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RESEARCH PAPER

Stimulation and Suppression of Innate Immune Function by American Ginseng Polysaccharides: Biological Relevance and Identification of Bioactives

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ABSTRACT

Background Polysaccharides constituting about 10% by weight of ginseng root are known to stimulate the immune system but have recently been shown to also suppress induced proinflammatory responses. Our study aims to determine whether American ginseng root polysaccharides (AGRPS) stimulates basal innate immune function and at the same time can suppress response to lipopolysaccharide (LPS) induced proinflammatory response. An *in vitro* mechanistic study was used to identify the bioactive fraction(s) responsible for AGRPS immunomodulatory effects.

Methods The *ex vivo* and *in vivo* immunomodulatory effects after oral administration of AGRPS extract was studied in adult rats by measuring cultured alveolar macrophage production of NO and changes of plasma cytokine level, modification of LPS proinflammatory immune response by AGRPS extract was also examined. To identify the bioactive fraction(s) responsible for AGRPS extract immunomodulatory effects, the immunobioactivities of the extract fractions (isolated by ion exchange and size exclusion chromatography) was investigated in an *in vitro* mechanistic study.

Results Culture of alveolar macrophages obtained from AGRPS extract treated rats resulted in an increase of *ex vivo* production of NO and also reduced alveolar macrophage responsiveness to *ex vivo* LPS challenge. Oral treatment with AGRPS extract elevated plasma TNF-α concentration *in vivo*. This treatment also suppressed LPS induced elevation of plasma TNF-α *in vivo*. AGRPS extract immunostimulatory and immunouppressive effects were mediated primarily by acid PS and its species with molecular weights ≥100 kDa and 50–100 kDa.

Conclusion : AGRPS extract exerted immunostimulation and suppressed LPS immune response under basal and LPS induced proinflammatory conditions respectively.

KEY WORDS ginseng polysaccharides · macrophage function · stimulation · suppression · bioactive fraction(s)

INTRODUCTION

The immune system consists of both innate and adaptive immunity components. Fast acting macrophage-mediated innate immunity is the first step in the recognition, destruction and removal of microbial pathogens in a host. Macrophages kill pathogens and cancer cells directly *via* phagocytosis and indirectly *via* the production of various proinflammatory mediators (*e.g.* nitric oxide [NO]) and cytokines (*e.g.* tumor necrosis factor-alpha [TNF-α]) [1–3]. During microbial infection caused by Gram negative bacteria, lipopolysaccharide (LPS) endotoxin stimulates innate macrophage cells by binding to their Toll-like receptor 4 (TLR-4), and this induces downstream intracellular events, activates kinases and transcription factors leading to production of proinflammatory mediators [4].

However, excessive macrophage stimulation by LPS during Gram negative bacteria infection generates uncontrolled production of proinflammatory mediators evoking harmful

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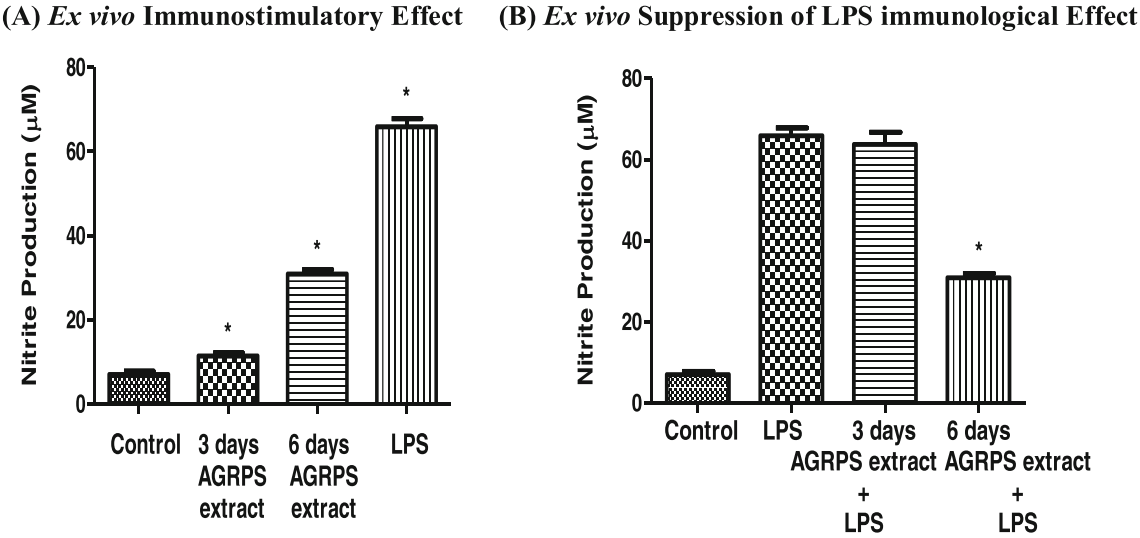


Fig. 1 Orally administered AGRPS extract: (**a**) elevated NO production and (**b**) reduced LPS-stimulated NO production *ex vivo* in cultured alveolar macrophages. Alveolar macrophages of rats ($n = 5$ per group) treated orally with saline (sham control) or 125 mg/kg AGRPS extract for 3 and 6 days were cultured for 24 h to measure production of NO (quantified as nitrite production). To determine responsiveness to LPS stimulation, alveolar macrophages of ginseng treated rats were exposed to 1 ug/ml LPS in culture to determine changes in 24 h NO production. The *ex vivo* alveolar macrophage NO production from each animal was determined in triplicate and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated control were statistically significant.

inflammatory response such as endotoxemia. Endotoxemia is characterized by fever, hypotension, myocardial dysfunction, acute respiratory failure, and multiple organ failure. Currently, antibiotics are used in the prophylaxis and therapy of endotoxemia [2,4–7]. The ability to detoxify or neutralize LPS immunotoxicity is a desired requirement for future bioactive compounds against endotoxemia [8,9]. Considering the problematic rise of microbial infections and their resistance to synthetic antimicrobial agents and naturally derived antibiotics, a key immunotherapeutic strategy to address this challenge will be to identify immunomodulatory agents which can interact with host immune response defense in such a way as to neutralize or inhibit LPS from triggering excess macrophage production of proinflammatory mediators [10]. The innate immunity (*e.g.* macrophage function) of a host which is responsive to LPS is also a known target for plant polysaccharides which are biological response modifiers that can up-regulate or down-regulate macrophage production of

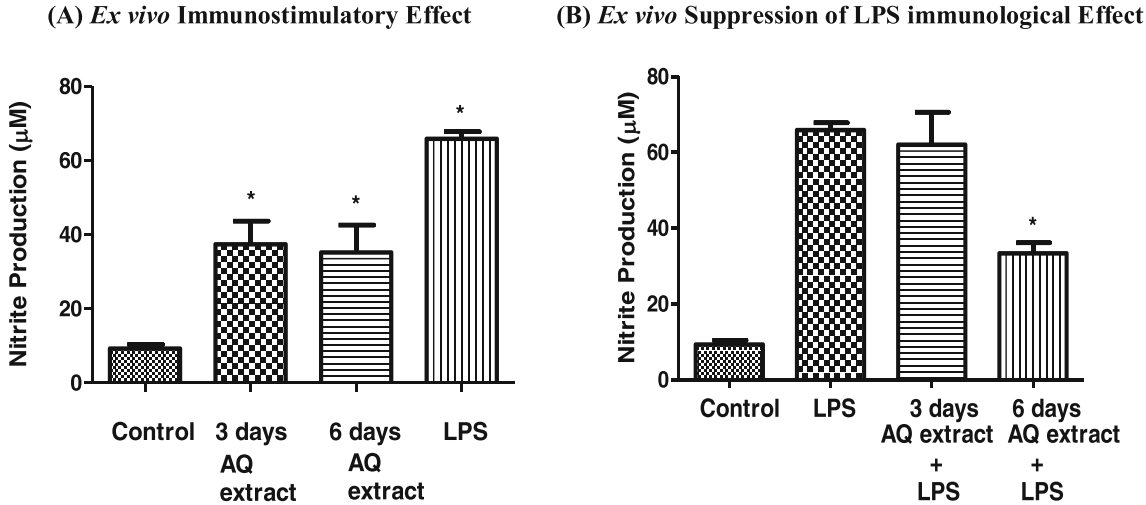


Fig. 2 Orally administered AQ extract: (**a**) elevated NO production and (**b**) reduced LPS-stimulated NO production *ex vivo* in cultured alveolar macrophages. Alveolar macrophages of rats ($n = 5$ per group) treated orally with saline (sham control) or 125 mg/kg AQ extract for 3 and 6 days were cultured for 24 h to measure production of NO (quantified as nitrite production). To determine responsiveness to LPS stimulation, alveolar macrophages of ginseng treated rats were exposed to 1 ug/ml LPS in culture to determine changes in 24 h NO production. The *ex vivo* alveolar macrophage NO production from each animal was determined in triplicate and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated control were statistically significant.

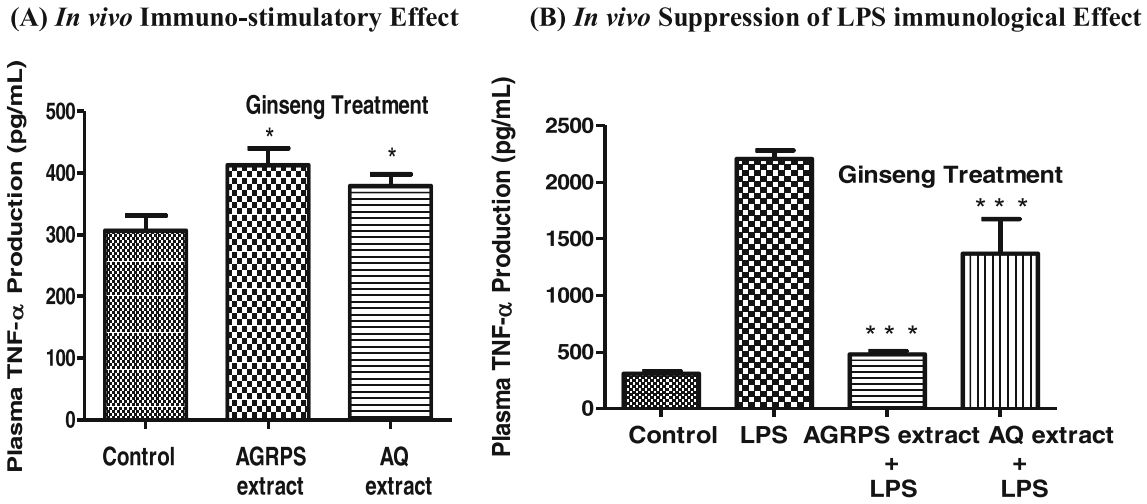
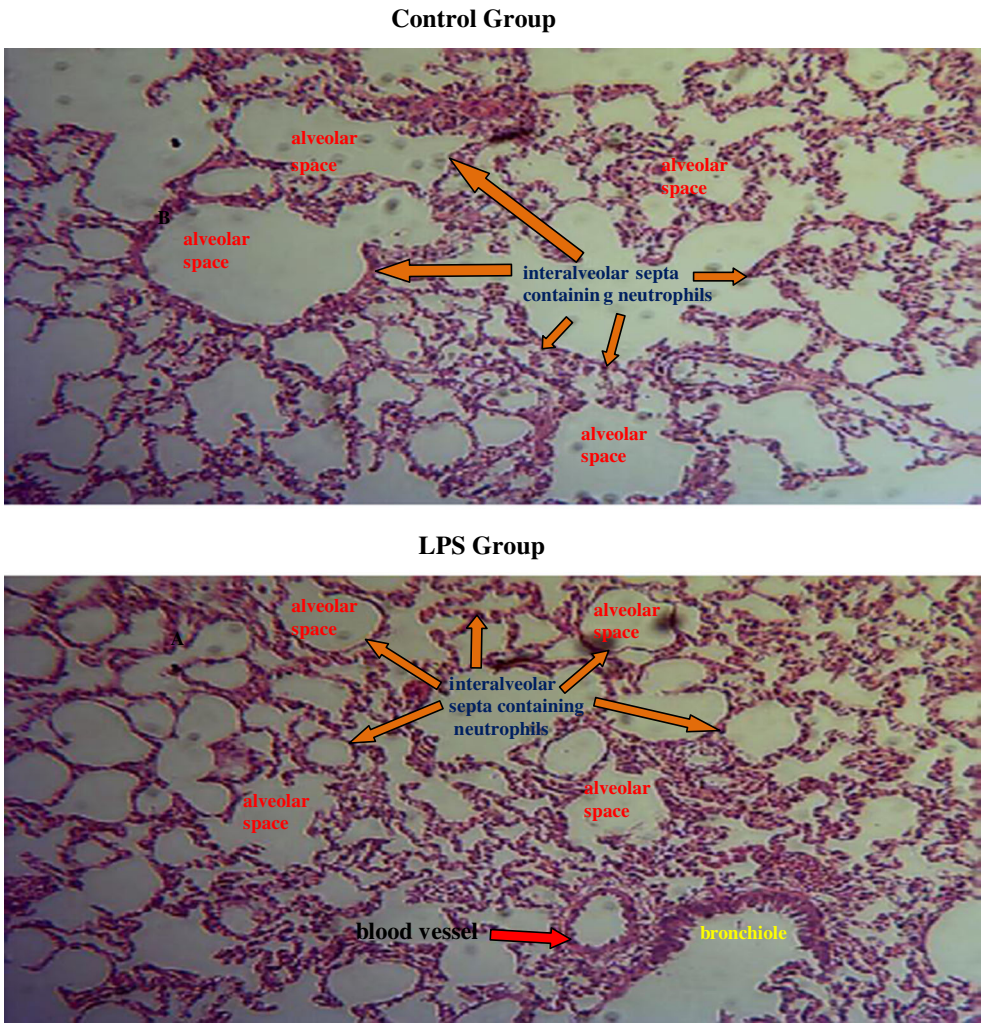


