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Fractionation and characterization of biologically-active polysaccharides from *Artemisia tripartita*

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Abstract

The leaves of Artemisia species have been traditionally used for prevention and treatment of a number of diseases. In this study, five polysaccharide fractions (designated A-I–A-V) were isolated from the leaves of *Artemisia tripartita* Rydb. by the sequential use of hot-water extraction, ethanol precipitation, ultra-filtration, and chromatography. The homogeneity and average molecular weight of each fraction were determined by high performance size-exclusion chromatography. Sugar composition analysis revealed that *Artemisia* polysaccharides consisted primarily of xylose, glucose, arabinose, galactose, and galactosamine. Moreover, all fractions contained at least 3.4% sulfate, and fractions A-II–A-V contained an arabinogalactan type II structure. All fractions exhibited macrophage-activating activity, enhancing production of intracellular reactive oxygen species and release of nitric oxide, interleukin 6, interleukin 10, tumor necrosis factor α, and monocyte chemotactic protein 1. In addition, all fractions exhibited scavenging activity for reactive oxygen species generated enzymatically or produced extracellularly by human neutrophils. Finally, fractions A-I and A-V exhibited complement-fixing activity. Taken together, our results provide a molecular basis to explain at least part of the beneficial therapeutic effects of Artemisia extracts, and suggest the possibility of using Artemisia polysaccharides as an immunotherapeutic adjuvant.

Keywords: *Artemisia tripartita* Rydb; Asteraceae; Immunomodulator; Polysaccharide; Macrophage; Neutrophil; Complement-fixing activity; Reactive oxygen species; Cytokines

1. Introduction

Processes that remove invading microbes and harmful foreign or endogenous substances are essential to maintain normal development and homeostasis in multicellular organisms (Beutler, 2004). Phagocytic leukocytes play multiple roles in these immune processes, serving as a link between the innate and acquired immune systems and contributing to the inflammatory response, angiogenesis, and the promotion of wound healing (Beutler, 2004; Hoebe et al., 2004). Importantly, phagocytes can directly kill invading microorganisms and tumor cells, using both oxidative and non-oxidative mechanisms (Tosi, 2005). Thus, the development of novel therapeutics to non-specifically augment macrophage immune responses represents an ideal strategy for enhancing defense against microbial infection (Finlay and Hancock, 2004).

A wide range of bioactive polysaccharides have been isolated from various medicinal plants, and these polysaccharides have been shown to possess immunomodulatory activity through their ability to modulate macrophage function [reviewed in (Schepetkin and Quinn, 2006)]. Indeed, botanical polysaccharides have been reported to increase macrophage cytotoxicity against tumor cells and microorganisms, activate phagocytosis, increase reactive oxygen species (ROS) and nitric oxide (NO) production, and enhance secretion of a variety of cytokines [reviewed in (Schepetkin and Quinn, 2006)]. Appropriate enhancement of these innate immune functions by bioactive

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compounds can then lead to improved host defense responsiveness (Finlay and Hancock, 2004). Moreover, most plant-derived polysaccharides are relatively non-toxic and do not cause severe side effects, which is a major problem associated with immunomodulatory bacterial polysaccharides and synthetic compounds (Finlay and Hancock, 2004). Thus, plant polysaccharides represent ideal candidates for therapeutics with immunomodulatory, anti-tumor, and wound-healing action.

The genus Artemisia belongs to the family Compositae (Asteraceae) and has been used in folk medicine to treat a variety of diseases, such as hepatitis (Hong et al., 2004), fever and malaria (Klayman, 1985), rheumatoid arthritis (Wang et al., 2005), and asthma (Kim et al., 2006). In the western United States, *Artemisia tripartita* (three-tip sagebrush) is a native species that has been used in the treatment of colds, sore throats, tonsillitis, headaches, and wounds by Native Americans (Moerman, 1998); however, the biologically-active components in *A. tripartita* extracts are unknown. Low-molecular weight compounds (e.g., lignans and sesquiterpene lactones) have been identified and isolated from *A. tripartita* (Kelsey and Shafizadeh, 1979), and similar compounds from other Artemisia species have been shown to have anti-fungal properties (Tan et al., 1998). Likewise, polysaccharides from various Artemisia species have been reported to exhibit therapeutic activities (Hayakawa et al., 1995; Hwang et al., 2003; Lee et al., 2003); however, potential immunomodulatory properties *of A. tripartita* polysaccharides have not been evaluated.

In the present study, we isolated five polysaccharide fractions from aqueous extracts of the leaves of *A. tripartita*. Analysis of these polysaccharide fractions showed that they had potent phagocyte immunomodulatory activity, as well as ROS scavenging and complement-fixing activity. Thus, the immunomodulatory activities of Artemisia polysaccharides likely contribute to the known therapeutic effects of Artemisia extracts.

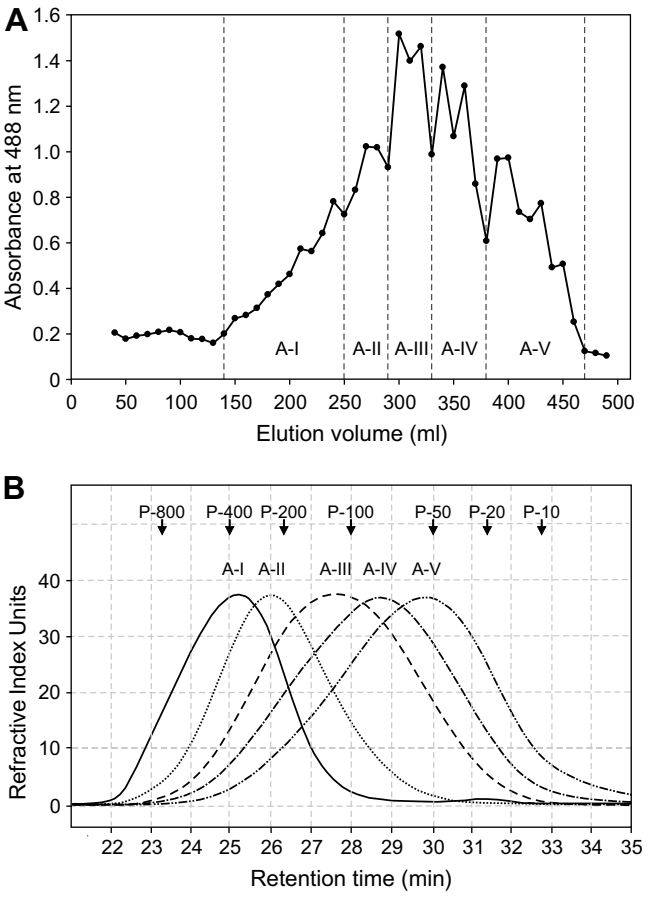

Fig. 1. Fractionation of Artemisia polysaccharides by size-exclusion chromatography. Panel A: Polysaccharides from crude Artemisia extract were isolated by DEAE-cellulose chromatography, and the eluate was further fractionated using Sepharose 6B column chromatography. Total carbohydrate content of the fractions was determined by a phenol-sulfuric acid method (detected at 488 nm). Five fractions (designated A-I to A-V) were selected for further analyses. Panel B: Fractions A-I–A-V were analyzed by HP-SEC and monitored with a refractive index detector, as described. The arrows show peak retention times of the indicated standards used for calibration.

