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Antioxidant and immunoregulatory activity of alkali-extractable polysaccharides from North American ginseng

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ABSTRACT

The alkali-extractable polysaccharide (AEP) was isolated from the root of North American ginseng. Two fractions, AEP-1 and AEP-2, were further purified by gel filtration column chromatography. Gas chromatography analysis identified that AEP-1 was composed of Glc, Gal and GalA. And AEP-2 mainly contained Ara, Man, Gal, Glc and GalA. Antioxidant assays indicated that AEP and AEP-2 exhibited significant antioxidant activities in a dose-dependent manner. AEP-2 also exhibited macrophage-activating activity by increasing NO, TNF-α and IL-6 production. The results suggest that AEP-2 could be used as potential antioxidants and immunomodulators.

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1. Introduction

North American ginseng (*Panax quinquefolium* L.), a plant native to North America, has been recognized as a valuable tonic similar to Asian ginseng (*Panax ginsen* C.A. Meyer) [1]. North American ginseng is reported to have many activities, such as anticarcinogenic, antiaging, immunostimulatory and antioxidant effects [2]. Various phytochemicals were found to be responsible for the health benefits of North American ginseng. Ginsenosides and polysaccharides in North American ginseng were recognized as the two major active components [3]. Many studies had proved that polysaccharides in North American ginseng showed strong immunoregulatory effect [4–7]. However, most of the reported polysaccharides were prepared from water extraction of North American ginseng. And, after water extraction, the residue was usually abandoned. Actually, there are still some components in the residue which are of great use, such as alkali-extractable polysaccharides. As far as we know, there was no report about the extraction and purification of alkali-extractable polysaccharides from North American ginseng, as well as the antioxidant and immune activity determination.

In this work, we isolated alkali-extractable polysaccharides from North American ginseng, identified their chemical characteristics, and determined the antioxidant and immunomodulatory effects.

2. Materials and methods

2.1. Materials and regents

Four-year-old roots of North American ginseng were obtained from Fusong County (Jilin Province, China). DEAE Sepharose Fast Flow and Sephacryl S-300 High Resolution were obtained from GE Healthcare Bio-Sciences Co. (Piscataway, NJ, USA). Monosaccharide standards including galacturonic acid (GalA), glucose acid (GlcA), glucose (Glc), rhamnose (Rha), arabinose (Ara), galactose (Gal), xylose (Xyl) and mannose (Man) were purchased from Sigma–Aldrich (Shanghai, China). DMEM, RPMI 1640 medium, lipopolysaccharide (LPS), Griess reagent, sodium fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[^{3}H],9′[^{9}H]-xanthen]-3-one, FL), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were also purchased from Sigma–Aldrich. Fetal bovine serum (FBS) was obtained from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Raw murine macrophages (RAW 264.7) were purchased from National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China). All other chemicals and solvents used were of analytical grade unless otherwise specified.

2.2. Extraction, separation, and purification of polysaccharide

The dried roots of North American ginseng were ground into powder. The powder passing through an 80 mesh sieve was

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extracted as follows. Step 1, removal of ethanol-soluble substances: The powder was extracted twice with 15 times of 70% EtOH at 50 °C for 5 h to get the residue-1. Step 2, removal of water-soluble polysaccharide: the dried residue-1 was extracted twice with 14 times of distilled water at 100 °C for 4 h to get the residue-2; Step 3, extraction of alkali-soluble polysaccharide: residue-2 was extracted twice with 10 times of 0.3 M NaOH solution which contained 0.3% (w/w) $NaBH_4$ at room temperature for 4 h. The supernatant was collected and adjusted to neutrality with hydrochloric acid (1 M), and then dialyzed, concentrated, and deproteinated by Sevag method [8]. The supernatant containing alkali-extractable polysaccharide (AEP) was precipitated with ethanol (1:4 v/v) at 4 °C for 24 h and then lyophilized to get AEP.

AEP was purified on an ÄKTA explore 100 purification system. AEP was dissolved in distilled water, centrifuged (15,000 rpm, 20 min), and then the supernatant was loaded on a DEAE Sepharose Fast Flow column (2.6 cm × 100 cm) equilibrated with ultrapure water. The column was first eluted with distilled water, then with a linear gradient from 0 to 2.0 M NaCl at a flow rate of 4 mL/min. Different fractions (8 mL/tube) were collected using an automatic fraction collector, then dialyzed and lyophilized. The fractions were purified further on a Sephacryl S-300 High Resolution column (1.6 cm × 100 cm) eluted with 0.15 M NaCl at a flow rate of 0.5 mL/min to yield two main final fractions, named AEP-1 and AEP-2, respectively. The fractions obtained were combined according to the total carbohydrate content quantified by the phenol-sulfuric acid method [9].