Fig. 3 Immunostimulatory and suppression of LPS induced immunological effects of AGRPS or AQ extract treatment *in vivo*. To evaluate imunostimulation, the rats ($n = 5$ per group) were treated orally with saline (sham control) or 125 mg/kg AGRPS extract or 125 mg/kg AQ extract for 6 days. To evaluate suppression of LPS, the 6 days AGRPS and AQ extract treated rats were challenged for 2 h with 5 mg/kg LPS (IP) injection 24 h after the last dose of saline or AGRPS extract or AQ extract. Plasma TNF-α cytokine concentrations were determined by ELISA. The plasma TNF-α level in each animal was determined in triplicate and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated control (immunostimulatory effect) were statistically significant. * **Values $P < 0.05$ compared to the LPS positive control.

Fig. 4 Lung histochemical study of Saline (Control) and LPS treated rats (magnification × 40). Each image is a representative of the rats ($n = 5$ per group) that received saline orally or 5 mg/kg LPS (IP) injection. Lungs were processed for histochemical study at the end of 2 h right after LPS challenge. The diagrams show the alveolar space surrounded by the interalveolar septa containing neutrophils are indicated by the arrows.



proinflammatory mediators [10,11]. Macrophage activation by plant polysaccharides is mediated *via* their binding to macrophage TLR-4 during initial phase of the immune response. Activation of this receptor leads to intracellular signaling cascades, resulting in transcriptional factor activation and the production of proinflammatory mediators which kills pathogenic microorganisms [10]. Interestingly, polysaccharides from medicinal plants such as *Tripterygium wilfordii* have been reported to suppress LPS induced macrophage stimulation [12]. The use of plant polysaccharides as a new approach for the prophylaxis of LPS mediated diseases like endotoxemia may be dependent on their ability to inhibit LPS signaling pathways, which will lead to the down-regulation of macrophage production of proinflammatory mediators.

Ginseng root is a multi-action herb, with ginsenosides and polysaccharides being the main bioactive components [13,14]. Previously, ginsenosides were considered to be responsible for most of ginseng's pharmacological effects; however, recent studies indicate that ginseng polysaccharides (PS) also possess a wide range of biological activities. These include immunomodulation, anti-tumor, anti-oxidation, anti-depression, and hypoglycemia [15–19]. Water soluble PS that can be extracted from ginseng root constitute about 10% of its dry weight. These hydrophillic macromolecules are formed from complex chains of monosaccharides, such as L-arabinose, D-galactose, L-rhamnose, D-galacturonic acid, D-glucuronic acid and D-galactosyl residues linked together through glycosidic bonds [20–22]. The monosaccharide composition of American ginseng root polysaccharides (AGRPS) used in this study have been reported to contain glucose, galactose, arabinose, rhamnose and galacturonic acid [23], which is similar to that described previously [21,24,25]. Ginseng PS are known to exist as either acidic or neutral forms, and are believed to be heterogeneous in nature, with molecular weights ranging from 3.5 to 2,000 kDa [16,21,22,24,26–28]. Ginseng root polysaccharides (GRPS) are generally known for their immunostimulatory effects [25,29–32], although recent studies suggests that they also suppress induced proinflammatory

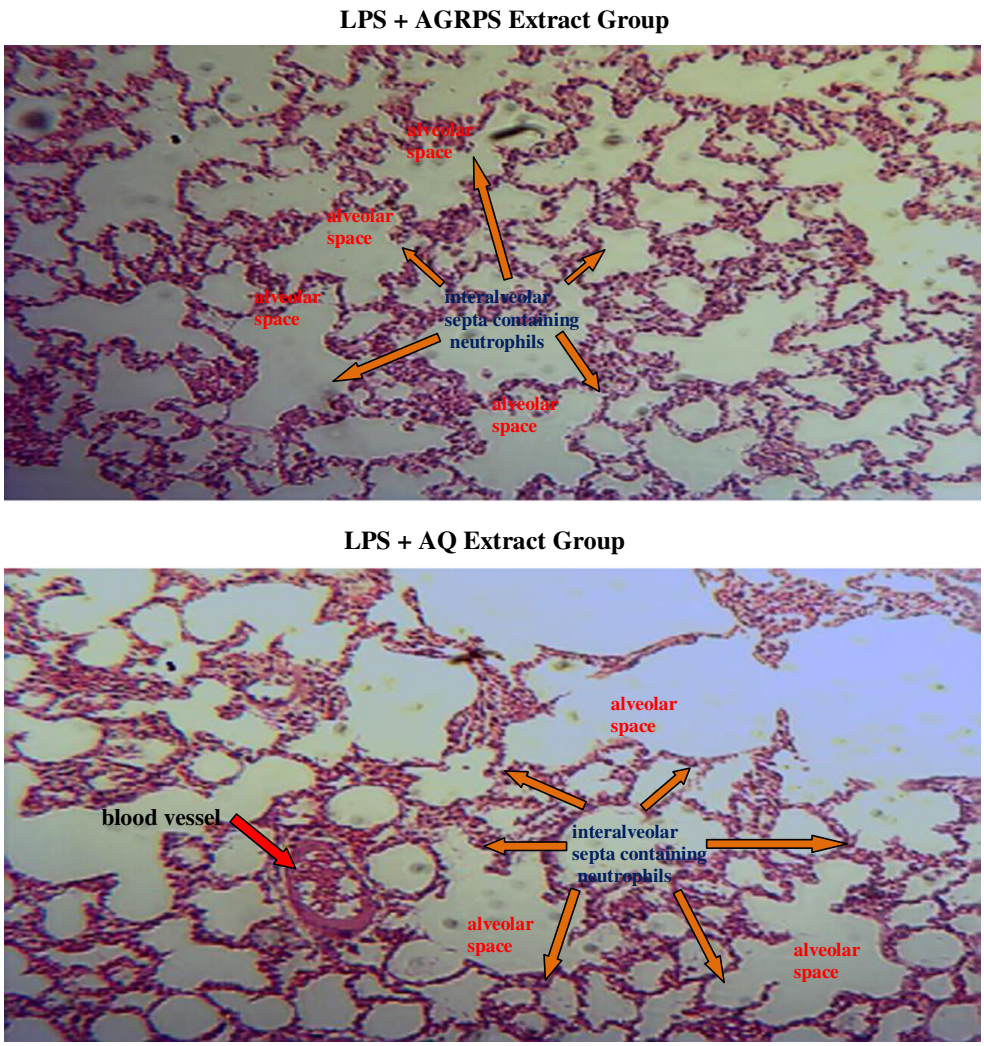


Fig. 5 Lung histochemical study of AGRPS and AQ extract treated rats after LPS Challenge (magnification × 40). Each image is a representative of the rats ($n = 5$ per group) that were orally administered with AGRPS or AQ extracts (125 mg/kg) prior to 5 mg/kg LPS (IP) injection. Lungs were processed for histochemical study at the end of 2 h right after LPS challenge. The diagrams show the alveolar space surrounded by the interalveolar septa containing neutrophils are indicated by the arrows.

responses. Zhao *et al.* (2011) reported GRPS inhibited immunological response associated with collagen-induced arthritis [33]. Intravenous treatment of mice with GRPS was reported to exert a protective effect against Staphylococcus aureus-infected septic mice by suppressing early acute inflammation [34,35]. One recent study has also revealed that intranasal and intravenous administered GRPS showed a protective effect on influenza viral infection by lowering levels of inflammatory cytokine (IL-6) and lung viral titers [36]. The examination of GRPS immunopharmacological effect under LPS induced proinflammatory condition will shed light if GRPS can exhibit a beneficial prophylactic effect against LPS mediated immune and inflammatory disease conditions like endotoxemia. It's possible that GRPS may inactivate signaling molecules which may account for its ability to suppress an induced proinflammatory immune response.

In view of the reported paradoxical immunomodulatory effects of ginseng polysaccharides, investigation was performed to determine the effects of AGRPS extract on basal immune function, and whether or not this treatment will also suppress LPS immunologic response *ex vivo* and *in vivo*. Physicochemical properties such as water solubility, ionic charge and molecular weight, are known to affect the biological activity of polysaccharides, including immunomodulation [37–39]. An *in vitro* mechanistic study was used to identify the bioactive fraction(s) responsible for AGRPS immunomodulatory effects. For the *in vitro* mechanistic study, AGRPS extract was separated into ionic charge (acidic and neutral) and molecular weight sub-fractions using ion exchange chromatography and ultrafiltration respectively. The different fractions of AGRPS extract was subsequently examined for their immunobioactivities.

MATERIALS

Ginseng test materials

Four-year-old American ginseng (*Panax quinquefolius*) roots collected in 2007 from five different farms in Ontario, Canada were provided by the Ontario Ginseng Growers Association [32].

Chemicals and biologicals

Superdex G-200 was purchased from GE Healthcare Bio-Sciences AB (Sweden). Amicon ultra-15 centrifugal filter units with molecular weight cut-off pore size of 100 KDa, 50 KDa, 30 KDa and 10 KDa were purchased from Millipore (USA). Cellu Sep H1® dialysis bags with a molecular weight cut-off pore size of 2 KDa were purchased from Membrane Filtration Products, Inc. (USA). The diethylaminoethyl (DEAE)-Cellulose

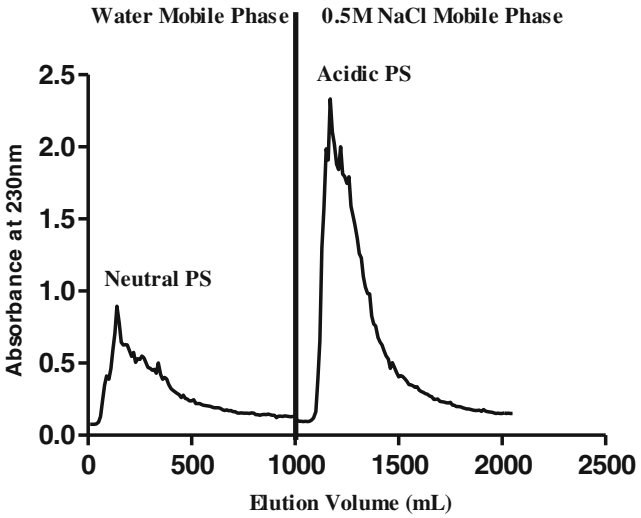


Fig. 6 DEAE-cellulose Ion exchange Column (40 × 2.5 cm) chromatographic fractionation of the AGRPS into neutral PS fraction and acidic PS fraction. The column was loaded with 3 g of AGRPS extract, and then eluted sequentially with 1,000 mL distilled water, followed by 1,000 mL 0.5 M NaCl at flow rate of 1 mL/min to obtain the neutral and acidic PS fractions respectively. The y-axis is the absorbance at 230 nm while the x-axis represents the elution volume (mL).

was purchased from Sigma (Oakville, Ontario). All other chemicals were of analytical grade and used as received. Cell culture medium and reagents were purchased from Gibco laboratories (USA). BD OptEIA ELISA kits tumour necrosis factor-α and interleukin-6 (BD Biosciences, USA). Purified LPS from Escherichia coli serotype 0111:B4 and Griess reagent were purchased from Sigma-Aldrich (USA). The RAW 264.7 (ATCC TIB 67) murine macrophage cell lines were provided by Dr. Jeff Dixon (Department of Physiology and Pharmacology, University of Western Ontario, Canada).