2. Results and discussion

2.1. Preparation and characterization of Artemisia polysaccharides

Acidic polysaccharides obtained from Artemisia extract were fractionated by preparative size-exclusion chromatography to obtain five main fractions, which were selected based on total carbohydrate elution profile (designated as A-I, A-II, A-III, A-IV, and A-V) (Fig. 1A). Each of the five fractions contained <2.4% protein (Table 1). Analysis of the fractions using the Yariv test showed that fraction A-I lacked arabinogalactan; whereas, all other fractions tested positive for the presence of type II arabinogalactan (Table 1). Type II arabinogalactans have a β-(1,3)-linked galactan backbone with side chains containing arabinose and galactose residues and have been reported to possess a variety of biological activities (Paulsen, 2001). All five fractions also contained at least 3.4% sulfate (Table 1), suggesting the possibility that Artemisia polysaccharides are also sulfated; however, further purification and analysis of the individual polysaccharides will be necessary to verify this issue. Note that the presence of sulfate groups has been reported to play a role in polysaccharide biological activity (Melo et al., 2002; Ghosh et al., 2004). For example, Schaeffer and Krylov (2000) reported that the presence of sulfate groups was necessary for anti-HIV activity of extracts from algae, and that potency increased with an increased degree of sulfation. Thus, it will be important in future studies to determine the role of sulfation in Artemisia polysaccharide biological activity.

Analytical high performance size-exclusion chromatography (HP-SEC) of the individual fractions showed that each fraction was represented by a broad, symmetrical peak on the chromatograms (Fig. 1B). Average molecular

Table 1
Chemical properties and molecular weights of polysaccharide fractions

Polysaccharide fraction	Protein content (%)	Arabinogalactan	Sulfate content (%)	Molecular weight (kDa)
A-I	0.85	Negative	5.49	355
A-II	1.04	Positive	4.29	251
A-III	1.54	Positive	3.53	126
A-IV	2.03	Positive	4.49	78
A-V	2.39	Positive	3.43	49

weights of the fractions were determined to be 355, 251, 126, 78, and 49 kDa for fractions A-I–A-V, respectively (Table 1).

Analysis of sugar composition of the Artemisia polysaccharide fractions showed that these polysaccharides consisted primarily of xylose, glucose, arabinose, galactose, and galactosamine, which accounted for the majority of monosaccharides present (Table 2). Small amounts of galacturonic acid, glucuronic acid, mannose, lyxose, and glucosamine were also detected in the fractions (Table 2). In polysaccharide fractions A-II–A-V, xylose was the dominant monosaccharide and represented >50 mol% of the total sugars in these fractions. In contrast, fraction A-I had a much higher glucose content (29.5 mol%), making it the predominant sugar in fraction A-I (Table 2). In comparison to *A. tripartita* polysaccharides, mannose was the predominant sugar in polysaccharides from *A. sphaerocephala* (Zhang et al., 2006), suggesting there are differences in polysaccharide structure between Artemisia species, which may confer distinct biological properties.

Very-high-field (600 MHz) ^{1}H NMR was used to characterize structure of the native Artemisia polysaccharides (Fig. 2). The spectra of fractions A-II, A-III, A-IV, and A-V were similar to each other, suggesting a common backbone structure, and resembled the spectra of native arabinogalactans isolated from other plant sources, which is consistent with the results of the Yariv test (Gane et al., 1995; Dong and Fang, 2001; Schepetkin et al., 2005). The chemical shifts of anomeric protons were evaluated according to data previously reported for the sugar composition of the arabino-3,6-galactans (type II) (Gane et al., 1995; Dong and Fang, 2001). The signals present at 3.40–4.38 and 4.95–5.17 ppm were consistent with the presence of β-D-galactopyranose (β-D-Gal *p*) and α-arabinofuranose (L-Ara *f*) residues, respectively (Gane et al., 1995). In comparison, a very weak signal was detected at these chemical shifts in the spectrum of fraction A-I, confirming our Yariv test that also showed the absence of arabinogalactan in this fraction. Fractions A-II–A-V also contained much stronger signals for *N*- and *O*-acetyl (1.97–2.08 ppm) and methyl groups (1.13–1.20 ppm). A paired doublet at 2.43–2.54 ppm was present in the spectra of all five fractions; however, we subsequently found this signal was due to the presence of sodium citrate bound to the polysaccharides. In all Artemisia fractions, signals corresponding to aromatic protons (5.45–6.25 ppm) were absent or very weak, suggesting the lack of aromatic groups or their presence in very low molar concentrations in the large polysaccharide structures.

Table 2
Sugar composition of polysaccharide fractions

	Polysaccharide fraction				
	A-I	A-II	A-III	A-IV	A-V
Arabinose	13.64	10.91	14.32	10.02	12.07
Fucose	0.14	0.00	0.00	0.00	0.00
Galactosamine	16.56	6.19	12.07	9.79	18.60
Galactose	7.56	8.93	18.15	15.68	12.41
Galacturonic Acid	1.57	1.79	1.46	0.74	0.74
Glucosamine	0.86	1.17	0.16	0.02	0.11
Glucose	29.47	10.01	0.43	4.73	1.32
Glucuronic Acid	0.04	0.24	0.22	0.14	0.16
Lyxose	3.39	1.86	1.66	1.14	0.98
Mannose	3.05	1.67	1.49	1.03	0.88
Xylose	23.71	57.22	50.04	56.71	52.73

The data are presented as mol% for each sugar. Individual components were identified and quantified based on elution of known standards.

