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Characterization and immunostimulating effects on murine peritoneal macrophages of a novel protein isolated from *Panax quinquefolius* L.

Bin Qi[1], Shan Wang[1], Qiu Wang[1], He Zhang[3], Xue-yuan Bai[2], Hui-nan He[1], Wei-jie Sun[1], Li Liu[1,*] , Da-qing Zhao[2,*]

[1] College of pharmacy，Changchun University of Chinese Medicine, Changchun, Jilin, 130117, China

[2]Center of Chinese Medicine and Bio-Engineering Research, Changchun University of Chinese Medicine, Changchun, Jilin, 130117, China

[3] The Affiliated Hospital to Changchun University of Chinese Medicine, Changchun, Jilin 130021, China

Abstract：

Ethnopharmacological relevance: Panax quinquefolius L. has been used as a proverbial tonic in oriental countries for hundreds of years. It is used as a traditional medicinal herb to nourish vitality.

Aim of the study: The purpose of our study was to inquiry the activation effects on murine peritoneal macrophages of a novel protein separated from the roots of *Panax quinquefolius* L.

Materials and methods: In our work, a novel protein of the roots of American ginseng (AGNP) was separated and purified from the roots of *Panax quinquefolius* L. The characteristic was investigated with SDS-PAGE, high pressure gel filtration chromatography (HPGFC) and

matrix-assisted laser desorption ionization/time-of-flight mass (MALDI-TOF-MS) spectrometry method. The method of neutral red was carried out to investigate the phagocytosis of peritoneal macrophages. And Griess method and colorimetry were executed to detect the level of nitric oxide and iNOS activity respectively. Tumor necrosis factor-α and interleukin-6 were analyzed by enzyme linked immunosorbent assay (ELISA).

Results: Our results demonstrated that the subunit molecular weight of AGNP determined by SDS-PAGE was 15kD and the content of proteins determined by Bradford assay was 2.31mg/mL. The molecular weight of the AGNP was15, 114 Da both of electrophoresis and MS purity. And the result of HPGFC showed that the molecular weight of AGNP was 31,086 Da, Immunological studied indicated that AGNP could conspicuously increase phagocytosis of macrophages, facilitate the nitric oxide production, Tumor necrosis factor-α and interleukin-6 production. What is more, AGNP dose-dependently stimulated NO formation through the up-regulation of iNOS activity.

Conclusions: In conclusion, AGNP had good immunoregulatory effects supporting the traditional claims and may provide a valuable therapeutic strategy to promoting immune function and metabolism.

Key words: *Panax quinquefolium*, Novel protein, Macrophages,

Immunostimulation

1. Introduction

Ginseng is one of most precious, popular, and renowned herbs used in traditional Chinese medicine. Ginseng has been used for thousands of years for its supposed healing properties (Attele et al., 1999; Wang and Yuan, 2008). The two commonly acknowledged species are Asian ginseng (*Panax ginseng* C. A. Meer) and American ginseng (*Panax quinquefolius* L.). Compared with the long history of use and extensive research on Asian ginseng, the study of American ginseng and its constituents has been much less extensive. American ginseng is planted mainly in Canada and the Eastern United States, and transplanted to northeast China as a medicinal herb similar to Asian ginseng (hereafter 'ginseng').

American ginseng has been used as a traditional medicine to treat many diseases including tumors and diabetes. It has a wide range of pharmacological activities that have been used in numerous clinical applications. It is reported that American ginseng has beneficial effects on the cardiovascular system, immune system, endocrine system, and central nervous system (Qi, Wang and Yuan, 2011; Ru et al.,2015). In traditional Chinese medicine, American ginseng is used as a tonic to nourish vitality—this is equivalent to promoting immune function and metabolism in Western medicine.

American ginseng contains numerous active constituents: ginsenosides, polysaccharides, peptidoglycans, phenolic compounds, fatty acids, nitrogen-containing compounds, and essential oils (Qi, Wang and Yuan, 2011; Sun, 2011). It is widely accepted that ginsenosides are the crucial active ingredients in ginseng and American ginseng. These triterpene saponins have a four *trans*-ring rigid steroid skeleton. More than 40 ginsenosides have been identified and isolated from ginseng (Liu, 2012). Ninety-eight ginsenosides have been identified from American ginseng including naturally occurring compounds and those resulting from steaming or biotransformation.

Studies have demonstrated that American ginseng has anti-tumor, antioxidant, immunomodulatory, and hypoglycemic activities as well as neuroprotective effects (Ni et al., 2009; Hofseth and Wargovich, 2007; Jin et al., 2010; Nguyen et al., 2012; Lee et al., 2013; Yang et al.,2014). In recent decades, ginseng and American ginseng have been used in Asian countries, particularly Korea and China, as dietary supplements.

Ginsenosides from American ginseng have exhibited multiple pharmacologic actions on the central nervous, cardiovascular, endocrine, and immune systems (Qi, Wang and Yuan, 2011; Ru et al., 2015). Nevertheless, few studies have focused on the biologic activities of the other constituents of ginseng and American ginseng. However, it has been reported that proteins isolated from ginseng and American ginseng have

anti-fatigue, anti-fungal and anti-viral activities (Wang and Ng, 2000; Yoon et al., 2002; Lam and Ng, 2001; Moon et al., 2010, Qi et al., 2014).