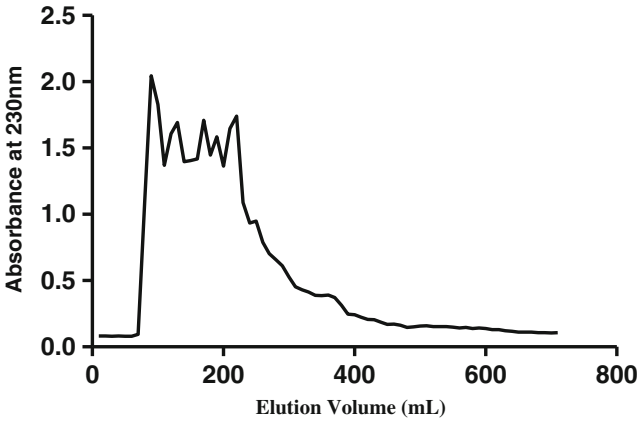
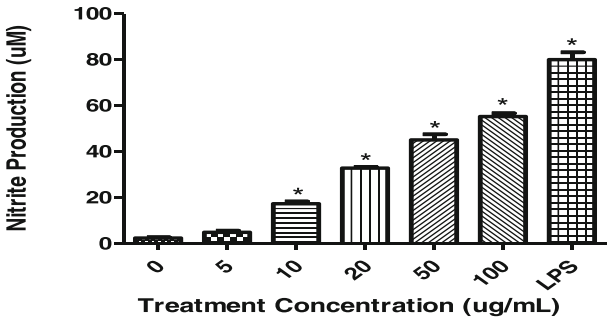


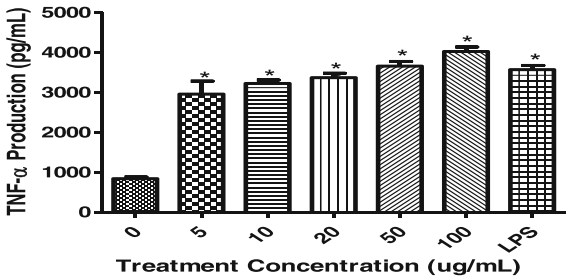
Fig. 7 Superdex G-200 size exclusion column (40 × 2.5 cm) chromatographic fractionation of acid PS. The column was loaded with 500 mg of acid PS, and then eluted with 700 mL distilled water at flow rate of 1 mL/min. The y-axis is the absorbance at 230 nm while the x-axis represents the elution volume (mL).

(A) NO Production

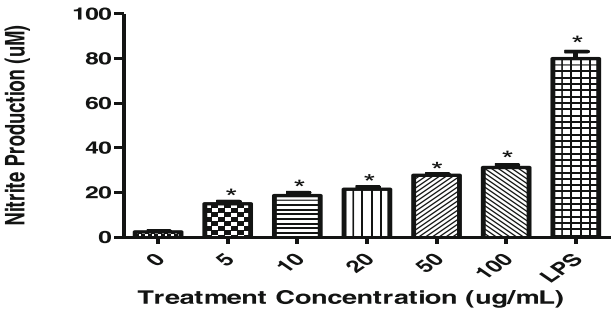
(B) TNF-α Production

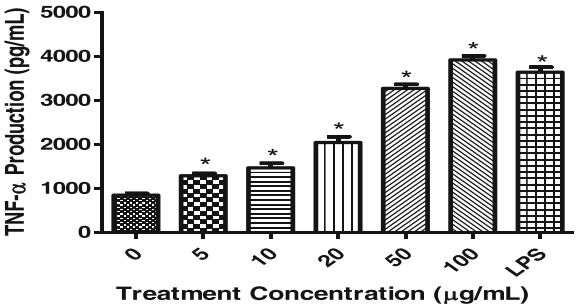
(i) AGRPS extract



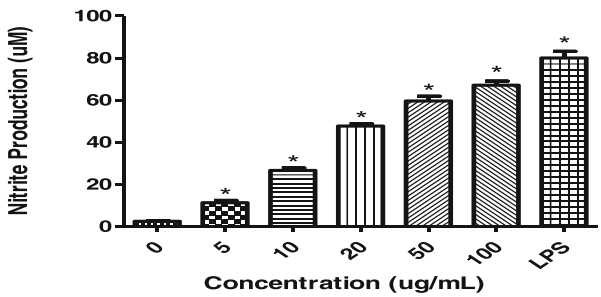


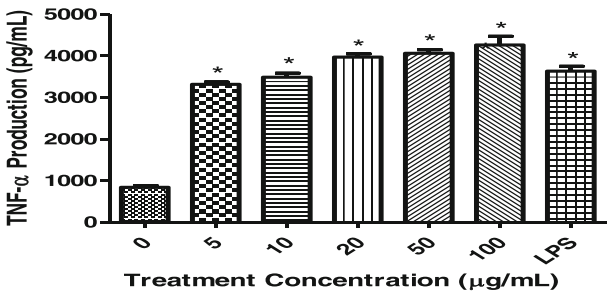
(ii) AQ extract



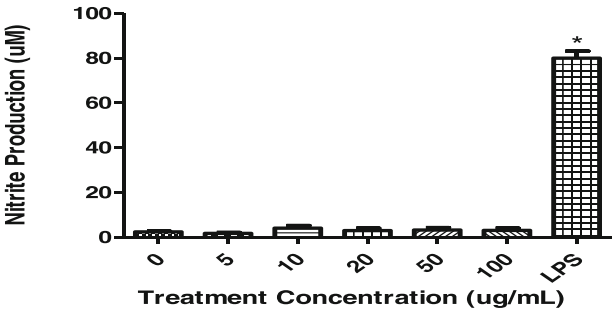


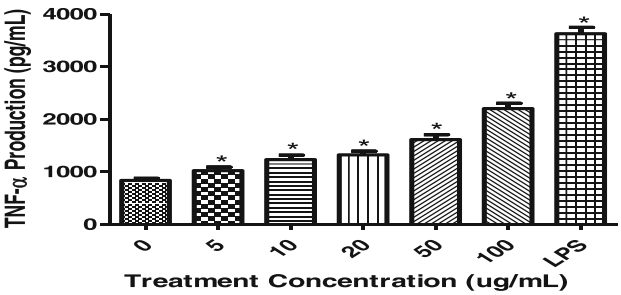
(iii) Acidic PS fraction





(iv) Neutral PS fraction





◀ **Fig. 8** Immunostimulatory effects of AGRPS and AQ extracts, acidic and neutral PS fractions on 24 h murine macrophage production of (A) NO and (B) TNF-α *in vitro*. Murine macrophages (RAW 264.7 cell line) were treated with 0, 5, 10, 20, 50, 100 μg/mL of AGRPS and AQ extracts, acidic and neutral PS fractions for 24 h. The culture supernatants were analyzed for NO and TNF-α by Griess reaction assay and ELISA, respectively. Cells treated with LPS (1 μg/mL) were used as positive controls. The experiments were performed in triplicate at three independent times and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated (vehicle) control were statistically significant.

Animals

Adult male Sprague–Dawley rats weighing 200–250 g (purchased from Charles River, St. Constant, QC, Canada) were used. The Animal Ethics Review Committee of the University of Western Ontario approved the study (Protocol No: 2009–070).

METHODS

Preparation of Aqueous and AGRPS Extracts

Dried ginseng root samples were shipped to Naturex (USA) for extraction. Samples were ground between ¼ and ½ inch and used to produce the aqueous (AQ) extract. Briefly, 4 kg ground ginseng roots were soaked three times during five hours in 16 L of water solution at 40°C. After extraction, the solution was filtered at room temperature. The excess solvent was then removed by a rotary evaporator under vacuum at 45°C. The three pools were combined and concentrated again until the total solids on a dry basis were around 60%. The Central Laboratory of Ontario Ginseng Research & Innovation Consortium (OGRIC) lyophilized these concentrates with a freeze dryer (Labconco, USA) at −50°C under reduced pressure to produce AQ ginseng extract in powder form. Yield of the powder extract from the concentrates was about 66%. The yield of the final extract (mean ± standard deviation of % extractive) from the initial ground root was 41.74 ± 4.92 for the AQ extract.

A solution of AQ extract in distilled water (1 g/10 mL) was prepared, and the crude AGRPS was precipitated by the addition of four volumes of 95% ethanol. The mixture was left for 1 h at room temperature for precipitation to occur. The crude AGRPS was collected by centrifugation at 350 × g (Beckman Model TJ-6, USA) for 10 mins and lyophilized to produce crude AGRPS fraction with a yield of about 10%. The crude AGRPS (10 g) was re-dissolved in 300 mL of water and partitioned five times with Sevag reagent (1:4 n-butanol:chloroform, *v*/*v*, 100 mL each) to remove proteins and produce AGRPS [40]. AGRPS was precipitated again by ethanol and lyophilized to produce a light brownish AGRPS extract which was used for the pharmacological and phytochemical studies.

EX VIVO AND IN VIVO PHARMACOLOGICAL EVALUATION

Ex vivo study to evaluate immunostimulation and suppression of LPS response

Adult male rats ($n = 5$ per group) were randomly divided into both a sham control and treatment groups. The treatment group received 125 mg/kg of AGRPS or AQ extract dissolved in saline by oral gavage once daily for 3 or 6 consecutive days. The sham control group received saline orally. Animals were anesthetized with intramuscular injection of ketamine 80 mg/kg and xylazine 5 mg/kg and the trachea was cannulated for lung BAL with Dulbecco's phosphate-buffered saline (PBS) at 37°C to collect alveolar macrophages which were then cultured without or with LPS for 24 h and maintained at 37°C in a humidified incubator with 5% CO2. The *ex vivo* alveolar macrophage NO production from each animal was determined in triplicates. All experiments were done only once.

In vivo study to evaluate immunostimulation and suppression of LPS response

The rats ($n = 5$ per group) were given a single daily dose of 125 mg/kg AGRPS or AQ extract in saline by oral gavage for 6 consecutive days. The sham control group ($n = 5$) received saline orally. Animals were anesthetized with intramuscular injection of ketamine 80 mg/kg and xylazine 5 mg/kg and blood was collected into heparinized tubes by intracardiac puncture, samples were then immediately centrifuged at 3,500 × g (Beckman Model TJ-6, USA) for 10 mins and the plasma was separated, aliquoted and stored at −20°C until use. Plasma TNF-α cytokine levels were measured as a marker for immunostimulation. To evaluate suppression of LPS induced immunological response, rats (10 treated with ginseng and 5 which received saline) were challenged with 5 mg/kg LPS intraperitoneal (IP) injection 24 h after the last dose of ginseng or saline. Animals were examined 2 h later based on studies revealing that systemic TNF-α cytokine production peaks 2 h after a LPS challenge [41–43]. The plasma TNF-α level in each animal was determined in triplicate. All experiments were done only once. This immunosuppression model was validated in past studies by other investigators to evaluate the immunomodulatory effects of orally administered pharmacological agents [44,45].

Quantification of NO and TNF-α

TNF-α in culture medium and plasma was measured by ELISA using rat cytokine-specific BD OptEIA enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, USA) according to the manufacturer's protocol. Nitrite accumulation in the culture media was measured as an estimate of NO production using Griess reagent (Sigma-Aldrich, USA) as previously described [32].

