), an uronic acid residue [39]. Moreover, there are two proton signals (δ 5.26, 1.33) and two corresponding carbon signals (δ 100.0, 17.2) which proposed that there is a residue of →2)-α-Rha$p$-(1→ (**F**) [39]. There are four proton signals (δ 5.58, 1.21, 4.84, 1.25) and four corresponding carbon signals (δ 97.6, 16.8, 99.5, 16.9) which are belonging to two sets of typical proton and carbon signals of the anomers and methyl groups of rhamnose residues. Those are attributed to the presences of →2,4)-α-Rha$p$-(1→ (**G**), and →2,4)-α-Rha$p$-(1→ (**H**) of different chemical environments in NFP-1 [36,39,43].

The HMBC spectrum of NFP-1 (Fig. 4B) showed correlations from δ 4.46 (H$_H$−2) to δ 78.1 (C$_D$-4), from δ 5.02 (H$_D$-1) to δ 78.7 (C$_F$-2), from δ 5.26 (H$_F$-1) to δ 75.9 (C$_G$-2), and from δ 3.51 (H$_G$-4) to δ 104.8 (C$_C$-1) which suggested that there was a structure fragment of NFP-1 consisting of →1,4)-α-Rha$p$-(2→4)-α-Gal$p$A-(1→2)-α-Rha$p$-(1→2)-α-Rha$p$-(4→1)-β-Gal$p$-T. Furthermore, the HMBC spectrum of NFP-1 showed correlations from δ 4.84 (H$_H$−1) to δ 68.6 (C$_A$-5), and from δ 5.04 (H$_A$-1) to δ 81.8 (C$_B$-3) which could be deduced that there is another structure fragment of NFP-1 consisting of →2,4)-α-Rha$p$-(1→5)-α-Ara$f$-(1→3)-α-Ara$f$-(1→. Moreover, the HMBC spectrum of NFP-1 showed a correlation from δ 5.58 (H$_G$-1) to δ 76.4 (C$_E$-4) which indicated that there was a structure fragment of NFP-1 consisting of →2,4)-α-Rha$p$-(1→4)-β-Gal$p$-(1→. Then, the structure unit of NFP-1 could be established as →4)-α-Rha$p$-(2→4)-α-Gal$p$A-(1→2)-α-Rha$p$-(1→2)-α-Rha$p$-(4→1)-β-Gal$p$-T with two branch chains of →2,4)-α-Rha$p$-(1→5)-α-Ara$f$-(1→3)-α-Ara$f$-(1→, and →2,4)-α-Rha$p$-(1→4)-β-Gal$p$-(1→ (Fig. 5).

3.6. Effect of NFP-1 on the viability of RAW264.7

NFP-1 could dose dependently enhance the viability of RAW264.7 cells in the concentration range from 5 to 120 μg/mL (Fig. 6). It was supposed that NFP-1 could dose dependently promote the proliferation of RAW264.7 macrophages without cytotoxic effects in the concentration range.
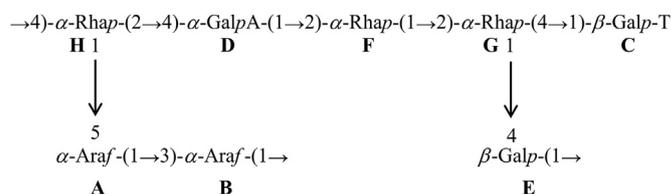
Fig. 5. The structure unit of NFP-1.

3.7. Enhancing effects of NFP-1 on the production of cytokines and NO from RAW264.7 macrophages

NFP-1 of 20 μg/mL significantly stimulated the production of TNF-α ($P < 0.01$, Fig. 7A), enhanced the productions of IL-6 and IL-1β ($P < 0.05$, Fig. 7B, 5C), and induced the production of NO ($P < 0.01$, Fig. 7E). In vitro studies showed that NFP-1 significantly induced NO production and enhanced the release of those cytokines in a dose dependent manner. It indicated that NFP-1 might have immunomodulatory potential. We found that NFP-1 cannot affect the secretion of IL-10 even at a high concentration of 100 μg/mL (Fig. 7D).