Our research team previously found that ginseng proteins can enhance cell-mediated immunity and monocyte–macrophage function, and increase the weight of immune organs in mice. We also determined that these proteins may increase anti-hypoxia and anti-fatigue activities. Ginseng proteins have therapeutic effects on experimental hyperlipidemia in rats, and protect against radiation injury in mice.

In the present study, we isolated and purified a novel protein from American ginseng. We investigated its characteristics initially with high-pressure gel filtration chromatography (HPGFC) and matrix-assisted laser desorption ionization–time-of-flight–mass spectrometry (MALDI–TOF–MS). We focused on a novel protein isolated from *Panax quinquefolius* L. to investigate the possible immunoregulatory effects on stimulation of peritoneal macrophages (PMs) using an *in vitro* tissue culture system.

2. Materials and methods

2.1. Materials

2.1.1. Plant material and its extraction

The fresh roots of 4-year-old *Panax quinquefolius* L. were purchased from the Fusong city of Changbai Mountains on September 15,2015,

and homogenized with 0.05 M Tris-HCl buffer solution (pH 7.4，the solvent ratio is 10:1) for 24 h at 4°C and extracted twice before centrifugation. The supernatant was applied to a hollow fiber membrane and the concentrated solution was lyophilized to yield American ginseng water-soluble proteins (AGWSPs). The content of AGWSPs determined by the Bradford assay was 80% (*w/w*) and the yield of AGWSPs was 4.0%.

2.1.2. Chemicals and reagents

DEAE Sepharose FF, SP Sepharose FF and Sephacryl S-100 HR were purchased from Amersham Biosciences (Amersham, UK). Trifluoroacetic acid (high-performance liquid chromatography (HPLC) grade) was purchased from Tedia (Fairfield, OH, USA). Acetonitrile (HPLC grade) was purchased from Sigma–Aldrich (Saint Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Experimental animals

Mice were treated in compliance with the *Guiding Principles for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA) as approved by the Animal Ethics Committee of China. A total of 120 male ICR mice (20±2 g) were purchased from the Pharmacology Experimental Center of Jilin University (Jilin, China). Mice were allowed to acclimatize to their new surroundings for 4 days,

and housed at room temperature of 22±2°C with a 12-h light–dark cycle. Standard chow and water were provided *ad libitum*. Ten mice were used at each time. Thirty mice were used for each experiment.

2.3. Methods

2.3.1. Purification of the novel protein

2.3.1. 1. Ion exchange chromatography

AGWSPS were applied to a SP Sepharose column (10 × 100 mm) pre-equilibrated with acetate buffer (pH 4.5). The column was washed with acetate buffer (pH 4.5) to remove unbound proteins, and then eluted with 0.3 M NaCl at 1.0 mL/min with 5 column volume. Fractions were collected, concentrated, and dialyzed against 0.05 M Tris-HCl buffer (pH 7.4).

The sample was loaded onto a pre-equilibrated DEAE column (10 × 100 mm) and washed with Tris-HCl buffer (pH 7.4) to remove unbound proteins. The target protein was eluted with a gradient of 0.2 M NaCl at 1.0 mL/min with 5 column volume. Fractions were collected and monitored at 280 nm to isolate the crude novel protein of American ginseng roots.

2.3.1. 2. Gel filtration chromatography

Fractions that had been separated after ion exchange chromatography

were collected and purified further using gel filtration chromatography. Proteins were dissolved in 2 mL of 10 mM sodium phosphate buffer (pH 7.4) to pass through a Sephacryl S-100 HR column (10 × 600 mm), eluted with 10 mM sodium phosphate buffer (pH 7.4) at 1 mL/min with 10 column volume. A single peak was observed at 280 nm, and the target protein was gathered and then lyophilized.

2.3.2. Determination of the molecular weight of the novel protein

The novel protein isolated from American ginseng roots (AGNP) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12.0% separating gel (Bradford, 1976). The isolated AGNP (30-mg each) was loaded in each lane. Bands were visualized by staining with Coomassie brilliant blue R-250 when electrophoresis was complete. Protein contents of AGNP were determined by the Bradford method using bovine serum albumin as a standard.

2.3.3. HPGFC

The ultraviolet spectrum of AGNP was scanned at 200–800 nm. Samples were dissolved in sodium phosphate buffer, filtered (0.22 μm) and analyzed by HPLC (1220 series; Agilent Technologies, Santa Clara, CA, USA) with a TSK-GEL G2000 SWXL column (7.8 × 300 mm; Tosoh, Tokyo, Japan). The protein markers that we used were

thyroglobulin (molecular weight, 669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa).