2.3. Analysis of monosaccharide composition

AEP-1 and AEP-2 were hydrolyzed by trifluoroacetic acid (2 M) at 120 °C for 4 h, respectively [10]. Derivatization of the released monosaccharides was carried out by the trimethylsilylation reagent according to the method of Guentas et al. [11]. The trimethylsilylated derivatives was further analyzed by gas chromatography (GC) on an Agilent 6890 instrument (Agilent Technologies, USA) equipped with HP-5 MS capillary column (0.25 mm × 30 m × 0.25 μm) and determined by a flame ionization detector (FID). The following program was adopted for gas chromatography analysis: injection temperature: 250 °C; detector temperature: 260 °C; column temperature programmed from 100 to 150 °C at 5 °C/min, holding for 5 min at 150 °C, then increasing to 240 °C at 5 °C/min and finally holding for 3 min at 240 °C. Nitrogen was used as the carrier gas and maintained at 40.0 mL/min. The speed of air and hydrogen gas was 400 and 40 mL/min, respectively. The injection was in spiltless mode.

2.4. Infrared spectrum analysis

Fourier transform infrared (FT-IR) spectra were obtained by using a PerkinElmer FT-IR spectrometer (PerkinElmer Spectrum 400 FT-IR, Massachusetts, USA) at the range of 4000–500 cm^{-1}.

2.5. Assays for antioxidant activity

2.5.1. Trolox equivalent antioxidant capacity assay

The method used was as described by Re et al. (1999), based on the capacity of a sample to inhibit the ABTS radical compared with a reference antioxidant standard (Trolox) [12]. Briefly, ABTS radical solution was produced by reacting 7 mM of ABTS aqueous solution with 2.45 mM potassium persulphate, and the mixture was kept in the dark at room temperature for 16 h. Then ABTS radical solution was diluted with PBS (pH 7.0) to an absorbance of 0.70 (±0.02) at 734 nm. Each sample (0.2 mL) with various concentrations (0.0–5.0 mg/mL) were added into 2 mL of ABTS radical solution and mixed vigorously. The mixture solution was incubated for 6 min at room temperature. The absorbance was measured at 734 nm. The antioxidant activity of crude polysaccharide extracts and polysaccharide fractions was measured by Trolox equivalent antioxidant capacity (TEAC) analysis as described by Fukumoto and Mazza (2000) [13].

2.5.2. Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay was conducted according to the method of Ou et al. with some modifications [14]. The ORAC assay was carried out on a FLUOstar OPTIMA plate reader (BMG LABTECH GmbH, Offenburg, Germany). The ORAC assay employs the area under the curve of the magnitude and time to the oxidation of fluorescein due to $ROO^{\bullet}$ radicals generated by the addition of AAPH in the presence of added antioxidants [15]. FL and AAPH were prepared with 75 mM phosphate buffer at pH 7.4. Samples (25 μL) at different concentrations (0–5 mg/mL) were mixed with 100 μL of 7.98×10^{-4} mM FL and incubated at 37 °C for 10 min than added 75 μL of 173 mM AAPH. Fluorescence was collected with 485 nm excitation and 515 nm emission, taking measurements from each sample at 2 min intervals for 2 h. The inhibition capacity was expressed as Trolox equivalents (μmol Trolox/g), and is quantified by integration of the area under the curve (AUC). All determinations were performed in triplicate.

2.6. Assay for immunomodulatory activity

2.6.1. Nitric oxide (NO) production

Nitric oxide (NO) production was assayed by measuring the nitrite concentration in the supernatant of cultured macrophages using the Griess reaction [16]. Briefly, cells were incubated in medium alone (control group) or medium containing various concentrations of polysaccharides fractions (1, 10, 50, 100, 150 μg/mL) or lipopolysaccharide (LPS, 1 μg/mL) as a positive control. Cells were incubated at 37 °C in 5% CO_2 for 24 h, and then the supernatants (50 μL) were pipetted from the medium and mixed with an equal volume of Griess reagent. After incubation for 15 min at room temperature, the absorbance was measured at 540 nm in an ELISA reader (Rayto RT-6000, Shenzhen, China). The concentration of nitrite was calculated with reference to a standard curve obtained with $NaNO_2$ (0–100 μM).