Lung Histochemical Study

Immediately after the collection of blood samples from the anesthetized rats used in the *in vivo* study, the lung samples of the right and left lobes from each rat were removed following animal sacrifice by thoracotomy. The lung samples (four from both lobes) were fixed with 10% formaldehyde solution at room temperature. The lung tissues were embedded in paraffin and cut into 5 mm sections and then stained with hematoxylin & eosin (H&E) by the London Laboratory Services Group of London Health Sciences Centre. Images of histopathologic changes in the lung tissues were observed under a light microscope.

Ion exchange Chromatography of AGRPS

AGRPS extract (3 g) was dissolved in water (200 mL) and loaded on a DEAE-Cellulose column (2.5×40 cm) pre-equilibrated with distilled water. The column was eluted first with 1.0 L of distilled water at a flow rate of 1 mL/min to obtain the unbound or neutral fraction and then with 1.0 L of 0.5 M sodium chloride (NaCl) to obtain the bound or acidic fraction. Absorbance of the eluates was monitored at 230 nm (wave length of maximum absorption for AGRPS determined from a UV scan) and 5 mL fractions were collected. The fractions were concentrated, dialyzed with Cellu Sep H1® dialysis bags (cut off pore size of 2 KDa) against water and freeze dried to give 0.9 g (30% yield) of the neutral PS fraction and 0.36 g (12% yield) of the acidic PS fraction.

Size Exclusion Chromatography *via* Superdex G-200 Fractionation of Acidic PS

Five hundred milligrams of acid PS was dissolved in 5 mL distilled water and then fractionated by lfcading onto a Superdex G-200 column (40×2.5 cm) equilibrated and eluted with distilled water mobile phase at 4°C with a flow rate of 1 mL/min. Absorbance of the eluates was monitored at 230 nm and 5 mL fractions were collected.

Size Exclusion Chromatography *via* Ultrafiltration Fractionation of Acidic PS

Acidic PS (20 mg) was dissolved in 20 mL of water and 15 mL was placed in a 100 kDa amicon ultra-15 centrifugal filter unit (15 mL capacity) and centrifuged at 3,500×g (Beckman Model TJ-6, USA) for 30 mins. The fraction with molecular weight of 100 kDa and above (≥100 kDa) were retained on the filter unit, while the fraction of less than 100 kDa in the filtrate obtained from this process was then placed in a 50 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. The fraction with molecular weight of less than 100 and above 50 kDa (50–100 kDa) were retained on the filter unit, while the fraction of less than 50 kDa in the filtrate obtained from this process was then placed in a 30 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. The fraction with molecular weight of less than 50 and above 30 kDa (30–50 kDa) were retained on the filter unit, while the fraction of less than 30 kDa in the filtrate obtained from the above mentioned process was then placed in a 10 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. The fraction with molecular weight of less than 30 and above 10 kDa (10–30 kDa) was retained on the filter unit. Ten mL of distilled water was used to wash and remove the acidic PS fractions with different molecular weights ≥100 kDa, 50–100 kDa, 30–50 kDa, 10–30 kDa retained on the filter units after which they were lyophilized at −50°C under reduced pressure (Labconco, USA).

In vitro Pharmacological Evaluation

Cell culture

Mouse macrophage cell line RAW 264.7 (grown to 60-80% confluence, and subjected to no more than 20 cell passages from Dr. Jeff Dixon) was cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum, 25 mM HEPES, 2 mM Glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were seeded in 96-well tissue culture plates at a density of 2×105 cells per well and maintained at 37°C in a humidified incubator with 5% CO2. To collect rat alveolar macrophages animals were anesthetized with intramuscular injection of ketamine 80 mg/kg and xylazine 5 mg/kg and the trachea was cannulated for lung bronchoalveolar lavage (BAL) using 10-mL syringe with three 10 ml washes of PBS at 37°C. Fluid recovered from BAL was then centrifuged at 1,000 rpm for 5 mins. Cells were immediately cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 25 mM HEPES, 2 mM Glutamine, 100 IU/mL penicillin and 100 µg/ml streptomycin in 96-well tissue culture plates, at a density of 2×105 cells per well at 37°C maintained in a humidified incubator with 5% CO2.

Cell treatment

Immunostimulatory effect

Macrophages were treated with 0, 5, 10, 20, 50, and 100 μg/mL (RAW 264.7 cell line) or 0, 50, 100 or 200 μg/mL (rat alveolar macrophages) of AGRPS and AQ extracts, acidic and neutral PS fractions for 24 h, maintained at 37°C in a humidified incubator with 5% CO2. Aliquots of culture medium were collected and frozen at −20°C until ready for NO and TNF-α analysis. The experiments were performed in triplicate on three independent occasions.

Indirect Suppression of LPS immunological response

To examine the indirect inhibitory effect on LPS stimulation *in vitro*, macrophages were pretreated with 0, 5, 10, 20, 50 and 100 μg/mL (RAW 264.7 cell line) or 0, 50, 100 or 200 μg/mL

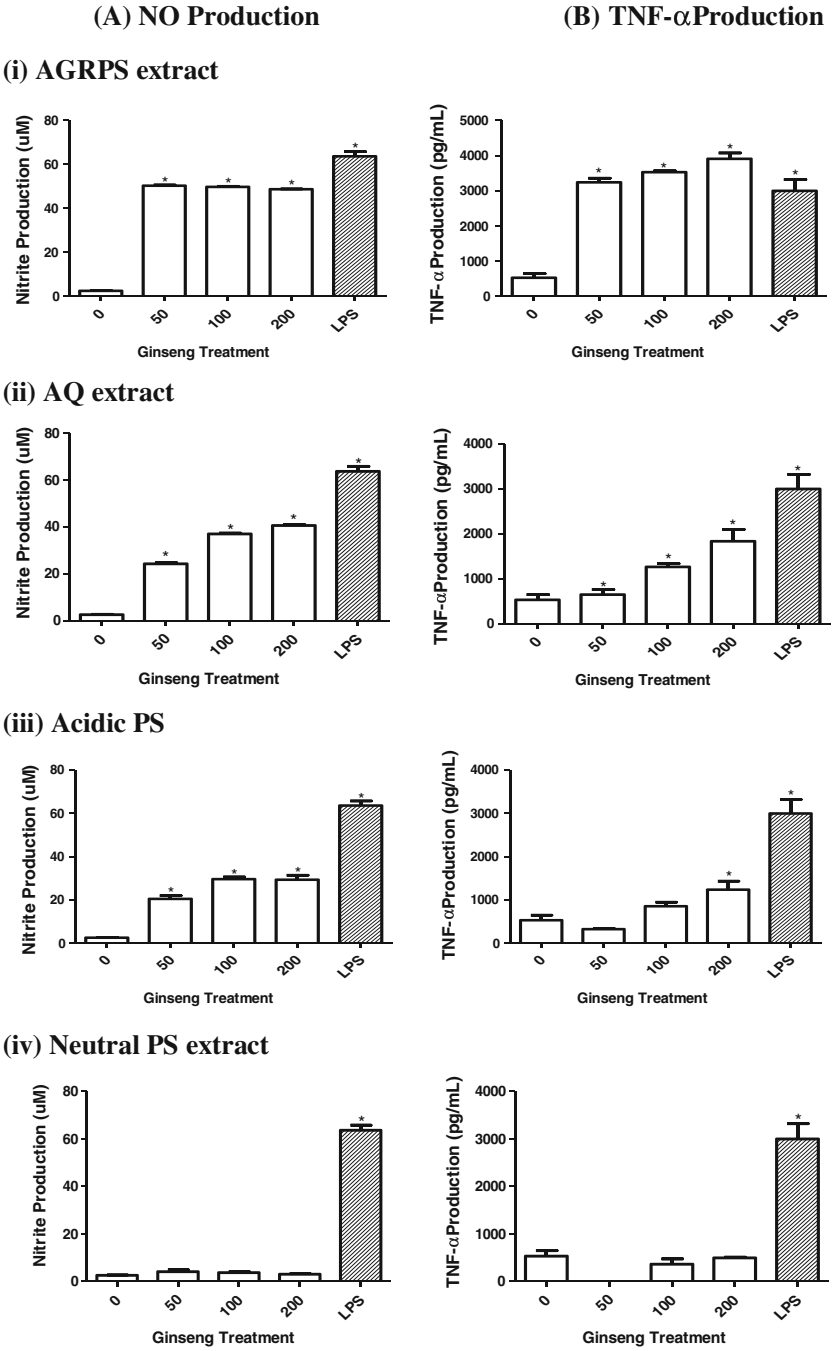


Fig. 9 Immunostimulatory effects of AGRPS and AQ extracts, acidic and neutral PS fractions on 24 h rat alveolar macrophage production of (**a**) NO (nitrite) and (**b**) TNF-α *in vitro*. Rat alveolar macrophages were treated with 0, 5, 10, 20, 50, 100 μg/mL of AGRPS and AQ extracts, acidic and neutral PS fractions for 24 h. The culture supernatants were analyzed for NO and TNF-α by Griess reaction assay and ELISA, respectively. Cells treated with LPS (1 μg/mL) were used as positive controls. The experiments were performed in triplicates at three independent times and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated (*vehicle*) control were statistically significant.

(rat alveolar macrophages) of AGRPS and AQ extracts, acidic and neutral PS fractions for 24 h and maintained at 37°C in a humidified incubator with 5% CO2. Thereafter macrophages were washed twice with culture medium and then treated with 1 μg/mL of LPS to determine the 24 h cytokine response. The experiments were performed in triplicates on three independent occasions.

STATISTICAL ANALYSIS

All statistical analyses were performed with GraphPad prism 4.0a Software (GraphPad Software Inc., USA). Data were presented as the mean ± standard deviation (SD) for all experiments. Data sets were evaluated by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. $P<0.05$ was considered to be statistically significant.

RESULTS

Ex vivo Immunomodulatory Effects of AGRPS and AQ extracts

Oral treatment of rats with AGRPS and AQ extracts for 3 and 6 days produced stimulation of 24 h *ex vivo* cultured alveolar macrophages (Figs. 1a and 2a) which are higher than sham control as determined by increased production of nitrite, a marker for NO. Only the 6 days AGRPS extract treatment