To directly evaluate possible lipopolysaccharide (LPS) contamination in our samples, we analyzed the Artemisia fractions for binding to rhodamine-labeled S3 peptide (Rh-S3), as described previously (Schepetkin et al., 2005). Analysis of the samples by HP-SEC showed free Rh-S3 was eluted as a single fluorescent peak with retention time of 25 min; whereas, addition of 5 ng LPS to Rh-S3 resulted in the appearance of a new fluorescent peak at 14 min, which represents Rh-S3 bound to LPS (Supplementary Fig. S1). In contrast, no new fluorescence peaks were observed after addition of the individual Artemisia polysaccharide fractions, even at 1000-fold higher concentrations of the fractions (Supplementary Fig. S1). Thus, these data directly demonstrate that LPS contamination was not present in the Artemisia fractions and, therefore, was not responsible for their immunomodulatory activity.

2.2. Effect of Artemisia polysaccharides on macrophage NO and ROS production

A minimum amount of NO was produced when murine J774.A1 macrophages were incubated with medium alone, whereas treatment of these cells with polysaccharide fractions resulted in a dose-dependent increase in NO production (Fig. 3A). Fractions A-I and A-V were the most potent inducers of macrophage NO production, enhancing the response >16-fold at concentrations of 800 μg/ml compared to the negative control (medium alone). In comparison, NO production induced by fractions A-I and A-V was a little over 50% of that induced by 50 ng/ml lipopolysaccharide (LPS).

In the absence of any treatment, murine macrophages generated very low levels of ROS; whereas, a dose-dependent enhancement of ROS production was observed in macrophages treated with 100–800 μg/ml doses of the

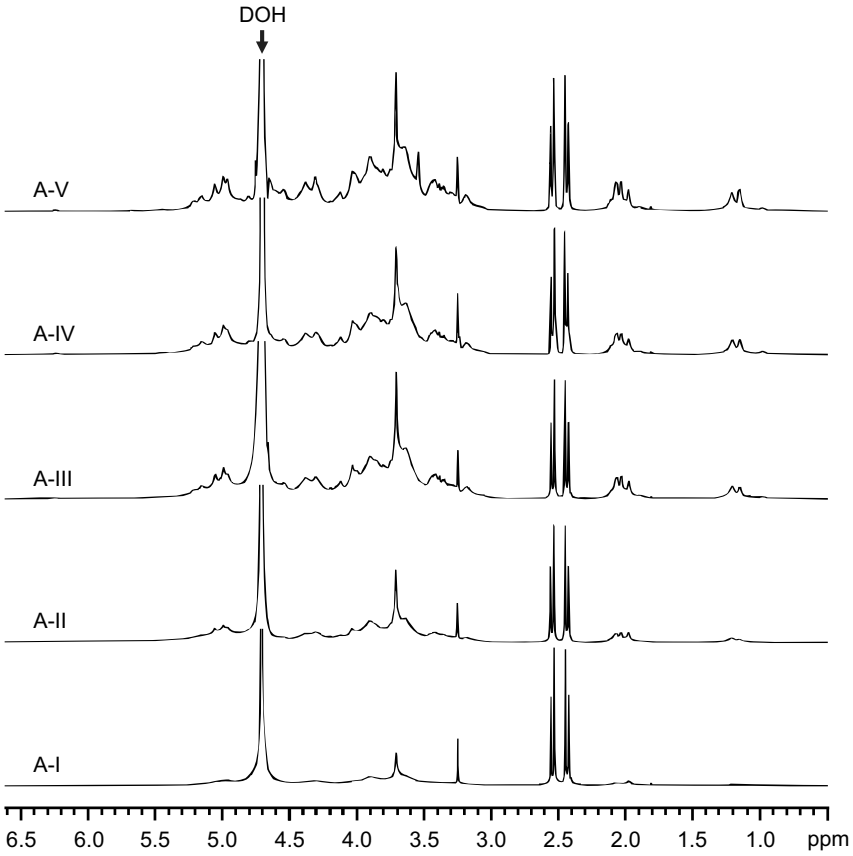

Fig. 2. ^{1}H NMR spectra of native Artemisia polysaccharide fractions. The fractions were dissolved in D_2O, and spectra were recorded at 20 °C, as described. The paired doublet at 2.48–2.55 ppm in the spectra is due to the presence of sodium citrate bound to the polysaccharides.

polysaccharide fractions (Fig. 3B and Supplementary Fig. S2), with fractions A-I and A-V being the most active fractions. Thus, the individual polysaccharide fractions showed a similar pattern with respect to their ability to induce NO and ROS production by murine J774.A1 macrophages. If we consider activity in terms of polysaccharide molar concentration, fraction A-I was still the most potent inducer of NO and ROS production (relative potency was A-I > A-II > A-III > A-IV ≈ A-V); whereas, fractions A-IV and A-V were the least active.

Macrophages play critical roles in host defense, including phagocytosis of pathogens and apoptotic cells, production of cytokines, and proteolytic processing and presentation of foreign antigens [reviewed in (Hume, 2006)]. Thus, the identification of agents that can modulate macrophages is of significant interest. Indeed, a variety of plant polysaccharides have been reported to exhibit beneficial pharmacological effects via their ability to modulate macrophage function [reviewed in (Schepetkin and Quinn, 2006)]. In this study, Artemisia polysaccharide fractions exhibited macrophage immunomodulatory properties, inducing NO and ROS production. These reactive oxidants play key roles host defense and various physiological processes and are also involved in the regulation of apoptosis and immune homeostasis [reviewed in (Serhan and Savill, 2005; Quinn et al., 2006)].

Although these functional assays suggested Artemisia polysaccharides were relatively non-toxic, we evaluated the potential cytotoxic effect of Artemisia polysaccharides to determine if the results might be influenced by background toxicity. Using an assay that measures ATP from viable cells, we found that none of the fractions significantly affected proliferation/viability of J774.A1 cells over the entire concentration range of Artemisia polysaccharides (100–800 μg/ml), confirming that these polysaccharides were not toxic to these cells (data not shown).