2.6.2. Measurement of cytokine production

RAW264.7 cells were cultured at a density of 2×10^5 cells/well for 24 h with polysaccharide fractions (10, 50, 150 μg/mL) or LPS (1 μg/mL), the control group was treated with medium alone. The supernatants were collected for the detection of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production using commercial ELISA kit (BD Biosciences Pharmingen, San Diego, USA) according to the instructions of kits. The absorbance was measured at 450 nm and 570 nm in an ELISA reader. Cytokine quantities in the samples were calculated from standard curves of recombinant cytokines using a regression linear method.

3. Results and discussion

3.1. Extraction and purification of alkali-extractable polysaccharide

AEP was isolated from the root of North American ginseng and the yield was about 2.85%. The purification was performed on a DEAE Sepharose Fast Flow column to obtain water-eluted and salt-eluted fractions, accounting for 64.2% and 25.1% of AEP by weight, respectively (Fig. 1a). And then based on molecular weight difference, the two fractions were further purified using a column of Sephacryl S-300. Each fraction yielded only one peak (Fig. 1b and c),

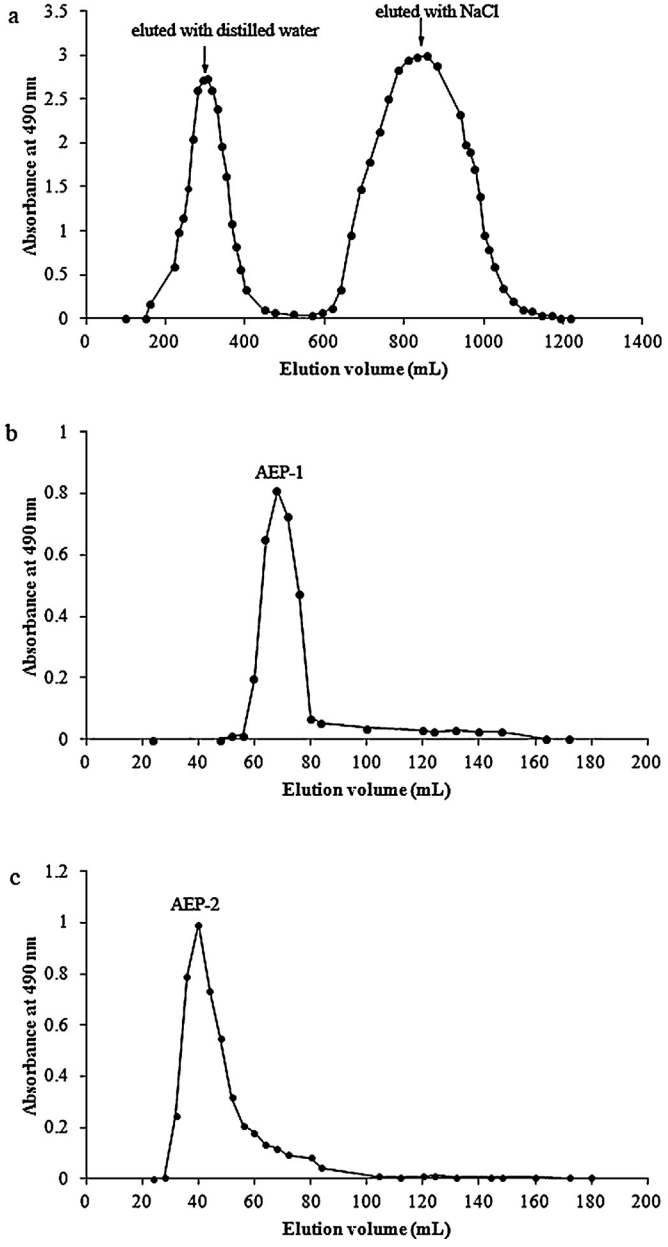

Fig. 1. (a) Elution profile of the crude polysaccharide (AEP) on DEAE Sepharose Flast Flow column, eluted with distilled water and NaCl (0–2.0 M). (b) and (c) Elution profile of AEP-1 and AEP-2 on Sephacryl S-300 column, respectively, eluted with 0.15 M NaCl.

designated as AEP-1 and AEP-2, respectively, basing on total carbohydrate elution profile. And AEP-1 and AEP-2 showed no absorption at 280 nm and 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid.