2.3.4 Identification of AGNP using MALDI–TOF–MS

The molecular weight of AGNP was measured by MALDI–TOF–MS (Voyager-DE STR; Tosoh). An acid solution A was prepared with acetonitrile, ultrapure water and trifluoroacetic acid (200:100:3 (*v/v*)). Then, sinapic acid was placed in solution A to obtain solution B (100 mg/mL). Samples (1 μL) were dissolved in solution B (1 μL), and stirred continuously (to ensure a uniform mixture) to obtain solution C. One-microliter of the latter was dried at room temperature and then applied to MALDI–TOF–MS corrected using myohemoglobin and cytochrome C.

2.3.5. Composition of amino acids of AGNP

AGNP was hydrolyzed in HCl (6 mol/L) for 24 h at 110±1°C in a vacuum for overall analyses of amino-acid composition. For cysteine analyses, samples were hydrolyzed in HCl (6 mol/L) for 24 h at 110±1°C after peroxidation with formic acid:hydrogen peroxide (10:1). For tryptophan analyses, samples were hydrolyzed in methanesulfonic acid (4 mol/L), and then NaOH (4 mol/L) was added. Amino acids converted to

phenylisothiocyanate derivatives were analyzed by HPLC (1220 series; Agilent Technologies) with a Wondasil-C18 (4.6 × 150 mm, 5 μm) column (Pyo et al., 2011).

2.3.6. Determination of immunostimulatory activities in vitro

2.3.6.1. PM preparation

PMs were acquired in accordance with methods described previously (Li and Xu, 2011) with slight modification. Briefly, each male ICR mouse received an intraperitoneal injection of sterile thioglycollate medium (2 ml) for every 72 h. After peritoneal washing with 10 mL of serum-free RPMI 1640 medium, PMs were obtained by centrifugation (500 g, 5 min, 4°C). Then, PMs were cultured in complete RPMI 1640 medium in a six-well plate for 2 h at 37°C in an atmosphere of 5% CO_2. Then, the plate was washed with phosphate-buffered saline (0.01 M, pH 7.4) to remove adherent cells. Purity and viability of PMs was 90%. PMs were cultured at 37°C in a humidified atmosphere of 5% CO_2. Number and viability of PMs was evaluated microscopically by Trypan blue exclusion (Felice et al., 2009).

2.3.6.2. Assay for PM phagocytosis

The phagocytic ability of PMs was tested using uptake of neutral red

dye, as described previously (Weeks et al. 1987) with slight modification. Briefly, PMs were plated at 2×10^5 cells/well in a final volume of 100 μL in a 96-well plate. They were incubated in medium alone or medium containing different concentrations of AGNP. Lipopolysaccharide (LPS; 20, 50 μg/mL) was used as the positive control. PMs were cultured at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Then, 100 μL of 0.075% germfree neutral red solution was added and the mixture incubated for 1 h. Media were removed, the plate washed thrice with phosphate-buffered saline (pH 7.2–7.4), and 150 μL of cell lysis buffer (1% glacial acetic acid:ethanol = 1:1,100 μL/well) added. The solution was blended adequately and measured at 550 nm using an ELISA reader (Infinite 200 Pro; Tecan, Geneva, Switzerland). The absorbance represented the phagocytic ability of PMs. All tests were carried out in triplicate.

2.3.6.3. Assays to measure nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) activity

NO production was measured by testing the nitrite concentration in the supernatant of cultured PMs using a colorimetric assay with Griess reagent, as reported previously (Jung et al., 2008; Wang et al., 2009). PMs were placed in a 96-well plate (2×10^6 cells/mL) and allowed to adhere for 2 h at 37°C. PMs were left overnight for attachment and AGNP (2, 10, 50,

100 and 200 μg/mL) added to wells. PMs were incubated in medium alone and LPS (20, 50 μg/mL) and used as a blank and positive control, respectively. PMs were incubated in an atmosphere of 5% CO_2 for 24 h at 37°C. Then, supernatants (50 μL) were pipetted from the medium and mixed with an equal volume of Griess reagent. Absorbance was measured at 540 nm using an ELISA reader (Infinite 200 Pro; Tecan). Nitrite products were calculated with reference to a standard curve obtained with $NaNO_2$.

PMs were treated with AGNP or LPS as stated above and cell supernatants collected to estimate iNOS activity using a colorimetric method according to manufacturer instructions (Nanjing Mindit Biochemistry, Nanjing, China). iNOS activity was calculated according to manufacturer instructions. Additionally, 1 nmol NO generated every minute was defined as one activity unit (Yang et al., 2008).

2.3.6.4. Measurement of cytokine production

PMs were cultured at 2×10^6 cells/well for 24 h with the purified AGNP (2, 10, 50, 100 and 200 μg/mL). PMs were incubated with LPS (20, 50 μg/mL) as the positive control; the control group was treated with medium alone. Supernatants were collected, and production of tumor necrosis factor (TNF)-α and interleukin (IL)-6 detected using ELISA kits according to manufacturer (BD Biosciences, San Diego, CA, USA)

instructions. Absorbance was measured at 450 nm in an ELISA reader (Infinite 200 Pro; Tecan). Cytokine quantities in samples were calculated from standard curves of recombinant cytokines using a regression linear method.