2.3. Effect of Artemisia polysaccharides on macrophage cytokine production

Many immunomodulatory compounds modulate cytokine and/or chemokine production. Thus, we analyzed the effects of Artemisia polysaccharide fractions on this response. As shown in Fig. 4, each of the five Artemisia polysaccharide fractions stimulated macrophage production of both pro-inflammatory and anti-inflammatory cytokines. Among the pro-inflammatory cytokines induced, interleukin 6 (IL-6) is one of the most important mediators of fever and the acute-phase response (Gabay, 2006). Tumor necrosis factor α (TNF-α) also plays an important role in inflammation and can act on monocytes and macrophages in an autocrine manner to enhance various func-

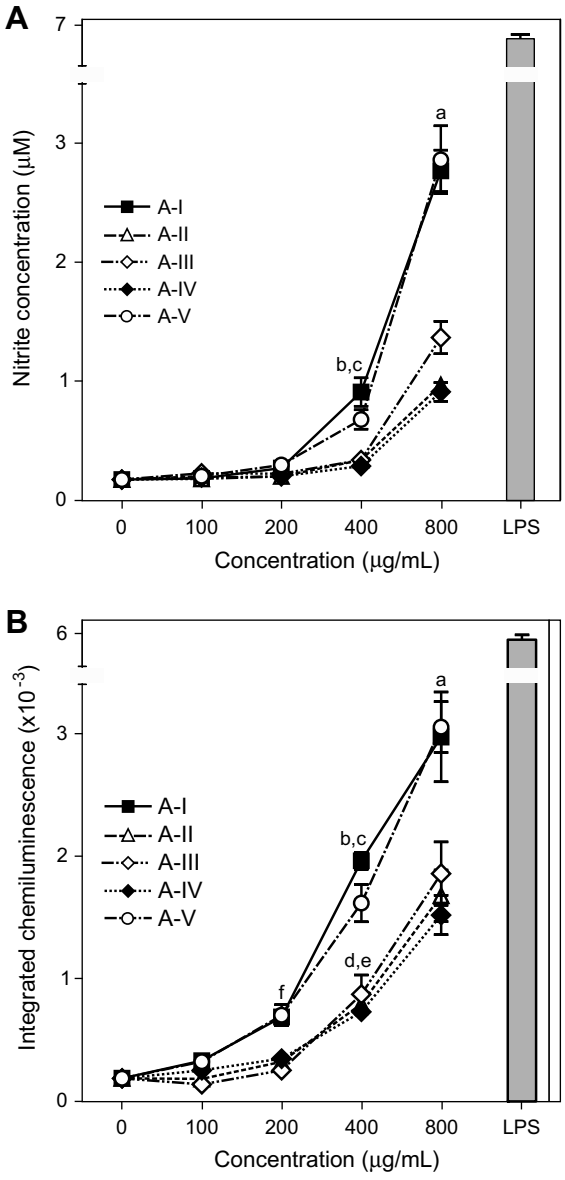

Fig. 3. Effects of Artemisia polysaccharide fractions on murine macrophage NO and ROS production. J774.A1 cells were cultured for 24 h with the indicated concentrations of polysaccharide fractions or 50 ng/ml LPS (positive control). NO production was quantified by measuring nitrite in the cell-free supernatants (Panel A), and chemiluminescence was monitored using an L-012 detection system, as described (Panel B). The data are presented as the means ± SEM of quadruplicate samples from one experiment that is representative of four independent experiments. Statistically significant differences between control (0 μg/ml) and the indicated concentrations of Fraction A-I (a,b: $P < 0.001$; f: $P < 0.05$), Fraction A-II (a: $P < 0.001$; c: $P < 0.01$), Fraction A-III (a: $P < 0.001$; d: $P < 0.01$), Fraction A-IV (a: $P < 0.001$; e: $P < 0.05$), and Fraction A-V (a,b: $P < 0.001$; f: $P < 0.05$) are indicated.

tional responses and induce the expression of additional immunoregulatory and inflammatory mediators (Baugh and Bucala, 2001). Production of the inflammatory chemokine, monocyte chemotactic protein 1 (MCP-1), was also induced by Artemisia polysaccharides at least 1.4-fold, as compared to untreated controls. Indeed, the enhancement of MCP-1 production was comparable to that obtained

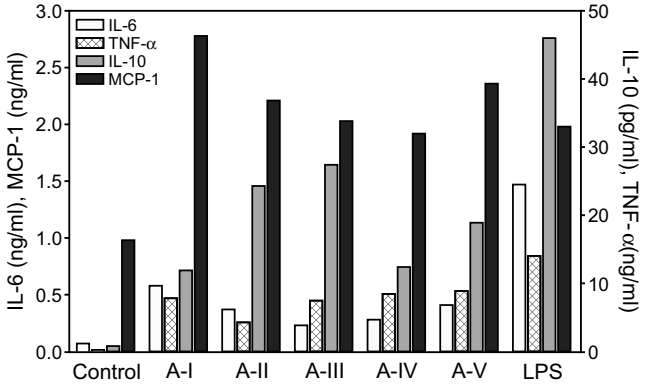

Fig. 4. Effects of Artemisia polysaccharide fractions on macrophage cytokine production. J774.A1 cells were cultured for 24 h with the indicated polysaccharide fractions (800 μg/ml) or LPS (50 ng/ml). The concentration of cytokines in cell-free supernatants was determined by a cytometric bead array analysis (IL-6, MCP-1 – left axis; TNF-α, IL-10 – right axis). The data are from one experiment that is representative of two independent experiments.

with LPS, which is well known as a strong cytokine/chemokine inducer (Fig. 4). This chemokine is involved in the recruitment of monocytes during acute and chronic inflammation (Conti and DiGioacchino, 2001). Along with these inflammatory mediators, the anti-inflammatory cytokine, IL-10, was also induced by Artemisia polysaccharides. IL-10 has been reported to inhibit cytokine synthesis and deactivate macrophages, thereby preventing or reducing potential detrimental effects from excessive macrophage activation during inflammation (Williams et al., 2004). Thus, the modulation of macrophage cytokine and chemokine production likely contributes to part of the therapeutic effects of Artemisia extracts. Note that previous studies have shown many plant-derived polysaccharides can induce the production of a range of cytokines [reviewed in (Schepetkin and Quinn, 2006)]; however, only polysaccharides from *Juniperus scopolorum* (Schepetkin et al., 2005) and Artemisia, as shown here, were found to induce IL-10 and MCP-1 production.