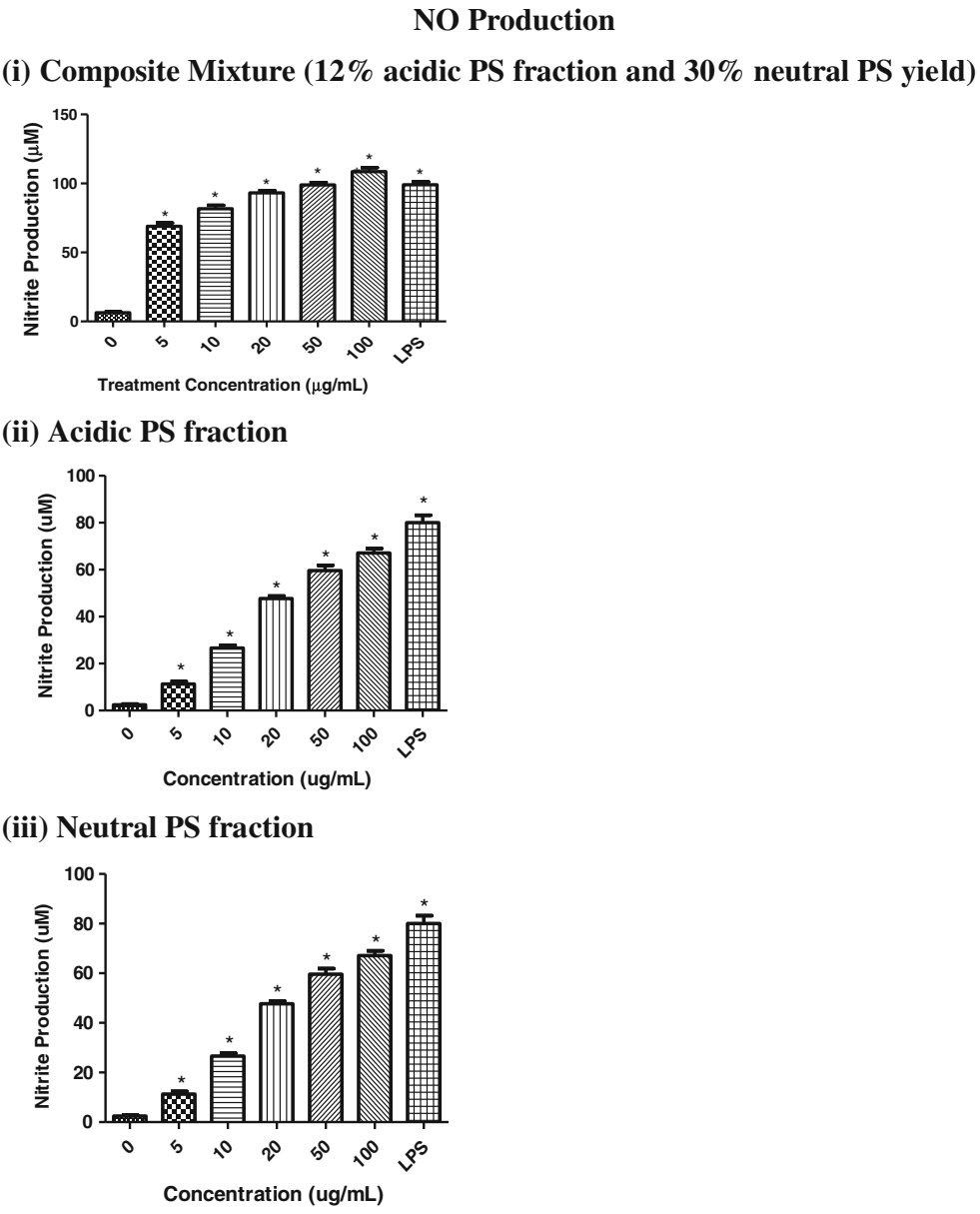


Fig. 10 Comparison of composite mixture (12% acidic PS fraction and 30% neutral PS yield), acidic PS, neutral PS fractions immunostimulatory effects on 24 h macrophage production of NO (nitrite) *in vitro*. Murine macrophages (RAW 264.7 cells) were treated with 0, 5, 10, 20, 50, 100 μg/mL of composite mixture, acidic PS, neutral PS fractions for 24 h. The culture supernatants were analyzed for NO by Griess reaction assay, respectively. Cells treated with LPS (1 μg/mL) were used as positive controls. The experiments were performed in triplicates at three independent times and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P<0.05$ compared to the untreated (*vehicle*) control were statistically significant.

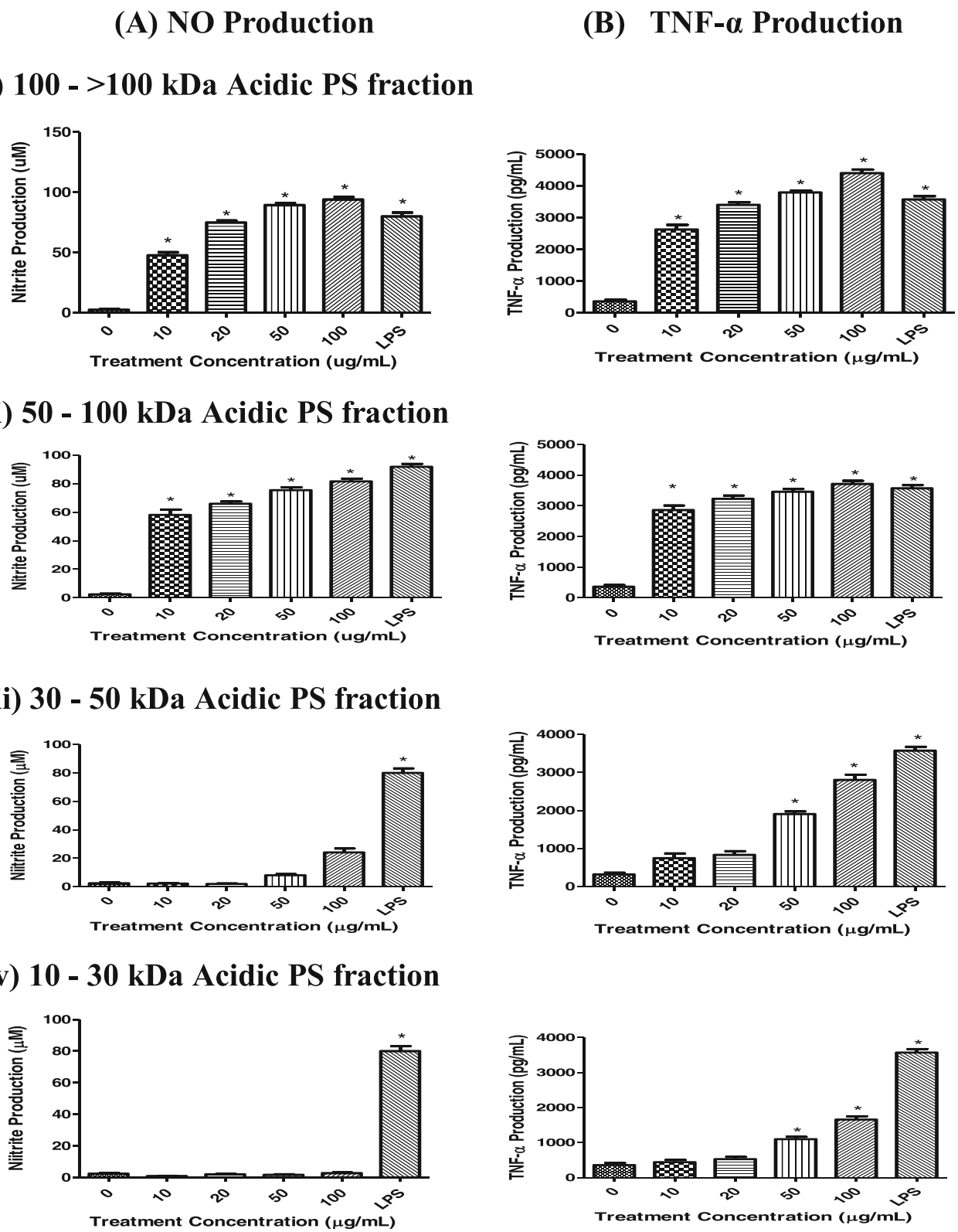
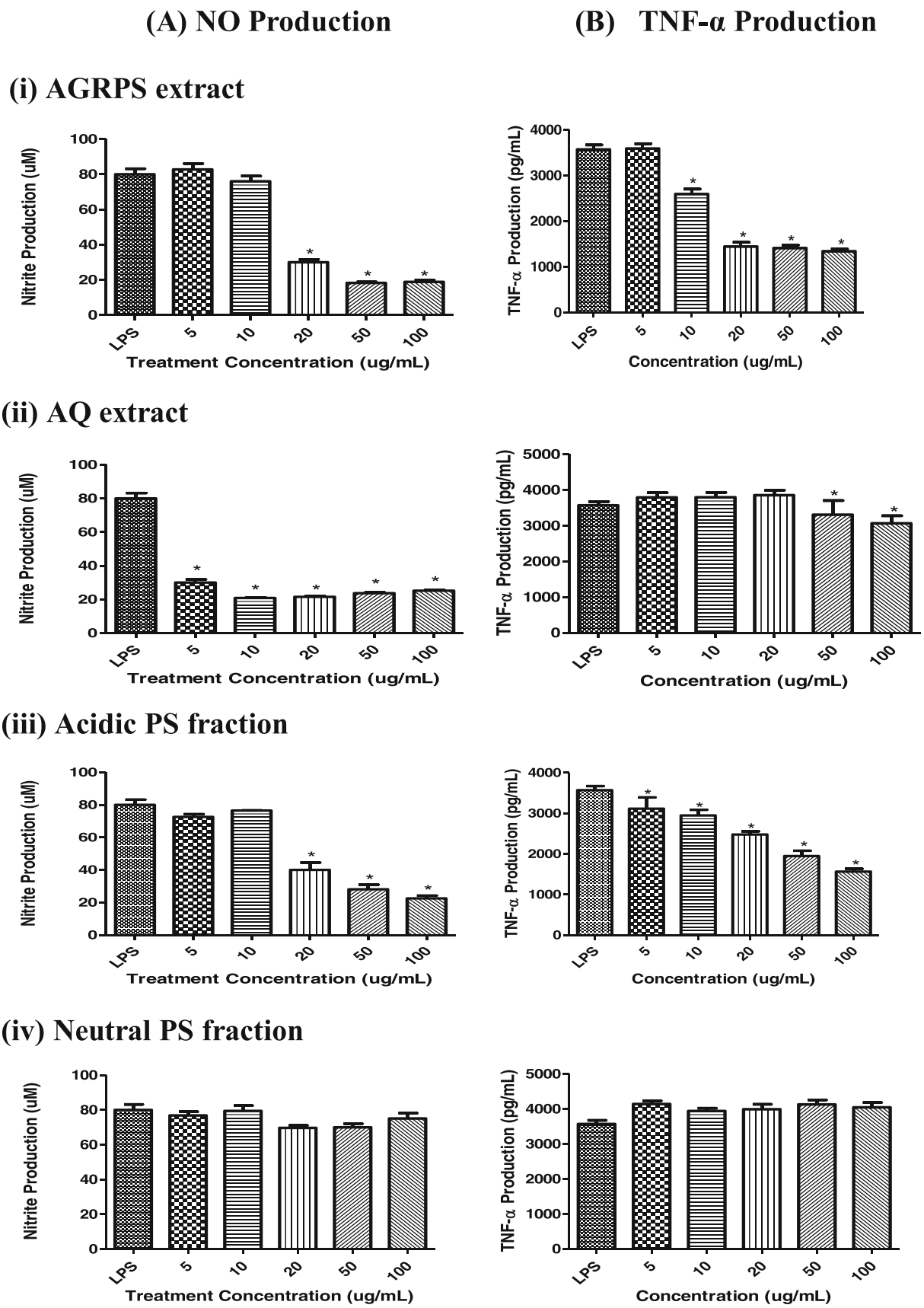


Fig. 11 Immunostimulatory effects of acidic PS fractions 100 - >100 kDa, 50–100 kDa, 30–50 kDa, 10–30 kDa on 24 h macrophage production of NO *in vitro*. Murine macrophages (RAW 264.7 cells) were treated with 0, 10, 20, 50, 100 μg/mL of acidic PS fractions 100 - >100 kDa, 50–100 kDa, 30–50 kDa, 10–30 kDa for 24 h. The culture supernatants were analyzed for NO (nitrite) by Griess reaction assay. Cells treated with LPS (1 μg/mL) were used as positive controls. The experiments were performed in triplicates at three independent times and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated (vehicle) control were statistically significant.


(A) NO Production
(B) TNF-α Production
(i) AGRPS extract
(ii) AQ extract
(iii) Acidic PS fraction
(iv) Neutral PS fraction
Nitrite Production (uM)
TNF-α Production (pg/mL)
LPS
5
10
20
50
100
Treatment Concentration (ug/mL)
Concentration (ug/mL)

◀ **Fig. 11** Immunostimulatory effects of acidic PS fractions 100 - >100 kDa, 50–100 kDa, 30–50 kDa, 10–30 kDa on 24 h macrophage production of NO *in vitro*. Murine macrophages (RAW 264.7 cells) were treated with 0, 10, 20, 50, 100 μg/mL of acidic PS fractions 100 - >100 kDa, 50–100 kDa, 30–50 kDa, 10–30 kDa for 24 h. The culture supernatants were analyzed for NO (nitrite) by Griess reaction assay. Cells treated with LPS (1 μg/mL) were used as positive controls. The experiments were performed in triplicates at three independent times and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated (vehicle) control were statistically significant.

production of NO reached 50% of the LPS positive control (Fig. 1a), while similar magnitude of response was observed in both the 3 and 6 days AQ extract treatments (Fig. 2a). The 6 days treatment of AGRPS extract gave two times higher NO production compared to that of the 3 days AGRPS extract treatment.