2.3.7. Statistical analyses

Data are the mean ± standard deviation (SD) of three replicate experiments. Results were evaluated by one-way analysis of variance followed by the Duncan's multiple-range tests. $P<0.05$ was considered significant. Statistical analyses were undertaken using SPSS v20.0 (IBM, Armonk, NY, USA).

3. Results and discussion

3.1. Determination of the molecular weight and protein contents of AGNP

3.1.1. Determination of the molecular weight of AGNP by SDS–PAGE

The molecular weight of the subunit of AGNP was determined using SDS–PAGE. A single band with a molecular weight of ≈15000 was seen on a desalted sample (Fig. 1). The protein content of AGNP determined by the Bradford assay was 2.31 mg/mL.

3.1.2. HPGFC of AGNP

The wavelength of maximum absorption for AGNP was 280 nm (Fig. 2). The elution time of AGNP using HPGFC was 8.665 min, which corresponded to a molecular weight of 31,086 Da.

3.1.3. Identification of AGNP by MALDI–TOF–MS

AGNP showed two major peaks according to MALDI–TOF–MS: peak 1 at *m/z* 7,562.24 (double charge) and peak 2 at *m/z* 15,114.70 (single charge). A protonated molecular ion was suggested. The relative molecular mass of AGNP was identified as 15,114.70 ± 50 Da for ten scans (Fig. 3). Our results demonstrated that the molecular weight of AGNP was 15,114 Da based on electrophoresis and MS. HPGFC revealed the molecular weight of AGNP to be 31,086 Da. All of these results suggested that the purified AGNP was a homodimer.

3.2 . Amino-acid composition of AGNP

Amino-acid composition of AGNP is shown in Table 1. Glycine, threonine and leucine were the main constituents, but tyrosine and tryptophan were absent, in AGNP. Eight essential amino acids for humans were detected in AGNP.

3.3. AGNP enhanced the phagocytic activity of PMs

A characteristic feature of activated macrophages is an increase in

phagocytosis (Cheng et al., 2008). First we tested the phagocytic activity of AGNP-activated PMs. Effects of AGNP on PM phagocytosis are shown in Fig. 4. Each concentration of AGNP increased uptake of neutral red dye by PMs compared with control. AGNP increased PM phagocytosis significantly (100 μg/mL, $p<0.05$; 200 μg/ml, $p<0.01$), as did LPS (20 μg/mL, $p<0.01$; 50 μg/mL, $p<0.01$). These results suggest that AGNP can activate the innate immune response.

3.4. Effects of AGNP on NO production and iNOS activity

NO acts in numerous tissues to regulate a diverse range of physiologic processes. Therefore, NO can be used as a quantitative index of macrophage activation (Lorsbach et al., 1993). The stimulatory effect of AGNP on NO production in PMs is shown in Table 2. AGNP could increase NO production in PMs in a dose-dependent manner. Compared with the untreated group, the NO concentration was increased significantly by treatment with AGNP (50–200 μg/mL, $p<0.01$) (Table 2). In PMs, NO is synthesized by iNOS. To ascertain whether NO production was related to increased iNOS activity, we measured the iNOS activity of AGNP-induced PMs. At 100 and 200 μg/mL, AGNP had obvious effects on increases in iNOS activity (Table 2). Our results suggest that AGNP can improve the ability of PMs to release NO significantly. Additionally, iNOS activity was in accordance with NO production.

3.5. Effect of AGNP on cytokine secretion

Activated macrophages play key roles in innate and adaptive immune responses by secreting secondary compounds such as TNF-α. The latter has been considered to be an important molecule during regulation of the immune response against tumors (Martinez. et al., 2008). We investigated the effects of AGNP on expression of TNF-α and IL-6 from PMs (Fig. 5, 6). AGNP elicited strong expression of TNF-α and IL-6 at 50–200 μg/mL. TNF-α expression induced by 50 μg/mL of AGNP was higher ($p<0.05$) than that of control, and at 100 and 200 μg/mL was significantly higher ($p<0.01$) than that of control. IL-6 production induced by 200 μg/mL AGNP was 1.08-fold greater than that induced by 10 μg/mL LPS. IL-6 expression in AGNP-treated PMs was lower than that induced by 50 μg/mL LPS, but AGNP also increased IL-6 expression in PMs significantly compared with the negative control.

Macrophages participate in specific and non-specific immune reactions. Activated macrophages maintain homeostasis, protect the host against tumors and bacterial infection by phagocytosis, present antigens to lymphocytes, and secrete numerous cell factors to regulate the activity of other cells (Hume, 2006). Studies undertaken with macrophages may be good indicators of immune activity.

Phagocytosis of macrophages is an important barrier in innate

immunity. Macrophages are crucial antigen-presenting cells (Schepetkin & Quinn, 2006). During phagocytosis, macrophages digest pathogens and present the antigens of the pathogens to T-lymphocytes, and these actions represent regulation of adaptive immunity (Schepetkin & Quinn, 2006).

Cytokines are signaling molecules that control homeostasis of the organism by regulating the differentiation, proliferation, and apoptosis of cells, as well as defense functions (e.g., immune responses) (Zhang, & Dai, 2011). Activated macrophages can secrete cytokines such as TNF-α and IL-6, which have important roles in immune functions and inflammatory responses.