3.8. Inhibition effects of NFP-1 on the production of cytokines and NO from RAW264.7 macrophages induced by LPS

LPS, a vital structural component of the outer membrane of Gram-negative bacteria, is a stimulator being widely used to excessively activate the macrophages to mimic inflammatory diseases at the cellular level in vitro [44]. The level of pro-inflammatory mediators releasing from RAW264.7 macrophages activated by LPS (1 μg/mL), including TNF-α, IL-6, IL-1β, IL-10, and NO, were dramatically increased as compared to those of control-group ($P < 0.01$, Fig. 8). Thereafter, TNF-α, IL-6, IL-1β and the radical NO releasing from LPS-activated RAW264.7 macrophages which have been treated with NFP-1 of different concentrations were significantly decreased as compared to those of LPS-vehicle group (Fig. 8A, B, C, E). It was worthy to be noted that IL-10 releasing from LPS-activated RAW264.7 macrophages which have been treated with NFP-1 of different concentrations were assuredly promoted as compared to that of LPS-vehicle group (Fig. 8D). It is well known that IL-10 is an anti-inflammatory cytokine which can predominantly inhibit LPS mediated induction of the pro-inflammatory cytokines, such as TNF-α, and IL-1β [45,46]. This assay further confirmed that NFP-1 has immunomodulatory potential, particularly on inflammation.
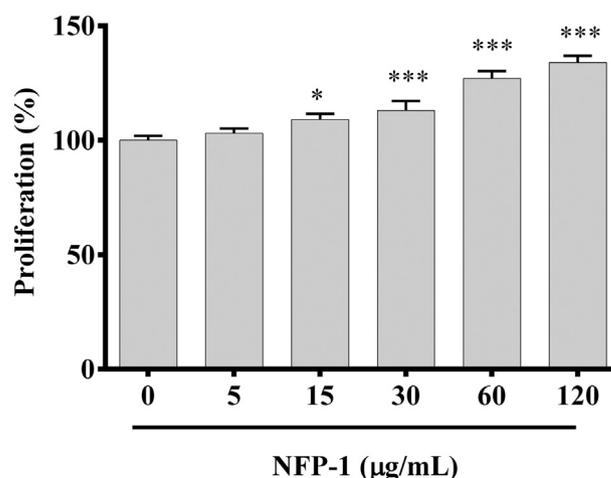
Fig. 6. Effect of NFP-1 on the viability of RAW264.7.
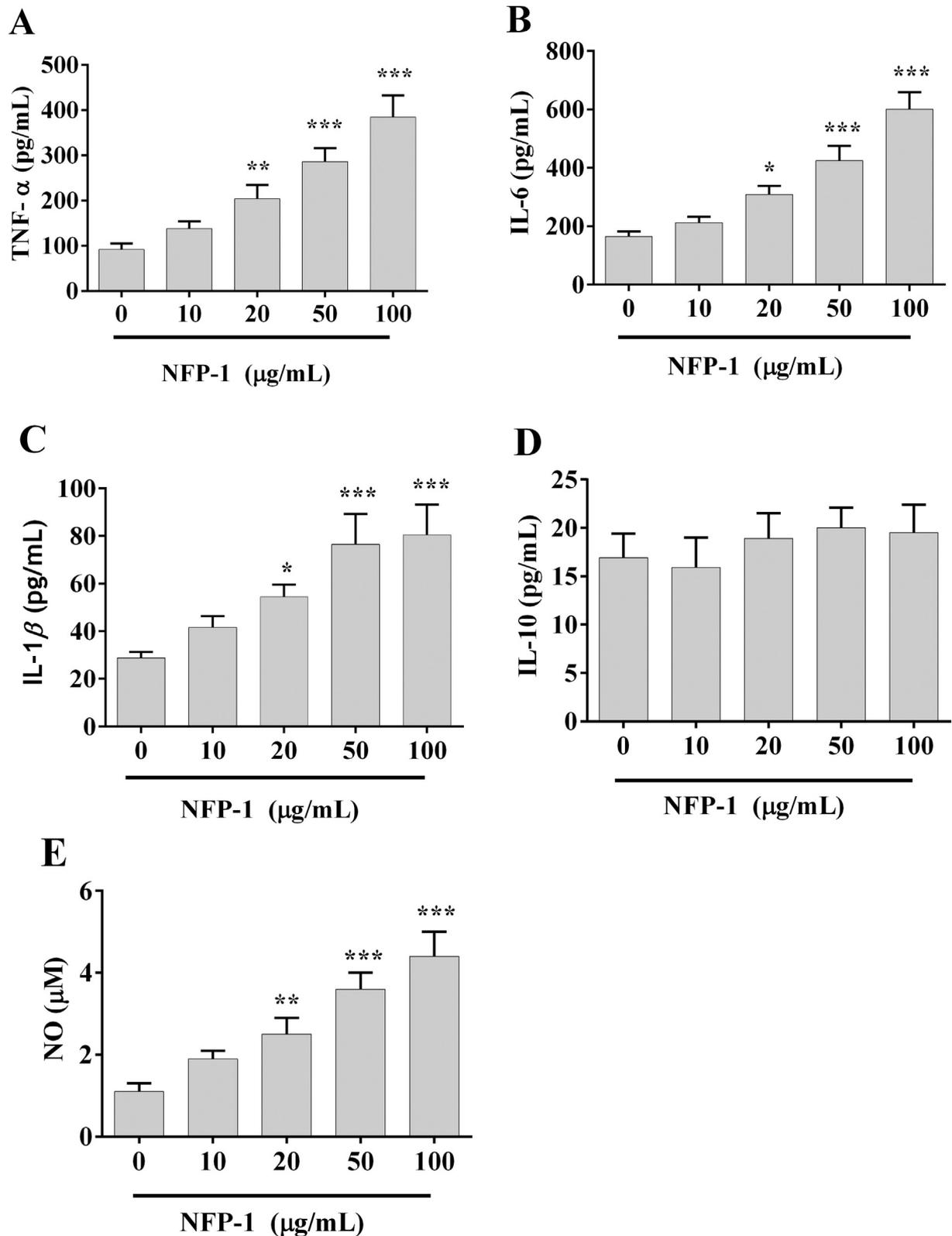
Fig. 7. Enhancing effects of NFP-1 on the production of cytokines and NO from RAW264.7 macrophages. Macrophages were cultivated for 24 h with culture media only (control group), and NFP-1 of different concentrations (10, 20, 50, 100 μg/mL). The culture media were harvested and assayed for the production of TNF-α (A), IL-6 (B), IL-1β (C), IL-10 (D), and NO (E). Data are presented as mean ± SD and are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with corresponding control group.