2.4. Effect of Artemisia polysaccharides on the complement system

Many plant-derived polysaccharides, including some arabinogalactans, have complement-fixing activity (Nergard et al., 2004). Therefore, Artemisia polysaccharide fractions were tested for their ability to fix complement, as compared to heparin, a known complement-fixing agent. As shown in Fig. 5, addition of each of the five fractions resulted in a dose-dependent inhibition of erythrocyte hemolysis, indicating that the isolated Artemisia polysaccharides exhibited complement-fixing activity. Fractions A-I and A-V showed the highest complement-fixing activity, whereas fraction A-IV was the least active (Fig. 5). All fractions inhibited hemolysis with $IC_{50} \geqslant 800$ μg/ml, which is similar to the activity of polysaccharides isolated from *Brassica oleracea* (Samuelsen et al., 2007). However,

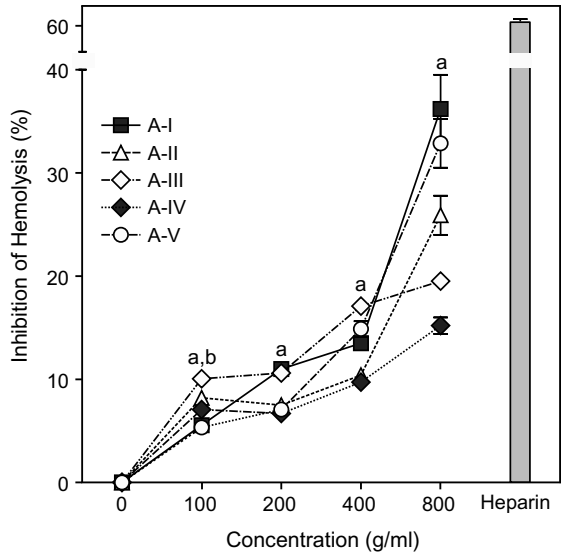

Fig. 5. Complement-fixing activity of Artemisia polysaccharide fractions. The indicated concentrations of each polysaccharide fraction were analyzed for their ability to fix complement, as described. Heparin (150 μg/ml) served as positive control. The data are presented as the means ± SEM of triplicate samples from one experiment that is representative of four independent experiments. Statistically significant differences between control (0 μg/ml) and the indicated concentrations of Fraction A-I (a: $P < 0.001$; b: $P < 0.01$), Fraction A-II (a: $P < 0.001$), Fraction A-III (a: $P < 0.001$), Fraction A-IV (a: $P < 0.001$), and Fraction A-V (a: $P < 0.001$; b: $P < 0.01$) are indicated.

it should be noted that polysaccharides isolated from other plants (e.g., *Biophytum petersianum* Klotzsch and *Veronia kotschyana*) exhibit higher complement-fixing activity with $IC_{50} < 100$ μg/ml (Nergard et al., 2006; Inngjerdingen et al., 2006). Thus, there appears to be quite a bit of variability in level of complement-fixing activity between polysaccharides extracted from different species. Nevertheless, our results suggest the possibility that complement-fixing activity could contribute to the immunomodulatory properties of Artemisia polysaccharides. Indeed, removal of complement by polysaccharide fixation has been proposed to be a potential therapeutic strategy for treating inflammatory diseases (Nergard et al., 2004), and various reports have shown that polysaccharides from different medicinal plants have anti-complement activity [reviewed in (Schepetkin and Quinn, 2006)].

2.5. Effect of Artemisia polysaccharides on neutrophil ROS production

To determine whether neutrophils also responded to Artemisia polysaccharides, we evaluated their effects on murine bone marrow neutrophils. In the absence of any treatment, murine neutrophils spontaneously generated significant levels of ROS (Fig. 6A). Surprisingly, all five polysaccharide fractions appeared to dose-dependently inhibit spontaneous ROS production by murine neutrophils, with fraction A-I being the most inhibitory (Fig. 6B). To evaluate whether this response was specific or not to murine neutrophils, we performed similar studies using human neutrophils and found that Artemisia polysaccharides also seemed to inhibit spontaneous ROS production by unstimulated human neutrophils (Fig. 6C,D). Treatment of human neutrophils with fractions A-I, A-II, A-IV and A-V resulted in dose-dependent loss of the ROS signal. Although fraction A-III was much less potent, it still inhibited ROS produced by human neutrophils at concentrations >200 μg/ml.

Since the inhibition of ROS by neutrophils treated with Artemisia polysaccharides was completely opposite to the activation observed in murine macrophages (see Fig. 3B), we considered the possibility that Artemisia polysaccharides might have ROS scavenging activity and that these cell types could be generating ROS in different cellular locations (i.e., intracellular vs. extracellular). We first analyzed the effect of Artemisia polysaccharides on intracellular ROS production in human neutrophils phagocytosing serum-opsonized zymosan. Phagocytosis of zymosan particles triggers intracellular ROS production, which can be specifically detected by the inclusion of superoxide dismutase (SOD) in the assay buffer to remove any extracellular ROS. In this system, Artemisia polysaccharides did not affect intracellular ROS production (Fig. 6E and F), suggesting the polysaccharides only had access to extracellular ROS.

To evaluate ROS scavenging activity of the polysaccharide fractions in the J774.A1 macrophage system, cells were pretreated with 50 ng/ml LPS for 20 h to elicit maximal ROS production, and chemiluminescence was measured immediately following treatment of the cells with 200 nM phorbol-12-myristate-13-acetate (PMA) with and without the indicated concentrations of polysaccharides. As shown in Fig. 7, none of the fractions reduced the ROS signal, suggesting ROS were being generated intracellularly and were, therefore, inaccessible to the polysaccharides located outside of the cell. To evaluate this possibility, we utilized cell-permeable and cell-impermeable ROS scavengers. Mannitol, a cell-permeable $OH^{\cdot}$ scavenger (Li et al., 1996), significantly reduced the ROS response in this system; whereas, SOD, a cell-impermeable $O_2^{\cdot-}$ scavenger (Dahlgren and Karlsson, 1999), and dextran, a cell-impermeable $OH^{\cdot}$ scavenger (Li et al., 1996), had no ROS scavenging effect (Fig. 7). Thus, we conclude that J774.A1 macrophages primarily produce intracellular ROS, which would be inaccessible to scavenging by cell-impermeable Artemisia polysaccharides.