The responsiveness of macrophages collected from the 6 days AGRPS and AQ extract treated animals to LPS stimulation *ex vivo* showed about 50% reduction in NO production as compared to the non-ginseng treated controls (Figs. 1b and 2b). The 3 days AGRPS and AQ extract treatments did not suppress LPS immunological response.

Macrophage stimulation is not a requirement to induce suppression of LPS since the 3 days AGRPS and AQ extract treatments was found to exert immunostimulation but did not suppress the LPS response as observed in the 6 days treatment. The 6 days AGRPS and AQ extract treatments exerted both immunostimulation and loss of LPS responsiveness. These data show that orally administered AGRPS and AQ extract has both immunostimulatory and suppression of LPS induced immunological effects.

In vivo Immunomodulatory Effect of AGRPS and AQ extracts

In light of the observed AGRPS and AQ extracts *ex vivo* effects on stimulation and suppression of LPS induced stimulation of alveolar macrophage immune function, additional studies were carried out *in vivo* to determine the pharmacological relevance in whole animal response. The 6 days treatment with AGRPS and AQ extracts elevated plasma TNF-α levels compared to the untreated control (Fig. 3a), suggesting AGRPS extract was more effective. The observed *in vivo* immunostimulatory effects of AGRPS and AQ extracts were very small when compared with that of 5 mg/kg LPS. The LPS treatment resulted in marked elevation of plasma TNF, while pretreatment with AGRPS and AQ extracts suppressed LPS induced plasma TNF-α level by 78% and 38% respectively (Fig. 3b).

To determine whether the suppression of LPS-induced plasma TNF-α level by ginseng extracts has any impact on organ dysfunction in LPS-treated animals, lung histology was evaluated. As shown in Fig. 4, 2 h LPS challenge (5 mg/kg) did not cause lung morphological and histopathological damages as demonstrated by the absence of neutrophils infiltration from the interalveolar septa into the alveolar space and the absence of alveolar hemorrhage. The 6 days oral AGRPS and AQ extract pretreatments prior to LPS exposure have similar morphology as the LPS challenge and sham control (Fig. 5). There is dissociation between the changes of plasma TNF-α cytokine production and the lung response (Fig. 6).

Mechanistic Identification of immunobioactive components of AGRPS

To identify the immunobioactive fractions of AGPS extract, the extract was fractionated into acidic and neutral species by ion exchange chromatography, which was then fractionated further according to molecular size by size exclusion chromatography *via* Superdex G-200 fractionation and ultrafiltration fractionation.

Ion Exchange Chromatography of AGRPS

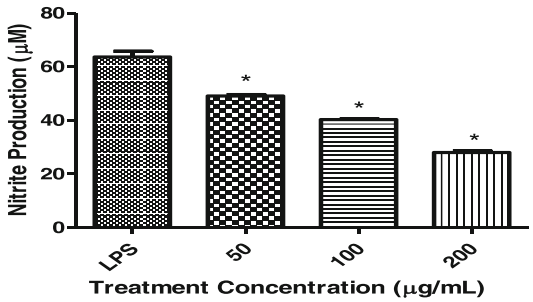
Ion exchange chromatography was used to separate AGRPS extract into both acidic (12.0%) and neutral fractions (30.0%). The chromatogram from the ion exchange chromatographic procedure showed the presence of two peaks. The first peak which eluted with the distilled water mobile phase corresponds to the neutral fraction, while the second peak which eluted with the 0.5 M NaCl mobile phase corresponds to the acidic fraction. After lyophilization, the resulting neutral fraction was a white cottony substance while the acidic fraction was a light brownish substance similar to AGRPS extract. About 58.0% of the AGRPS materials were not recovered from the chromatographic procedure.

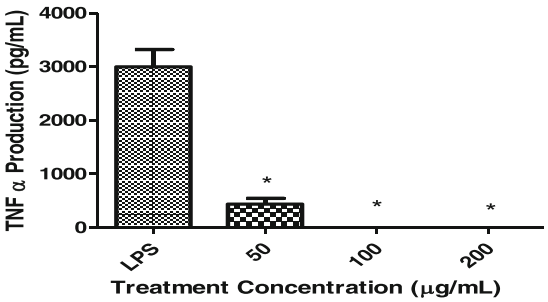
Size Exclusion Chromatography of Acidic PS

To perform activity guided fractionation of acidic PS, size exclusion chromatography with the aid of a Superdex G-200 column was then used as an analytical technique to examine in more detail the acid PS component. As shown in Fig. 7, the acidic PS constitutes phytochemicals of different molecular weights with peaks which were poorly separated by the Superdex G-200 column chromatographic technique. Ultrafiltration with molecular weight cut-off of 100 kDa, 50 kDa, 30 kDa

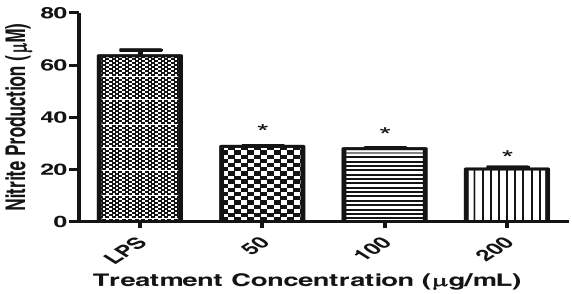
(A) NO Production **(B) TNF-α Production**

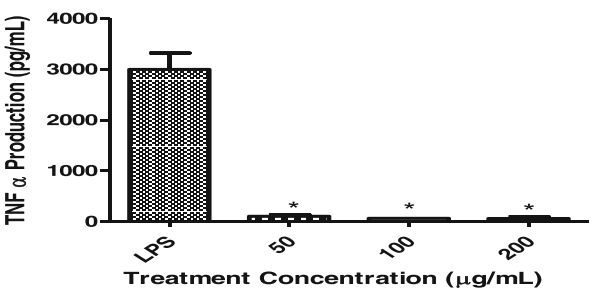
(i) AQ Extract



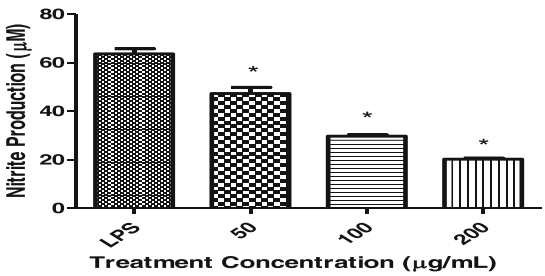


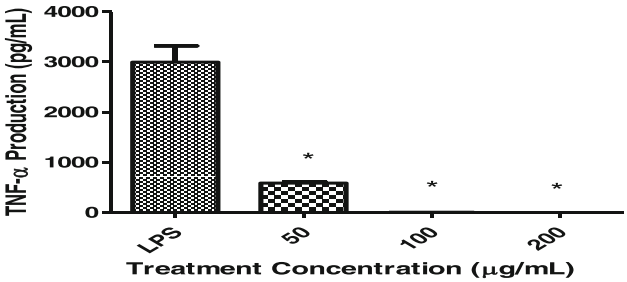
(ii) AGRPS extract



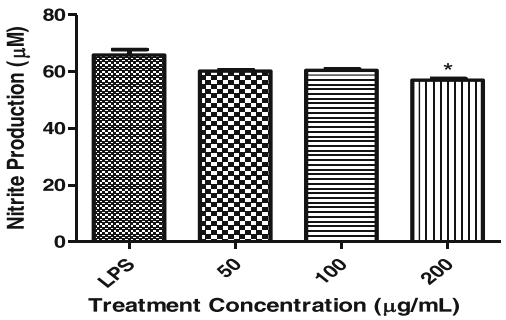


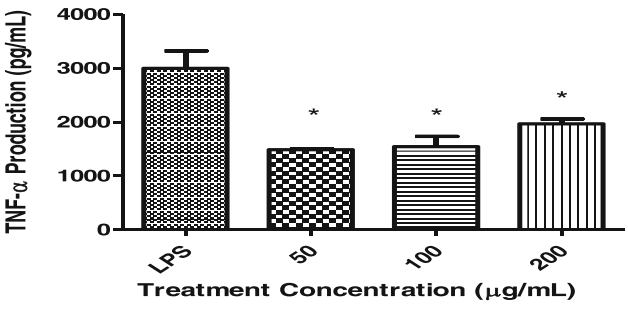
(iii) Acidic PS Extract





(iv) Neutral PS Extract





◀ **Fig. 13** Effects of AGRPS and AQ extracts, acidic and neutral PS fractions on LPS-stimulated 24 h rat alveolar macrophage production of (**a**) NO (nitrite) and (**b**) TNF-α *in vitro*. Rat alveolar macrophages were pretreated AGRPS and AQ extracts, acidic and neutral PS fractions (0, 5, 10, 20, 50, 100 μg/mL) for 24 h and were washed before challenge with LPS 1 μg/LI. The LPS-stimulated 24 h production of NO and TNF-α were determined by Griess reaction assay and ELISA, respectively. The experiments were performed in triplicates at three independent times and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the LPS positive control were statistically significant.

and 10 kDa was used as an alternative tool to separate the acid PS into 4 major fractions of different molecular weights (≥100 kDa, 50–100 kDa, 30–50 kDa, 10–30 kDa). The immunobioactivities of these fractions were then evaluated.

In vitro immunostimulatory effect of AGRPS and AQ extracts, Acidic and Neutral fractions isolated from AGRPS

The *in vitro* mechanistic study revealed that AGRPS extract, AQ extract and acidic PS significantly up-regulated murine cell line and rat alveolar macrophage production of NO (nitrite) and TNF-α compared to the untreated control in a dose-dependent manner. Figs. 8 and 9 showed the influence of ionic charge (acidic PS and neutral PS fractions) on AGRPS modulation of macrophage function *in vitro*. The immunostimulatory effects of AGRPS extract, AQ extract and acidic PS show a biphasic dose–response relationship, exhibited by a marked increase in effect at the low dose range. The mechanistic study also indicated that neutral PS did not stimulate production of NO, but showed some significant stimulation of TNF-α production. Fig. 10 shows the interaction between acidic PS and neutral PS using the composite mixture that simulates the 12% acidic PS fraction and 30% neutral PS yield (ratio 1:2.5 of acidic PS: neutral PS) obtained from the ion exchange chromatographic procedure. This investigation revealed that neutral PS enhances the immunostimulatory effect of acidic PS.

In vitro immunostimulatory effect of different molecular weight fractions isolated from Acidic PS

Since the acidic PS showed more immunostimulatory effect in the bioassay results as shown in Figs. 8–9, ultrafiltration was used to fractionate the acidic PS into different molecular weight fractions (*i.e.* ≥100 kDa, 50–100 kDa, 30–50 kDa and 10–30 kDa) to evaluate the influence of molecular weight on AGRPS extract immunobioactivities. As a continuation of the mechanistic study, Fig. 11 shows the influence of different molecular weight fractions of acidic PS on AGRPS extract modulation of macrophage function *in vitro*. The ≥100 kDa and 50–100 kDa molecular weight fractions of acidic PS enhanced macrophage production of NO (nitrite) and TNF-α compared to the untreated control. The 30–50 kDa and 10–30 kDa molecular weight fractions of acidic PS were devoid of stimulating NO production, though they did slightly exhibit some TNF-a production. Hence, high molecular weight (≥100 kDa and 50–100 kDa) is a vital property for AGRPS extract to exert an immunostimulatory effect.

In vitro suppression of LPS-induced stimulation by AGRPS and AQ extracts, Acidic and Neutral fractions isolated from AGRPS

The mechanistic study data presented in Figs. 12 and 13 shows the inhibitory effect of 24 h pre-conditioning with ginseng on 24 h accumulation of NO (nitrite) and TNF produced by murine cell line and rat alveolar macrophage induced by LPS challenge. In the absence of ginseng precondition, LPS induced a marked NO and TNF response. AGRPS extract, AQ extract and acidic PS significantly suppressed LPS stimulated 24 h production of NO and TNF-α. Neutral PS had no significant effect suggesting that acidic PS is critical in AGRPS extract suppression of LPS induced immunological response.