NO is a novel signaling molecule and a key mediator of signal transduction in the immune system. If macrophages are activated, the amount of NO produced by iNOS can be quantified. We studied iNOS activity in activated PMs using a colorimetric method. Our results showed that AGNP could improve the ability of PMs to release NO significantly. TNF-α produced by activated macrophages, T-lymphocytes, and natural killer cells has a wide variety of effects and is a key cytokine in immune and inflammatory reactions (Kouakou, et al., 2013; Zhao, et al., 2013). TNF-α plays an important part in host defense and induces expression of many other immunoregulatory and inflammatory mediators (Baugh and Bucala, 2001). We found that AGNP could increase TNF-α production in PMs considerably.

American ginseng has multiple pharmacological actions including antioxidant, anti-inflammatory, and immunostimulatory activities. Increasingly studies are reporting biological activities for the other constituents of American ginseng. Previous studies have reported that proteins isolated from ginseng and American ginseng have multiple biological activities, including anti-fatigue, antifungal and antiviral properties. In one of our previous studies, we found that ginseng proteins can enhance cell-mediated immunity and monocyte-macrophage function and increase the weight of immune organs in mice. Furthermore, we determined that ginseng proteins may increase anti-hypoxia and anti-fatigue activities. Additionally, we found that ginseng proteins have beneficial effects on murine models of hyperlipidemia. Finally, we observed that ginseng proteins have protective effects against radiation injury in mice.

In this study, we isolated a novel protein from American ginseng and investigated its characteristics initially with HPGFC and then MALDI-TOF-MS. We focused on the effect of AGNP on murine peritoneal macrophages to determine the immunomodulatory effects of this novel protein.

4. Conclusions

Our study reports for the first time the immunomodulatory effects of AGNP. AGNP can significantly increase PM phagocytosis, promote NO

production, and increase expression of TNF-α and IL-6. AGNP stimulated NO formation through up-regulation of iNOS activity in a dose-dependent manner. Further research should be carried out to investigate the methods of the chemical modification and enzyme engineering to retain the original biological activity, enhance the stability of the protein conformation after oral intake and pass through the gastrointestinal system. Based on our present report and traditional claims, AGNP might be developed as a potential agent for promoting immune function and metabolism.

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Fig.1. Determination of molecular weight of AGNP through SDS-PAGE. M represents molecular markers. Coomassie brilliant blue staining was used to stain protein moieties of AGNP. The numbers represent standard proteins used: 1) rabbit phosphorylase b (97kDa), 2) bovine serum albumin (BSA) (66kDa), 3) rabbit actin (43kDa), 4) bovine carbonic anhydrase (31kDa), 5) trypsin inhibitor (20kDa), 6) hen egg white lysozyme (14kDa).

Fig.2. The UV spectrum of AGNP.

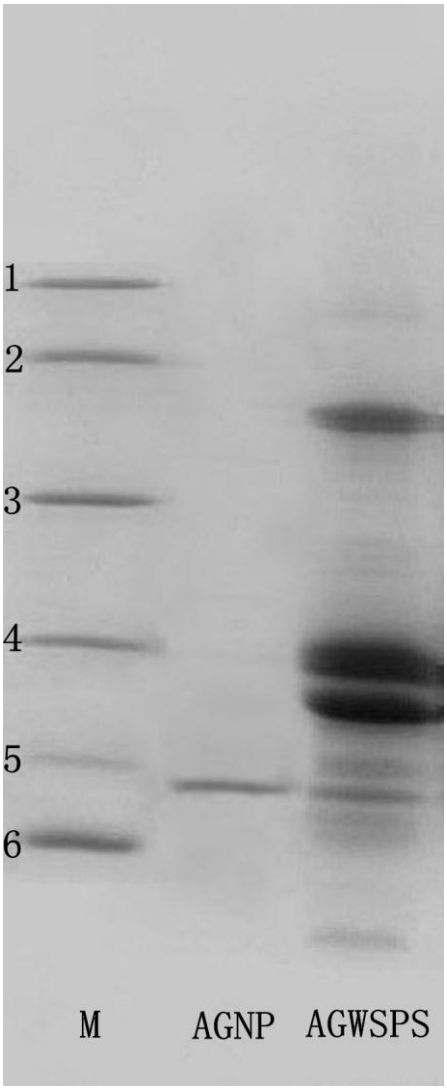
Fig.3. The mass spectrum for AGNP.