To directly confirm ROS scavenging activity of Artemisia polysaccharides, we evaluated their ability to scavenge enzymatically-generated $O_2^{\cdot-}/H_2O_2$ (xanthine/xanthine oxidase system) and $OH^{\cdot}$ [H_2O_2/horseradish peroxidase (HRP) system]. Artemisia polysaccharide fractions A-II–A-V exhibited ROS scavenging activity in the xanthine/xanthine oxidase system (Fig. 8A). As a positive control, SOD also inhibited most of the chemiluminescence signal. Addition of Artemisia polysaccharide fractions to an $OH^{\cdot}$-generating system (H_2O_2/HRP) also resulted in

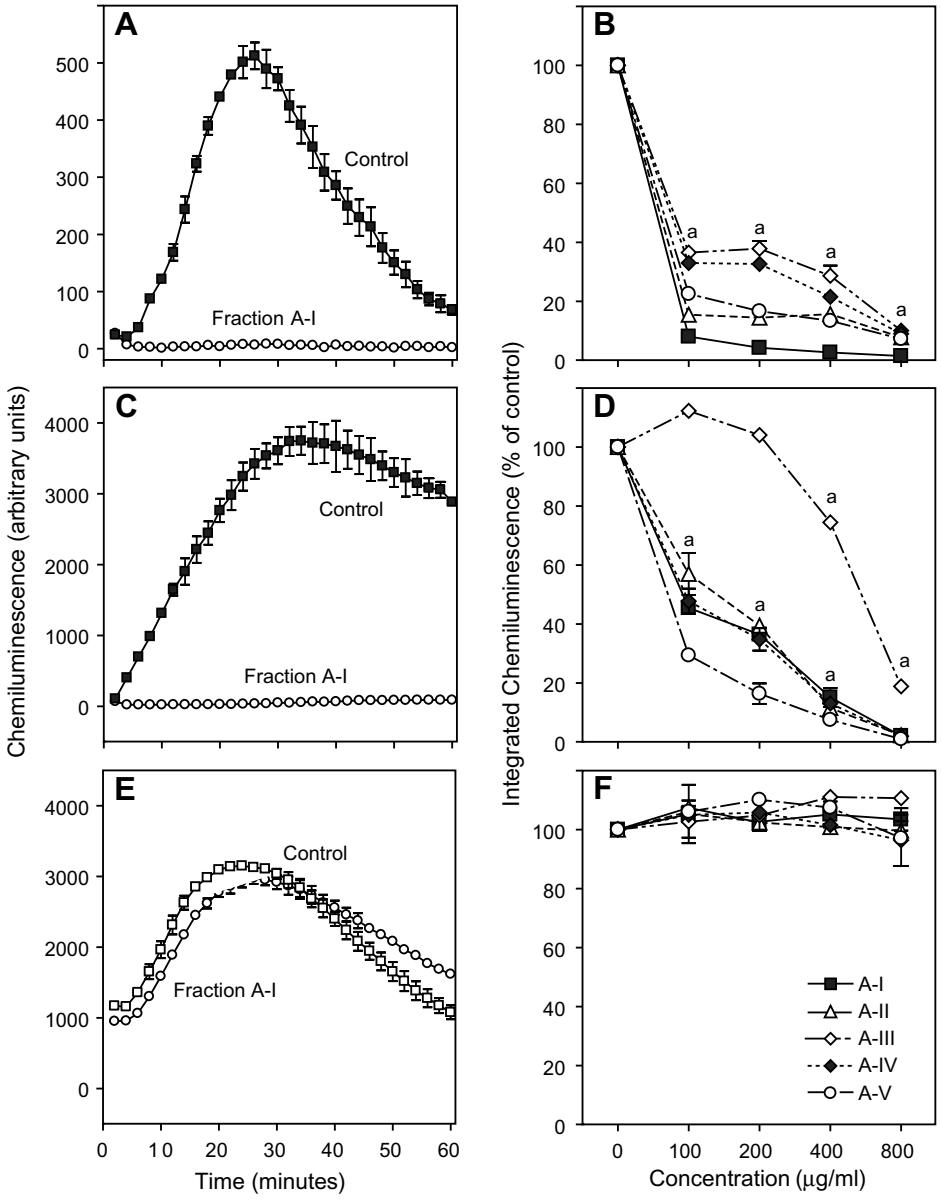

Fig. 6. Effects of Artemisia polysaccharides on ROS production by murine and human neutrophils. Murine neutrophils (5×10^4 cells/well) (Panels A and B) or human neutrophils (10^5 cells/well) (Panels C and D) were treated with 800 μg/ml polysaccharide fraction A-I (Panels A and C) or the indicated concentrations of polysaccharide fractions, and chemiluminescence was monitored immediately for 1 h in the presence of 25 μM L-012 (symbol legend is shown in Panel F). Panels E and F: Human neutrophils (10^5 cells/well) were treated with 800 μg/ml polysaccharide fraction A-I (Panel E) or the indicated concentrations of polysaccharide fractions plus serum-opsonized zymosan particles (100 μg/ml), and chemiluminescence was monitored immediately for 1 h with 25 μM L-012 and 50 U/ml SOD. The data are presented as the means ± SEM of triplicate samples from one experiment that is representative of three independent experiments. Statistically significant differences between control (0 μg/ml) and the indicated concentrations of Fraction A-I (a: $P < 0.001$), Fraction A-II (a: $P < 0.001$), Fraction A-III (a: $P < 0.001$), Fraction A-IV (a: $P < 0.001$), and Fraction A-V (a: $P < 0.001$) are indicated.

dose-dependent scavenging of $OH^{\bullet}$, which was similar to that of the positive controls, mannitol and dextran (Fig. 8B). Thus, these data support the conclusion that the apparent inhibition of neutrophil ROS production by Artemisia polysaccharides was actually due, at least in part, to direct scavenging of neutrophil-generated ROS. Indeed, when neutrophils treated with Artemisia polysaccharides were washed to remove the polysaccharides prior to measuring ROS, we found that fractions A-II and A-III actually enhanced human neutrophil ROS production >6-fold and >8-fold, respectively; whereas, fractions A-I, A-IV, and A-V had little effect on this response (Fig. 9A). In comparison, none of the polysaccharide fractions stimulated murine neutrophil ROS production (Fig. 9B). The reason for the differences in responses between human and murine neutrophils is not clear; however, this may simply reflect differences between bone marrow and peripheral blood neutrophils.