Bioassay of the composite mixture (simulating the 12% acidic PS fraction and 30% neutral PS yield obtained from the ion exchange chromatographic procedure) as seen in Fig. 14, revealed that neutral PS enhances acidic PS suppression of LPS immunological response.

In vitro suppression of LPS-induced stimulation by different molecular weight

Fractions isolated from Acidic PS

The mechanistic study data also indicates that ≥100 kDa and 50–100 kDa molecular weight fractions of acidic PS suppressed LPS induced macrophage immunological response. The 30–50 kDa and 10–30 kDa molecular weight fractions of acidic PS did not suppress LPS immunological response (Fig. 15). Hence, high molecular weight (≥100 kDa and 50–100 kDa) is a vital property for AGRPS extract to suppress LPS induced immunological response.

DISCUSSION

Medicinal plants are used as prophylactic and therapeutic agents in herbal medicine. Polysaccharides have recently been recognized as a major contributor to the bioactivity of herbal medicines. Polysaccharides from plant sources with immunomodulatory, anti-oxidant, anti-hyperglycemic, anti-bacterial,

anti-inflammatory, anti-viral and anti-tumor activities have been reported [10,11,17,19,26,33,36,46]. Polysaccharides, incluing lentinan, a (1,3)-β-D-glucan, isolated and purified from Lentinus edodes has been licensed as an over-the-counter (OTC) dietary supplement and immunostimulatory drug in China [47]. In Canada, CVT-E002™ (sold commercially as COLD-FX®) a poly-furanosyl-pyranosyl polysaccharide-rich herbal product of the root of American ginseng was licensed in 2007 as a natural health product to 'help reduce the frequency, severity and duration of cold and flu symptoms by boosting the immune system' [21,48].

The paradoxical immunomodulatory effect of AQ and AGRPS extracts was examined *ex vivo* and *in vivo*. In the *ex vivo* study, Figs. 1–2 shows that there was an increase in proinflammatory production in cultured alveolar macrophages obtained from AGRPS and AQ extracts treated rats. The AGRPS and AQ extract treatments reduced responsiveness of alveolar macrophages collected from ginseng-treated animals to LPS *ex vivo* challenge (Figs. 1–2). As shown in Fig. 3, the *in vivo* study suggests that orally administered AGRPS and AQ extracts possess *in vivo* immunostimulatory and suppression of LPS induced immunological effects. This was evidenced by the extracts up-regulation and down-regulation of plasma TNF-α cytokine production under basal and LPS induced proinflammatory conditions respectively. Overproduction of early and primary cytokines like TNF-α in the inflammatory process contributes significantly to the pathological complication observed in infectious and inflammatory diseases such as endotoxemia [49–51]. There was a significant difference in cytokine production between LPS challenge and ginseng treatments exposed to LPS. However, in the lung histochemical study described in Figs. 4–5, no difference was observed between ginseng treatments exposed to LPS and the LPS challenge. It may take a longer duration of exposure or a higher dose of LPS to observe lung pathological complication observed in infectious and inflammatory diseases like sepsis induced acute lung injury.

An *in vitro* mechanistic study was performed to identify the underlying bioactive fraction(s) responsible for AGRPS and AQ extracts *ex vivo* and *in vivo* immunobioactivities. For this study, AGRPS and AQ extracts and the fractions of AGRPS extract immunomodulatory effects were evaluated *in vitro* using murine cell line and rat alveolar macrophages. The relationship between physicochemical properties (ionic charge and molecular weight) of AGRPS fractions and their influence on AGRPS modulation of macrophage function was examined. The pharmacological effects of ginseng polysaccharides including immunomodulation can be attributed to its acidic and neutral polysaccharide components [26]. There have been different reports of ginseng acidic and neutral polysaccharide immunomodulatory effects in the literature. Kim *et al.* (1990) reported that acidic PS and neutral PS of *Panax ginseng* (Asian ginseng) may stimulate B cells and macrophages [16]. While the study by Fan *et al.* (2010) showed that neutral PS of *Panax ginseng* stimulated the proliferation of lymphocytes; increased natural killer cell cytotoxicity; enhanced the phagocytosis and NO production by macrophages and increased the level of TNF-α in serum [52]. The *in vitro* mechanistic study also delineated the influence of AGRPS (*Panax quinquefolius*) ionic charge fractions on AGRPS and AQ extracts modulation of macrophage function. The data revealed that acidic PS was the major bioactive species responsible for the immunostimulatory and suppression of LPS induced immunological effects of AGRPS and AQ extracts (Figs. 8–9 and 12–13). This outcome suggests that acidic (and not the neutral) PS physicochemical property is vital for AGRPS and AQ extracts macrophage-mediated immunomodulatory effects. Because neutral PS did not stimulate production of NO, but showed some significant stimulation of TNF-α production, investigation was done to determine if there was any interaction between acidic PS and neutral PS. Bioassay of a composite mixture of 12% acidic PS fraction and 30% neutral PS (Figs. 10 and 14) revealed that neutral PS enhances acid PS macrophage-mediated immunostimulatory and suppression of LPS induced immunological effects. The observed enhancement of acidic PS immunostimulatory effect by neutral PS supports the previous finding by Fan *et al.* (2010) which reported that neutral PS of *Panax ginseng* enhanced macrophage production of NO [52]. While the observed enhancement of acidic PS suppression of LPS immune response by neutral PS is a novel outcome. One study by Lemmon *et al.* (2012) [that used human peripheral blood mononuclear cells and ginseng concentrations similar to that utilized in this study] reported that the immunomostimulatory effects of AGRPS and AQ extracts are mediated by PS with a molecular weight higher than 100 kDa [53]. Data from this study showed that ≥100 kDa and 100–50 kDa molecular weight fractions of acidic PS possess both immunostimulatory and suppression of LPS induced immunological effects which are a novel outcome. This indicates that the PS high molecular weight physicochemical property plays a significant role in AGRPS and AQ extracts macrophage-mediated immunostimulatory and suppression of LPS induced immunological effects (Figs. 11 and 15).

The *in vitro* mechanistic study revealed the importance of the acidic nature and high molecular weight of the polysaccharide fractions in AGRPS and AQ extracts modulation of macrophage function. Outcomes of this study correlate well with what has been previously reported regarding ionic charge and molecular weight physicochemical properties being critical factors that affect the biological activity of polysaccharides [37–39]. Macrophages are activated through their recognition and binding of plant polysaccharides *via* Toll-like receptor 4 (TLR4). The activation of these receptors leads to intracellular signaling cascades, resulting in transcriptional activation and the production of proinflammatory mediators

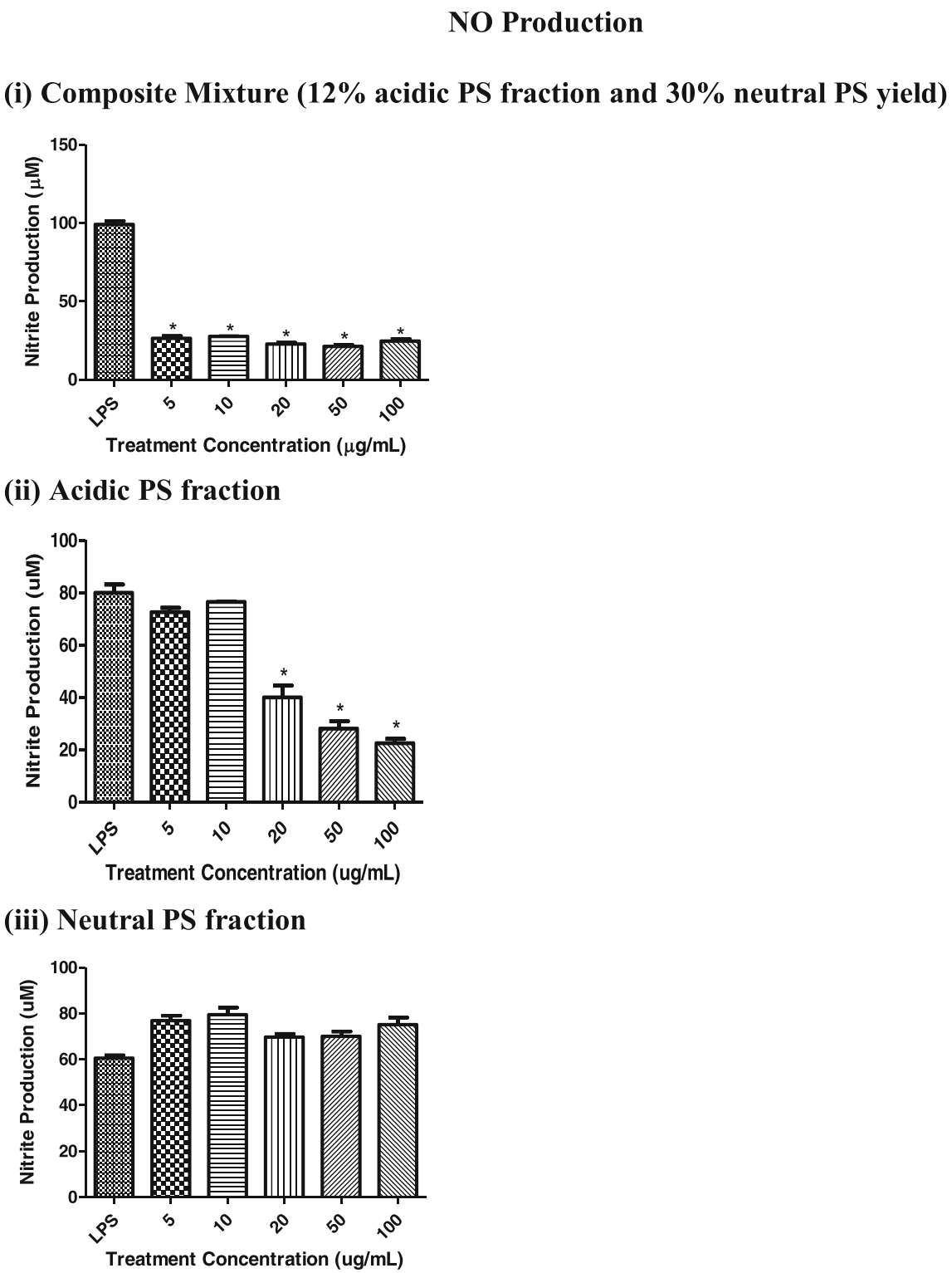


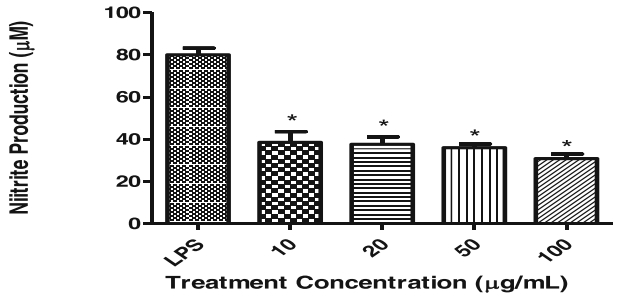
Fig. 14 Comparison of composite mixture (12% acidic PS fraction and 30% neutral PS yield), acidic PS, neutral PS fractions on LPS-stimulated 24 h macrophage production of NO (nitrite) *in vitro*. Murine macrophages (RAW 264.7 cells) were pretreated composite mixture, acidic PS, neutral PS fractions (0, 5, 10, 20, 50, 100 μg/mL) for 24 h and were washed before challenge with LPS 1 μg/mL. The LPS-stimulated 24 h production of NO and TNF-α were determined by Griess reaction assay and ELISA, respectively. The experiments were performed in triplicates at three independent times and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the LPS positive control were statistically significant.