3.2. Characterization of polysaccharide fractions

Analysis of GC indicated that AEP-1 was composed of Glc, Gal and GalA in a molar ratio of 4.67:0.97:3.92. AEP-2 consisted of Ara, Man, Gal, Glc and GalA with a relative molar ratio of 1.03:0.76:1.68:3.02:3.65. Both AEP-1 and AEP-2 had GalA. Wenjin Zhu et al. purified one water-soluble polysaccharide (eluted with NaCl) from the roots of *P. quinquefolium* L., which was composed of Glc and Gal in a molar ratio of 2.1:1. The monosaccharide composition of glucogalactan was different from that of the AEP-2, but similar to AEP-1 [7]. The monosaccharide composition of AEP-2

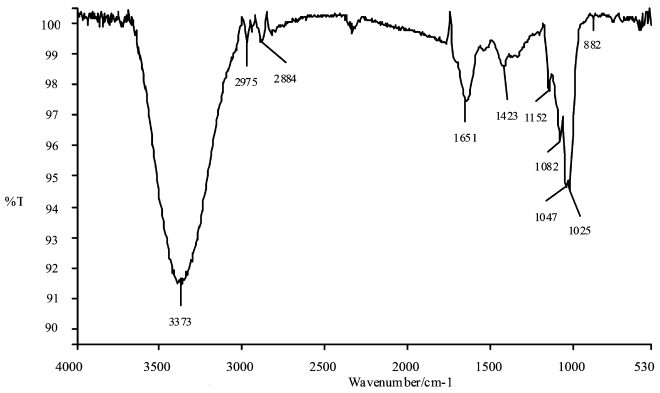

Fig. 2. FT-IR spectrum of AEP-1.

were similar to that of *P. ginseng*, which was composed of Glc, Gal, Man and Ara in the molar ratio of 3.3:1.2:0.5:1.1 [17]. Besides, Cold-fX, a polysaccharide-rich extract of North American ginseng, has been standardized to contain in excess of 80% (by weight) poly-furanosyl-pyranosyl-saccharides, and does not contain ginsenosides. The resulting extract is rich in Ara, Gal, Rha, GalA, GlcA, and D-galactosyl residues [18].

FT-IR spectroscopy is typically used for the qualitative measurement of organic functional groups. The infrared spectra of AEP-1 and AEP-2 were presented in Figs. 2 and 3. Both infrared spectra displayed a broadly stretched intense peak at 3300–3500 cm^{-1} characteristic of hydroxyl groups and a weak C—H band at 2920–2980 cm^{-1}.The prominent band between 1010 and 1100 cm^{-1} was representative of pyran structure. The band at about 890 cm^{-1} was ascribed to β-pyranoses in the polysaccharides [19]. The peak around 1650 cm^{-1} in Fig. 1 was due to the bound water [20]. In the IR spectrum of AEP-2, the absorption near 1730 cm^{-1} indicated the presence of uronic acid. Two absorptions around 1615 cm^{-1} and 1415 cm^{-1} were the antisymmetric and symmetric COO— stretches [21].

3.3. Antioxidant activity

Antioxidant activities of AEP, AEP-1 and AEP-2 were evaluated by TEAC and ORAC assays. Values determined by ORAC method were higher than that of the corresponding TEAC assay, which was due to the different nature of the two methods [22]. As shown in Table 1, all of the three polysaccharides exhibited antioxidant activity. Previous study showed that acidic polysaccharide from *Panax japonicus* C.A. Meyer had good ability to scavenge hydroxyl and DPPH free radical in a dose-dependent manner [23]. In this study, AEP-2 showed the highest TEAC and ORAC value, followed

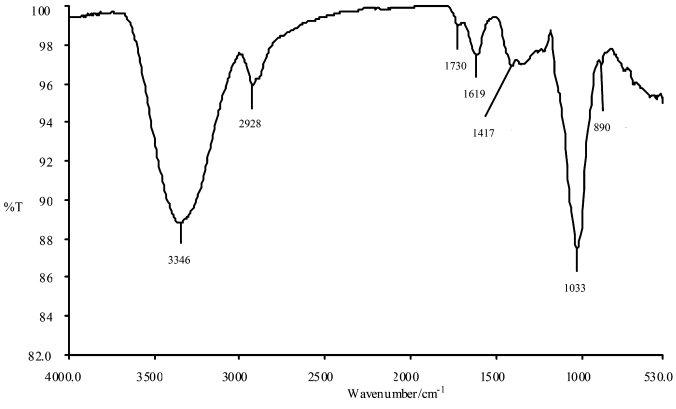

Fig. 3. FT-IR spectrum of AEP-2.