The ability of Artemisia polysaccharides to scavenge ROS may play an important role in the therapeutic

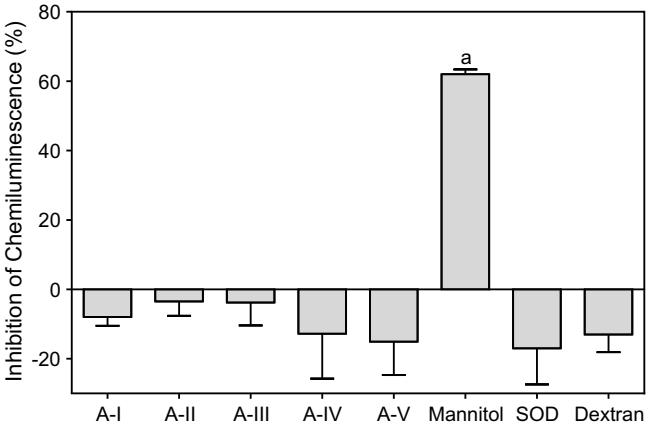

Fig. 7. Analysis of ROS scavenging by Artemisia polysaccharides in J774.A1 macrophages. J774.A1 cells were pre-incubated for 20 h with 50 ng/ml LPS. Following the culture, the cells were treated with 200 nM PMA plus Artemisia polysaccharide fractions (800 μg/ml), mannitol (200 mM), SOD (25 U/ml), or dextran (5 mM). Chemiluminescence was monitored immediately after addition of PMA, and the results are presented as % inhibition of chemiluminescence compared to the response of control PMA-stimulated cells. The data are presented as the means ± SEM of triplicate samples from one experiment that is representative of three independent experiments. Statistically significant differences (a: $P < 0.001$) compared to control samples treated with PMA alone are indicated.

properties of Artemisia extracts. Various other botanical polysaccharides have also been reported to possess ROS scavenging activity. For example, protein-bound polysaccharide from the mycelium of *Coriolus versicolar* (Sakagami et al., 1992) and polysaccharide extracts from other mushroom species (Liu et al., 1997) were found to possess ROS-scavenging activity. Moreover, dextran was reported to function as an $OH^{\cdot}$ scavenger to protect proteins from metal-catalyzed oxidation (Li et al., 1996). Thus, our data are consistent with the putative role of various botanical polysaccharides as ROS scavengers. It should be noted, however, that the large Artemisia polysaccharides were only effective scavengers of extracellular ROS, and ROS generated intracellularly were not accessible.

Neutrophils are principal effectors of the initial host response to injury or infection and constitute a significant threat to invading bacterial pathogens (Tosi, 2005). Thus, the ability to regulate neutrophil function represents an important immunomodulatory property of Artemisia polysaccharides. A number of other plant-derived polysaccharides have been reported to stimulate neutrophil functional activity. For example, chitosan, a polysaccharide of D-glucosamine, accelerates the wound-healing process by enhancing neutrophil phagocytic activity as well as ROS production (Morimoto et al., 2001). Likewise, polysaccharides from *Acacia cyanophylla* stimulated ROS production by murine neutrophils (El Abbouyi et al., 2004), and polysaccharides purified from *Ganoderma lucidum* enhanced human neutrophil phagocytosis and chemotaxis (Hsu et al., 2003).

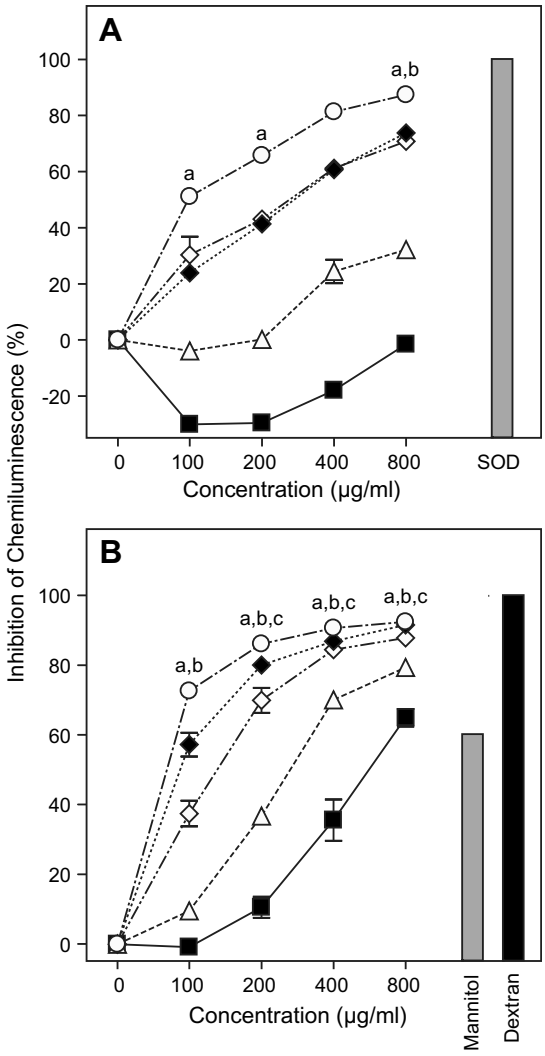

Fig. 8. Scavenging of ROS by Artemisia polysaccharide fractions in cell-free systems. The indicated concentrations of polysaccharide fractions [A-I (■), A-II (△), A-III (◇), A-IV (♦), A-V (○)] were added to the xanthine/xanthine oxidase system (Panel A) or H_2O_2/HRP system (Panel B) containing 25 μm L-012, and chemiluminescence was monitored for 20 min. SOD (25 U/ml) was used as a positive control in the $O_2^{-\cdot}/H_2O_2$-generating system, while mannitol (200 mM) and dextran (5 mM) were used as positive controls in the $OH^{\cdot}$-generating system. The data are presented as the means ± SEM of triplicate samples from one experiment that is representative of two independent experiments. Statistically significant differences in inhibition between control (0 μg/ml) and the indicated concentrations of Fraction A-I (c: $P < 0.001$), Fraction A-II (b: $P < 0.001$), Fraction A-III (a: $P < 0.001$), Fraction A-IV (a: $P < 0.001$), and Fraction A-V (a: $P < 0.001$) are indicated.

3. Conclusion

Although Artemisia has been widely used in traditional remedies, little is known regarding the active component(s) responsible for its therapeutic properties. While previous studies indicated that some of the biological properties of Artemisia were due to the presence of low-molecular weight compounds, such as lignans and sesquiterpene lactones, we have demonstrated here that polysaccharides of Artemisia have potent immunomodulatory properties.