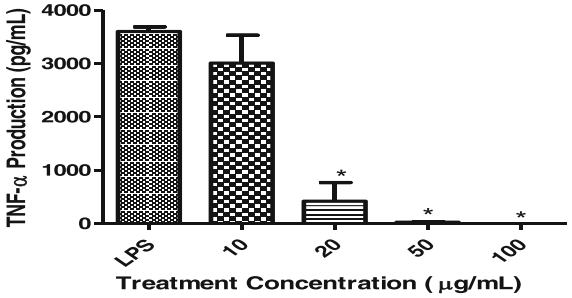
[10]. Results from this study suggests that acidic nature and high molecular weight (≥100 kDa and 100–50 kDa) polysaccharide fractions are vital for AGRPS and AQ extracts activation and suppression of macrophage function. These

(A) NO Production

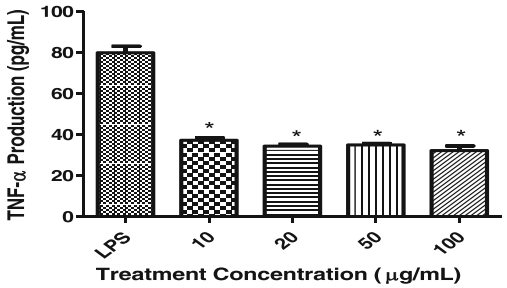
(B) TNF-α Production

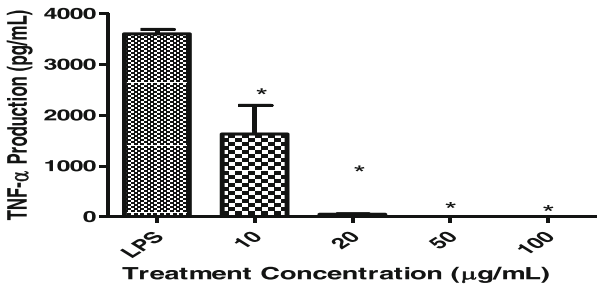
(i) >100 - 100 kDa Acidic PS fraction



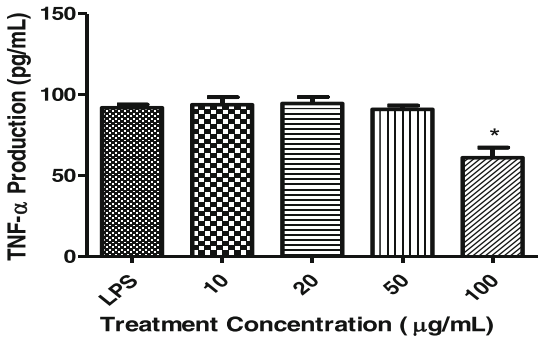


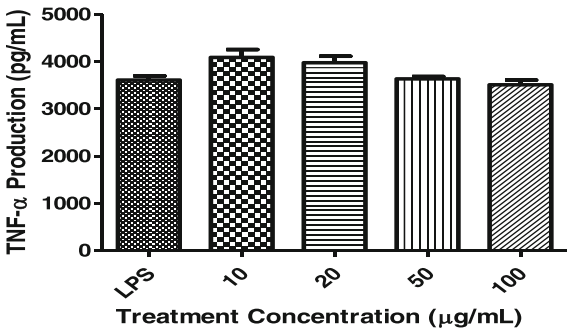
(ii) 100 - 50 kDa Acidic PS fraction



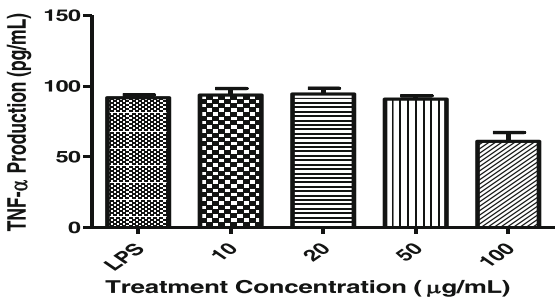


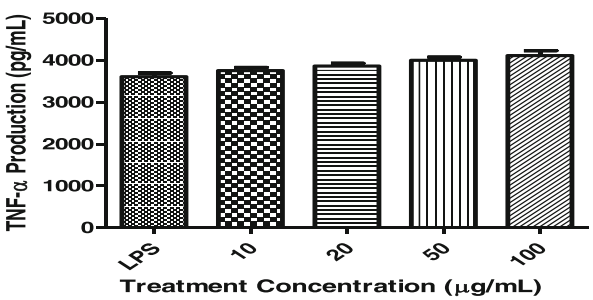
(iii) 30 - 50 kDa Acidic PS fraction





(iv) 10 - 30 kDa Acidic PS fraction





◀ **Fig. 15** Effects of acidic PS fractions 100 - >100 kDa, 50–100 kDa, 30–50 kDa, 10–30 kDa, on LPS-stimulated 24 h macrophage production of (**a**) NO (nitrite) and (**b**) TNF-α *in vitro*. Murine macrophages (RAW 264.7 cells) were pretreated with acidic PS fractions 100 - >100 kDa, 50–100 kDa, 30–50 kDa, 10–30 kDa (0, 10, 20, 50, 100 μg/mL) for 24 h and were washed before challenge with LPS 1 μg/mL. The LPS-stimulated 24 h production of NO and TNF-α were determined by Griess reaction assay and ELISA, respectively. The experiments were performed in triplicates at three independent times and the data were shown as mean ± SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the LPS positive control were statistically significant.

physicochemical properties may be associated with the ability of the extract's polysaccharide fractions to bind to TLR4 receptor and up-regulate or down-regulate TLR4 receptor expression, which triggers or inhibits intracellular signaling cascades and the production of proinflammatory mediators under basal or LPS induced proinflammatory conditions respectively. Data from this study opens the door for future investigation of the possible association between the extracts polysaccharide fractions physicochemical properties and the extracts ability to up-regulate or down-regulate TLR4 receptor expression. TLR4 has been shown to be expressed in murine macrophages activated by *Carthamus tinctorius* polysaccharides [54]. Studies which used TLR 4-deficient mice indicates that TLR4 is involved in murine macrophage recognition and activation of *Platycodon grandiflorum* polysaccharides [55] and Levan polysaccharide fraction from fermented soybean mucilage [56]. The expression of Toll-like receptors including TLR2, TLR4 and the adaptor molecule MyD88 which were increased in murine macrophages stimulated with *Staphylococcus aureus* bacteria has been reported to be significantly reduced by ginseng polysaccharide pretreatment *in vitro*. Ginseng polysaccharide intravenous pretreatment was also reported to enhance macrophage-mediated bactericidal activity in mice by reducing the number of *S. aureus* bacteria present in the spleen, kidney, and blood [34,35]. The iNOS (inducible nitric oxide synthase), MAPK (mitogen-activated protein kinase) kinases such as p38, ERK1/2 (extracellular signal-regulated kinases 1/2), PI3K (phosphoinositide 3-kinase), and NF-kB (nuclear factor kappa B) signaling pathways may mediate the immunostimulatory effect observed in AGRPS and AQ extracts and high molecular weight acidic PS, as studies by Friedl *et al.* (2001) and Lemmon *et al.* (2012) using AQ and PS extracts of P ginseng and American ginseng AQ and PS extracts [30,53] demonstrated. The signaling pathways that mediate the extracts suppression of LPS induced NO and TNF-α production requires further investigation.

The ion exchange chromatography was used to fractionate AGRPS extract into fractions of neutral and acidic charge. The 30.0% recovery for the neutral fraction of AGRPS (*Panax quinquefolius*) obtained from this study is close to the 33.0% recovery reported for Panax notoginseng polysaccharides [57]. The AGRPS (*Panax quinquefolius*) neutral fraction recovery is different from the 60.5% recovery reported for *Panax ginseng* polysaccharides [24]. In this study 12.0% recovery obtained for AGRPS (*Panax quinquefolius*) acidic fraction is close to the 17.1% recovery reported for *Panax ginseng* polysaccharides [24]. This was different from the 31.0% recovery reported for *Panax notoginseng* polysaccharides [57]. The difference in species of the ginseng herb may account for the variations in the recovery data of the neutral and acidic fractions between this study and other studies [24,57].

Our previous study and that of Lemmon *et al.* (2012) suggests that concurrent treatment of AQ and AGRPS extracts did not inhibit LPS induced immune response [32,53]. While data from the *in vitro* study shows that direct treatment and 24 h pretreatment with AGRPS and AQ extracts exerts a direct immunostimulatory effect and an indirect suppression of LPS induced immune response respectively as shown in Figs. 8–9 and 12–13. A similar situation was observed in the *ex vivo* study (Figs. 1–2), where the 6 days AQ and AGRPS extract pretreatments was more effective than that of the 3 days in suppressing the LPS induced immune response.

The *ex vivo* and *in vivo* immunomodulatory effects of AGRPS and AQ extracts (Figs. 1, 2 and 3) were validated *in vitro* (Figs. 8–9 and 12–13). The *ex vivo*, *in vivo* and *in vitro* studies support the observations from our previous study, indicating that bioactive AGRPS isolated from AQ extract and its parent AQ extract exerted immunostimulatory effects [32]. The *in vivo* immunostimulatory effect of 125 mg/kg AGRPS and AQ extracts was very small as compared with 5 mg/kg LPS toxic stimulatory effect, suggesting that American ginseng extract and its natural health product containing polysaccharides like CVT-E002™ are safe with health benefits with regards to immunostimulation [48]. The results from this study suggests that under LPS mediated excessive stimulatory infectious condition like endotoxemia; pretreatment with AGRPS and AQ extracts will not cause additive immunostimulatory effects, but rather the extracts will reduce and neutralize LPS immunologic stimulatory effect. The ability of AGRPS and AQ extracts to neutralize LPS immunological is a desired requirement for future prophylactics against endotoxemia. A possible mechanism by which AGRPS suppresses LPS immunotoxic stimulatory effect may be through its ability to desensitize immune cells (*e.g.* macrophages) from LPS toxic stimulation, similar to the tolerance ability of LPS pre-exposure to desensitize subsequent LPS challenge [58]. AGRPS and AQ extracts are not antagonists of LPS because they have low stimulatory effect and also requires pretreatment before they can suppress LPS stimulation. The data from this study supports the reported CVT-E002^{TM} immunostimulatory [22,29,48,59–62] and suppression of proinflammatory effects [21,63] under basal and induced proinflammatory conditions respectively. Therapies directed at the neutralization of proinflammatory mediators

or LPS that were promising in experimental models have been largely ineffective in clinical trials. Therefore, the development of new therapies is of major interest [64–66].

The *in vitro* stimulatory effect was stronger than that observed *in vivo* which may be due to low bioavailability of the orally-administered extracts. Our newly developed method for plasma analysis of AGRPS, detected low levels of AGRPS in the plasma of rats orally fed with AGRPS [67]. The low plasma levels of AGRPS are pharmacologically in close agreement with the *in vitro* 50–200 μg/mL immunobioactive concentrations of AGRPS in cultured murine cell line and rat alveolar macrophages reported in this study and that of our previous study [32]. Evaluation of the total AGRPS concentration at C_{max} in total plasma volume revealed that an estimated 9.49% of the orally administered AGRPS was available in systemic plasma. Polysaccharides of *Radix ophiopogonis* have been reported to have low bioavailability of 1.7% in rats after oral administration respectively, and this can be attributed to their large molecular size and hydrophilic character [68]. The 125 mg/kg daily oral dose of American ginseng used in the *ex vivo* and *in vivo* studies, is in agreement with the 9 g of ginseng daily dose [69] recommended for human consumption (assuming an average adult weighs 70 kg).

The merit of this study is that a modern day approach of evaluating herbal medicines [70,71] was utilized *via* the phytochemical and immunopharmacological screening of AGRPS and AQ extracts, obtained from good agricultural practice quality American ginseng roots randomly collected from five different farms by the Ontario Ginseng Growers Association in Ontario, Canada. The acidic PS and its ≥100 kDa and 100–50 kDa molecular weight fractions were identified as the major bioactives responsible for the extracts immunomodulatory effects. AGRPS and AQ extracts *in vivo* stimulation was small in magnitude as compared to its suppression of LPS induced immunologic response, suggesting attention should be focused more on their beneficial immunosuppressive effect which may be useful in the prophylaxis of LPS related disease conditions such as endotoxemia.

CONCLUSION

From this study it can be concluded that AGRPS and AQ extracts possess *ex vivo*, *in vivo* and *in vitro* stimulatory and suppressive effects in basal immune function and LPS-induced endotoxic conditions respectively, which can be attributed primarily to acidic PS and its higher molecular weight fractions and not neutral PS or the lower molecular weight fractions of acidic PS. Hence AGRPS and AQ extracts may have beneficial suppressive effect against LPS related disease conditions such as endotoxemia.

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