Table 1
TEAC and ORAC values for different polysaccharide fractions.

Sample	TEAC (μmol Trolox equivalent/g sample)	ORAC (μmol Trolox equivalent/g sample)
AEP	46.57 ± 0.88b	669.40 ± 47.85b
AEP-1	3.96 ± 0.45c	9.67 ± 2.18c
AEP-2	51.89 ± 1.66a	791.89 ± 345.18a

Values within a row followed by different letters are significantly different at $P < 0.05$. Data are expressed as mean ± SD ($n = 3$).

by AEP and AEP-1. The antioxidant activity of polysaccharides was supposed to be related to the configuration of sugar units and monosaccharide compositions [20]. In present study, Ara and Man were observed in AEP-2, but not in AEP-1. And the amount of Gal in AEP-2 was higher than AEP-1. It might be concluded that Ara, Man, and Gal would play an important role in the antioxidant activity of polysaccharide. The results of TEAC and ORAC assays indicated that the alkali-extractable polysaccharides from North American ginseng could be explored as potential antioxidants.

3.4. Immunomodulatory activity analysis

3.4.1. Effect of polysaccharides on NO production

NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological process [24]. Nitrite concentrations in the supernatant of polysaccharide stimulated macrophages were determined as a reflection of NO production. In this study, AEP and AEP-2 were able to stimulate macrophages to produce NO in a dose-dependent manner (Fig. 4). AEP-2 appeared to be the most potent and induced significantly higher ($P < 0.05$) NO production at the concentration of 10 μg/mL, comparing to control. AEP also induced significant increase of NO release at the concentration of 150 μg/mL. However, AEP-1 stimulated the least NO, demonstrating its weak effect on macrophages activation. These results were similar to that of the antioxidant assays.

3.4.2. Effect of polysaccharides on cytokine secretion

Activated macrophages play a key role in the innate and adaptive immune responses by secreting secondary compounds such as cytokines and IL-1β, which are the inhibitory factors of cancer [25]. In this work, we investigated the effect of polysaccharides on the production of TNF-α and IL-6 from RAW264.7 macrophages

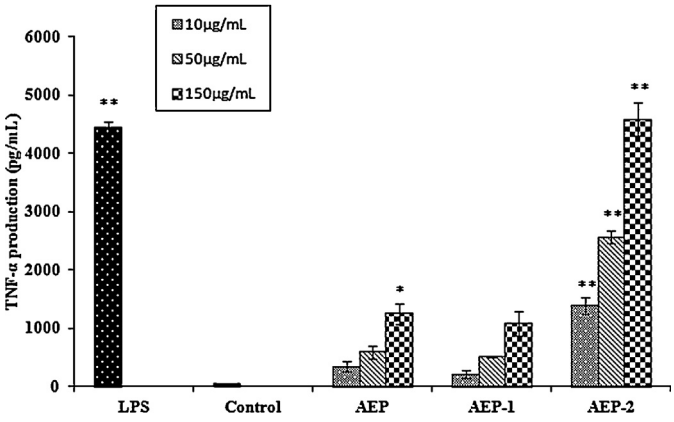

Fig. 5. Effects of different concentrations of North American ginseng polysaccharides on TNF-α production in macrophages RAW 264.7. Cells were incubated for 24 h with the indicated concentrations of polysaccharide fractions or LPS (1 μg/mL). Control cells were incubated with medium alone. Values are mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

(Figs. 5 and 6). As shown in figures, AEP-2 showed the strongest activation of TNF-α and IL-6 at the whole concentration range. On a dose-response basis, TNF-α releasing induced by 10 μg/mL of was significantly higher ($P < 0.05$) than that of control, and at the high dose of 150 μg/mL was comparable to that elicited by LPS at 1 μg/mL. By contrast, AEP (10–50 μg/mL) and AEP-1 had no apparent immuno-stimulatory effect. It has been extensively shown that the immunomodulating activity of polysaccharides is dependent on their chemical composition, molecular weight, conformation, glycosidic linkage, degree of branching, etc. [26]. In our study, the monosaccharide composition of AEP-1 differed from AEP-2. And the immunomodulatory activity of them was also different. As shown in Figs. 5 and 6, polysaccharides up-regulated macrophage production of TNF-α and IL-6 in a concentration dependent manner. Moreover, the maximum stimulatory response pertaining to TNF-α was much greater than that of IL-6.