4. Discussion

It has been shown that a fair amount of polysaccharides derived from plants could enhance immunity by activating the macrophage and complement system [47,48]. Botanical polysaccharides could induce a variety of immune responses in macrophages, such as promoting the proliferation, inducing the production of NO, promoting the secretion of pro-inflammatory cytokines. Activated macrophages play an
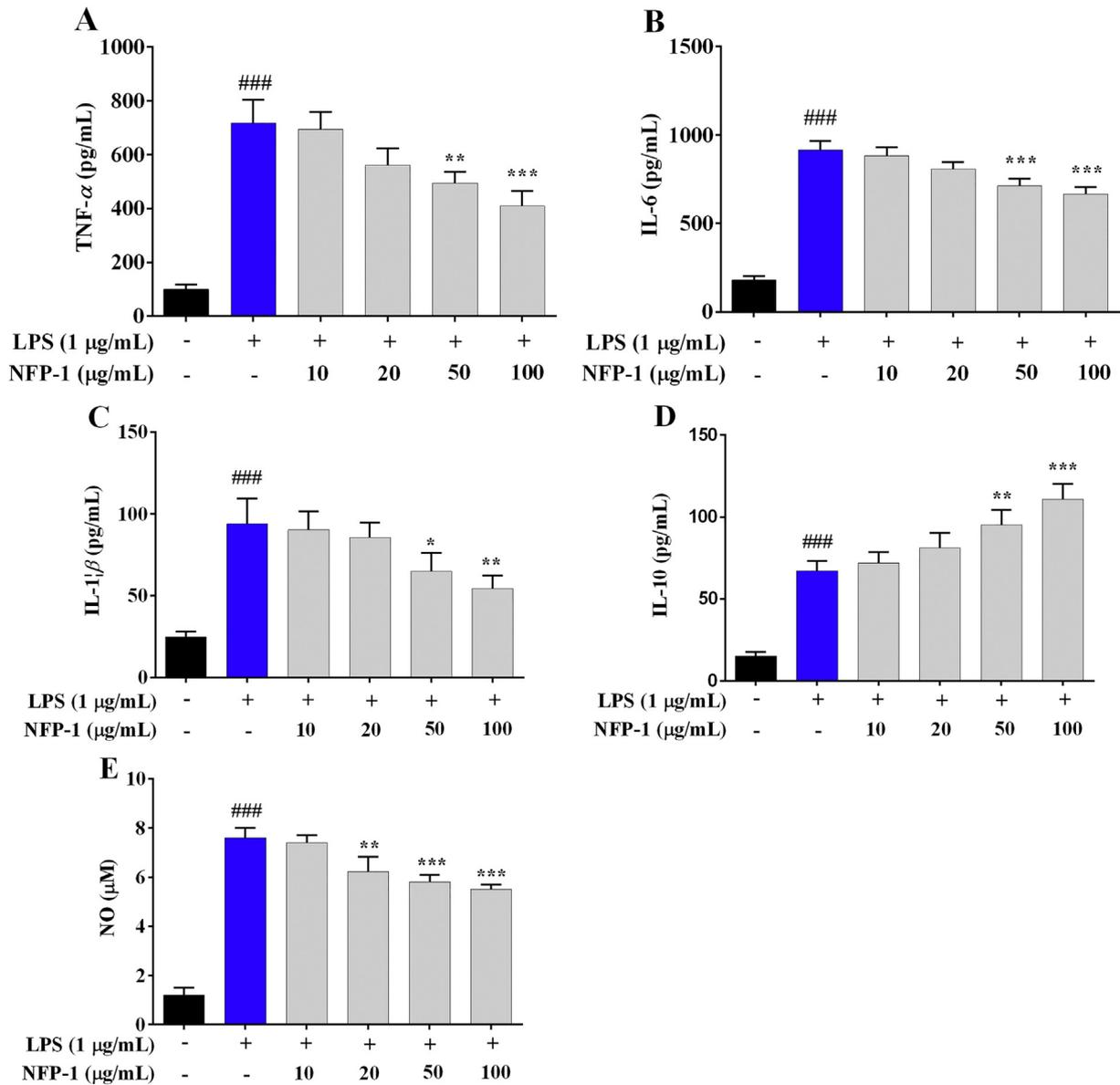
Fig. 8. Inhibition effects of NFP-1 on the production of cytokines and NO from RAW264.7 macrophages induced by LPS. Macrophages were cultivated for 24 h with culture media only (control group), LPS (LPS-vehicle group), and LPS in addition with NFP-1 of different concentrations (10, 20, 50, 100 μg/mL). The production of TNF-α (A), IL-6 (B), IL-1β (C), IL-10 (D), and NO (E) in the Supernatants were analyzed by ELISA and Griess reaction assays, respectively. Data are presented as mean $\pm$ SD and are representative of three independent experiments. ### $P < 0.001$ compared with control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with LPS group.

important role in the occurrence and development of inflammation disease. However, the pro-inflammatory mediators cascade would break out when macrophages were excessively activated by the severe infection, such as bacteria and viruses [49]. In vitro studies have demonstrated that NFP-1 could modulate the immune responses in macrophages with or without LPS. Therefore, it was considered that NFP-1 possessed dual function in immunomodulatory.

It is known that monosaccharide compositions of polysaccharide have influence on its biological activities. Polysaccharide SNLWP-1 from *Solanum nigrum* L. mainly containing galactose and arabinose had significant antitumor and immunomodulatory activities, whereas polysaccharide SNLWP-2 from *Solanum nigrum* L. being rich in glucose hardly demonstrated the activity [50]. Polysaccharide BPs from the roots of *Bupleurum smithii* var. *parvifolium* is immunomodulatory active which mainly composed of arabinose and galactose [51]. Here, NFP-1 is an acidic heteropolysaccharide which mainly composed of arabinose,

galactose, rhamnose and galacturonic acid, and its dual function in immunomodulatory activity which might be associated with its characteristic monosaccharide composition.

Conflict of interests

Authors declare that there is no conflict of interests.

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