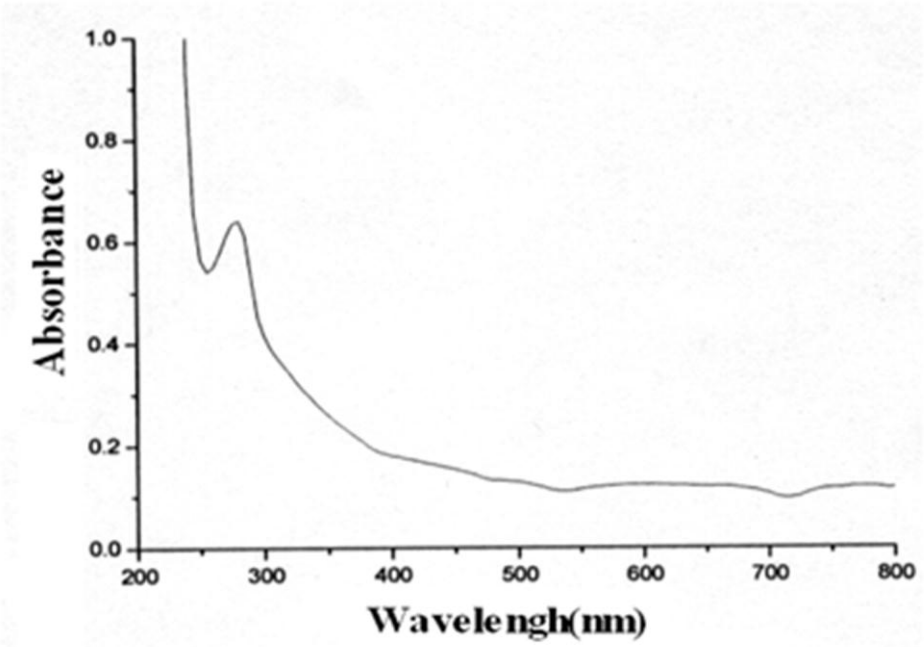
Fig.4. Effects of AGNP on phagocytosis of murine peritoneal

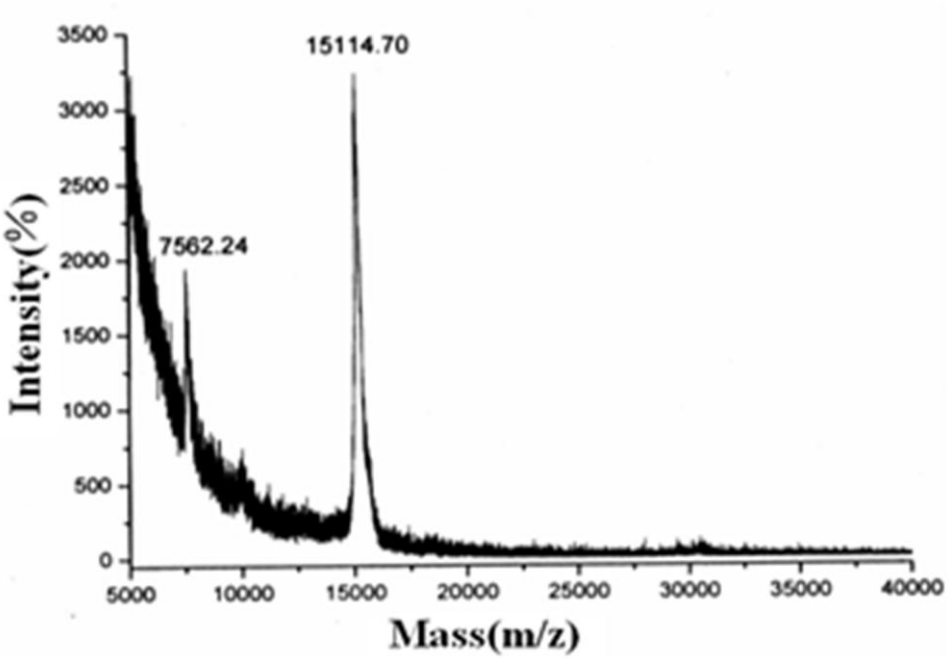
macrophages. Macrphages were treated with various concentrations of AGNP or LPS (20, 50 μg/mL). The peritoneal macrophages were cultured at 37℃ in humidified 5% CO_2 for 24 h, phagocytosis was measured as OD 550 nm. Values are means± S.D. for three independent experiments. * $p<0.05$ and ** $p<0.01$ compared with control groups.

Fig.5. Effects of different concentrations of AGNP on TNF-α production in macrophages. Peritoneal macrophages were cultured for 24h with the purified AGNP sample in different concentrations. Cells were incubated with LPS (20, 50 μg/mL) were used as a positive control, and the control group was treated 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 AGNP on IL-6 production in macrophages. Peritoneal macrophages were cultured for 24h with the purified AGNP sample in different concentrations. Cells were incubated with LPS (20, 50 μg/mL) were used as a positive control, and the control group was treated with medium alone. Values are mean ± SD (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.