In present study, the alkali-extractable polysaccharides were derived from the residue recovered after the isolation of the water-soluble polysaccharides. Many reports proved water-extracted polysaccharides had immunomodulatory and anti-tumor activity. Chike Godwin Azike et al. reported that the crude polysaccharides

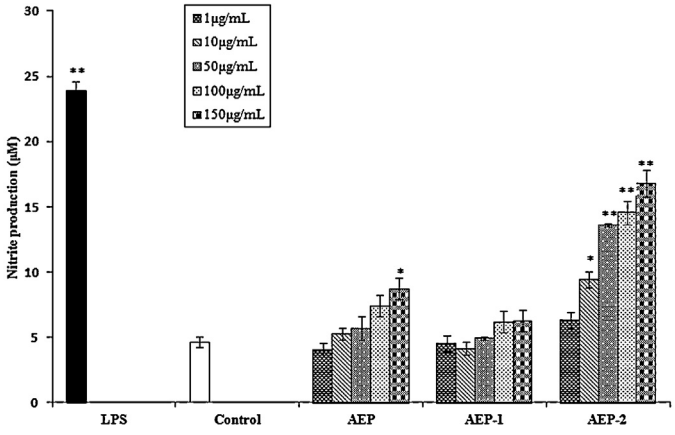

Fig. 4. Effects of different concentrations of North American ginseng polysaccharides on NO production in macrophages RAW 264.7. Cells were incubated for 24 h with the indicated concentrations of polysaccharide fractions or LPS (1 μg/mL). Control cells were incubated with medium alone. Values are mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

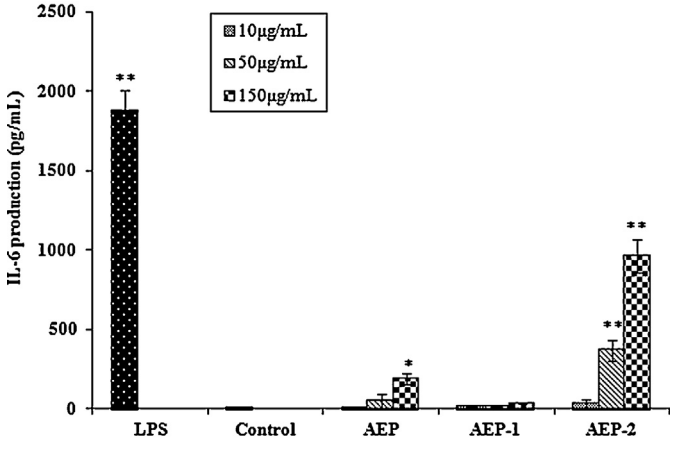

Fig. 6. Effects of different concentrations of North American ginseng polysaccharides on IL-6 production in macrophages RAW 264.7. Cells were incubated for 24 h with the indicated concentrations of polysaccharide fractions or LPS (1 μg/mL). Control cells were incubated with medium alone. Values are mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

could modulate the immune function of macrophages, including immuno-stimulatory and immuno-suppressive effects [6].

Indeed, a variety of plant polysaccharides have been reported to exhibit beneficial pharmacological effects to modulate macrophage function [27]. Stimulated macrophages also release a broad spectrum of cytokines including interleukins, TNF-α and NO [28], which are the inhibitory factors of cancer. Our experiments demonstrated that AEP-2 strongly increased the production of IL-6 which could promote the proliferation of T-lymphocytes and B-lymphocytes. Besides, AEP-2 induced the production of TNF-α and NO in a dose-dependent manner. However, AEP-1 had a weak effect on the production of cytokines. The results indicated that the antitumor factors induced from macrophage by polysaccharides fractions were required for AEP-2 antitumor activity.

4. Conclusion

In this study, we obtained two alkali-extractable polysaccharide fractions from the root of North American ginseng by DEAE Sepharose Fast Flow and Sephacryl S-300 HR column chromatography. Antioxidant test indicated that the crude polysaccharide and AEP-2 had significant radical scavenging effect. AEP-2 could significantly increase NO, TNF-α and IL-6 release from macrophages, whereas AEP-1 did not work to activate macrophages. The present results suggested that AEP-2 would be expected as potential antioxidants and immunomodulators.

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