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Intensity(%)
Mass(m/z)
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1500
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500
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15000
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30000
35000
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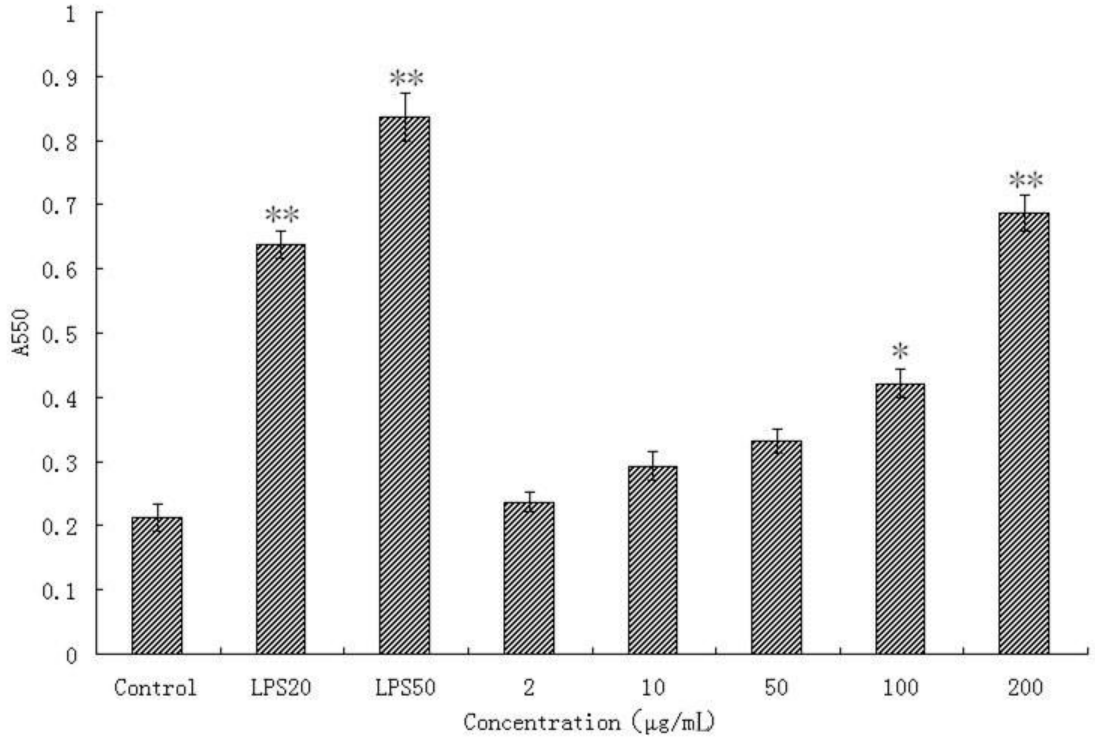
A550
Concentration (μg/mL)
Control
LPS20
LPS50
2
10
50
100
200
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**
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*
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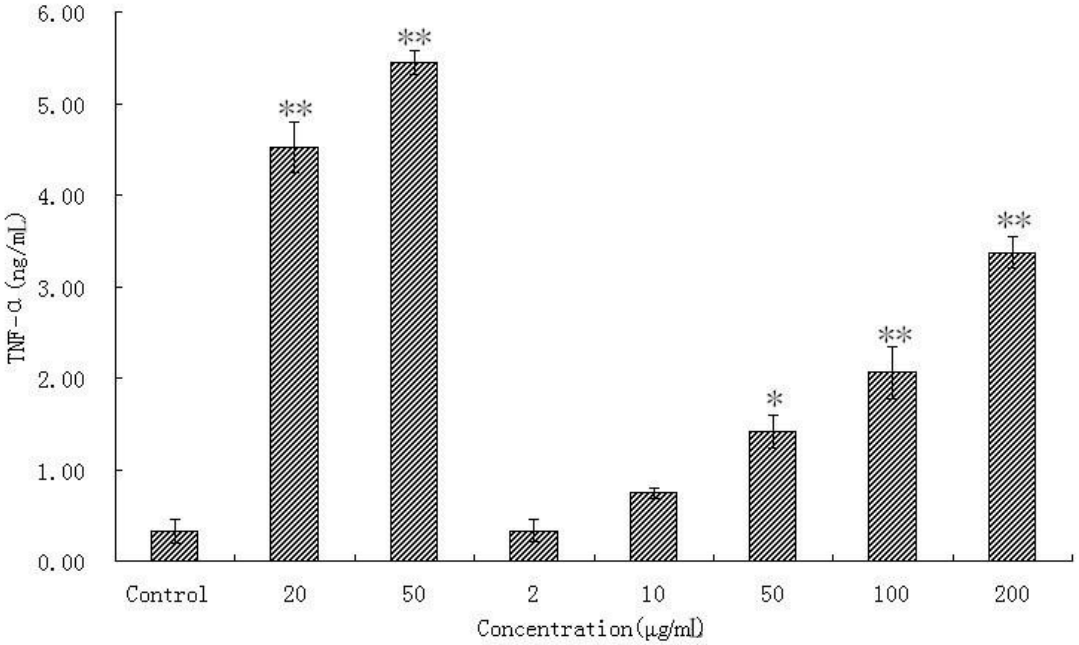
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0


6.00
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TNF-α (ng/ml)
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Control
20
50
2
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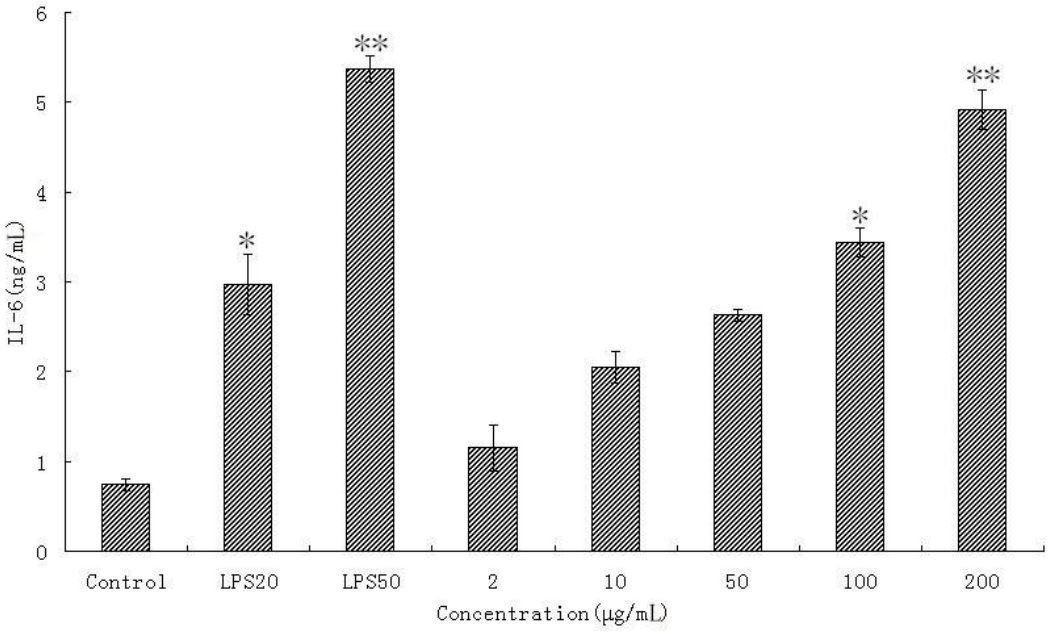
200
Concentration(μg/ml)


6
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4
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IL-6(ng/mL)
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Control
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LPS50
2
10
50
100
200
Concentration(μg/mL)

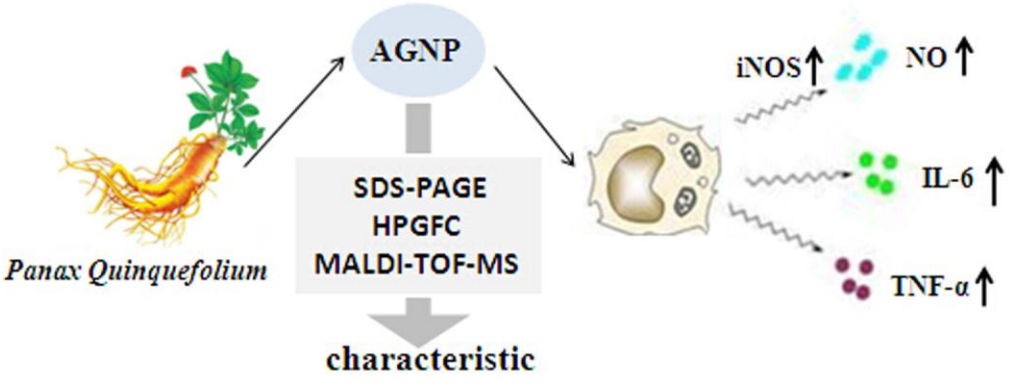

Table 1

Amino acid composition			
Amino acid	Amino acid composition (mg/g)		
	AGWSPS	AGCNP	AGNP
Asp	17.53	18.65	19.21
Glu	12.92	17.21	18.89
Ser	28.02	17.54	18.75
His	19.21	13.44	14.45
Gly	20.15	60.25	61.85
Cys	66.38	8.21	9.54
Thr	76.96	28.15	30.86
Arg	168.28	4.87	5.21
Ala	126.38	16.42	17.21
Pro	80.42	13.01	13.97
Tyr	14.48	0	0
Val	34.85	23.25	24.35

Met	10.25	2.03	2.42
Ile	57.35	11.32	12.00
Leu	5.92	27.65	28.94
Phe	35.21	6.15	7.23
Lys	73.48	14.02	15.12
Trp	4.02	0	0

Table 2

Effect of AGNP on NO production and iNOS activity in macrophages (values are means ± S.D.).

Group	Dose (μg/mL)	NO production（μmol/L)	iNOS activity (U/g prot)
Control LPS		3.30±0.96	1747.34±233.11
	20	61.52±1.37**	4947.91±105.62**
	50	70.22±2.13**	5447.91±205.62**
AGNP	2	10.33±1.37	1830.13±99.78
	10	21.44±0.95*	2084.51±112.30
	50	32.41±1.32**	2332.48±173.24
	100	38.06±2.48**	3158.40±118.31*
	200	45.10±1.47**	4454.47±215.32**

In vitro activation of peritoneal macrophages stimulated by different concentrations of the sample in terms of NO production and iNOS activity. The sample AGNP in the concentration range from 2μg/mL to 200μg/mL was used in the test.

* $p<0.05$ compared with control groups,

** $p<0.01$ compared with control groups.