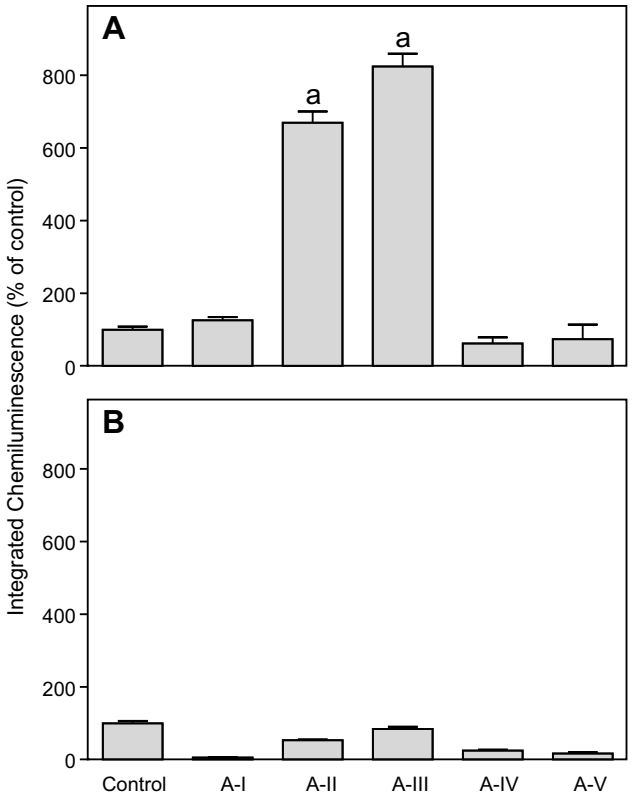

Fig. 9. Analysis of ROS production by polysaccharide-treated neutrophils after polysaccharide removal. Human neutrophils (10^5 cells/well) (Panel A) or murine neutrophils (5×10^4 cells/well) (Panel B) were pre-incubated with polysaccharides (800 μg/ml) for 15 min at 25 °C. The cells were pelleted by centrifugation, the media was aspirated, and the cells were washed again. The washed cells were placed in assay buffer, and L-012 chemiluminescence was monitored for 1 h, as described. The data are presented as the means ± SEM of five replicate samples from one experiment that is representative of four independent experiments. Statistically significant differences (a: $P < 0.001$) compared to control samples are indicated.

We isolated five sulfated polysaccharide fractions from *A. tripartita* and demonstrated that these polysaccharides exhibited immunomodulatory and anti-inflammatory activities, including modulation of macrophage and neutrophil functions, scavenging of extracellular ROS, and complement-fixation. Collectively, these activities could lead to enhanced phagocyte microbicidal responses, while protecting host tissues from excessive oxidant injury and enhancing wound–healing.

4. Experimental

4.1. Reagents

β-Glucosyl Yariv reagent [1,3,5-tri-(4-β-D-glucosopyranosyloxyphenyl-azo)-2,4,6- trihydroxybenzene] was purchased from Biosupplies Australia (Parkville, Australia). Gum arabic was purchased from Fluka BioChemica (Buchs, Switzerland). 8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4-(2H,3H)dione (L-012) was purchased from Wako Chemicals (Richmond, VA). The cytometric bead array kit was purchased from BD Biosciences Pharmingen (San Diego, CA). DEAE-cellulose, Sepharose-6B, dextran from *Leuconostoc mesenteroides*, *N*-(1-naphthyl)ethylenediamine, sulfanilamide, horseradish peroxidase (HRP), Histopaque 1077, zymosan A from *Saccharomyces cerevisiae*, LPS from *Escherichia coli* K-235, xanthine oxidase, xanthine, SOD from bovine erythrocytes, PMA, antibody-sensitized sheep erythrocytes, sodium nitrite ($NaNO_2$), and gelatin veronal buffer (GVB) were purchased from Sigma Chemical Co. (St. Louis, MO). Pullulan standards were purchased from Phenomenex (Torrance, CA).

4.2. Fractionation of polysaccharides

Leaves of *A. tripartita* were collected in the mountain areas of Montana and were taxonomically confirmed. Dried and ground leaves (1.2 kg) were extracted with 10 L boiling distilled H_2O for 1 h, and the aqueous extract was centrifuged at 2000*g* for 15 min. A 4-fold volume of ethanol was added to the supernatant to precipitate the polysaccharides overnight at 4 °C. The precipitate was pelleted by centrifugation, re-dissolved in distilled H_2O, sonicated for 10 min, and centrifuged at 80,000*g* for 1 h. The supernatant was then filtered through a 0.22 μm filter and concentrated in an Amicon concentrator with a 5 kDa PM5 membrane to obtain a crude extract (yield of 1.2% by weight).

The crude polysaccharide extract was further purified using ion-exchange chromatography on a DEAE-cellulose column equilibrated with 0.05 M Tris–HCl buffer (pH 8.0). The column was washed with equilibration buffer, and bound material was eluted with equilibration buffer containing 2 M NaCl. The eluate was concentrated to obtain the crude acid polysaccharide extract, which was then fractionated by size-exclusion chromatography on a Sepharose-6B column (2.5×95 cm) equilibrated with 0.01 M sodium citrate buffer (pH 7.0) containing 0.15 M NaCl and eluted with the same buffer at a flow rate of 21 ml/h. The carbohydrate elution profile was determined by the phenol–H_2SO_4 method (Dubois et al., 1956), modified to a microplate format, and absorbance was measured at 488 nm using a SpectraMax Plus microplate reader (Molecular Devices, Palo Alto, CA). The relevant fractions were pooled and concentrated. For analysis of biological activity, the fractions were diluted in Hanks balanced salt solution (HBSS) to a concentration of 5 mg/ml and filtered through sterile 0.22 μm filters.

4.3. Characterization of polysaccharide fractions

Samples were analyzed for protein content using a modified Lowry assay (Sigma) with bovine serum albumin as a standard. Sulfate determination was carried out by the sodium rhodizonate method (Terho and Hartiala, 1971).

For nuclear magnetic resonance (NMR) analysis, samples (5 mg) were dissolved in deuterium oxide (0.5 ml), and ^{1}H NMR spectra were recorded on a Bruker DRX-600 spectrometer (Bruker BioSpin, Billerica, MA) at 20 °C using 3-(trimethylsilyl)-propionic 2,2,3,3,-d_4 acid sodium salt as an internal reference.

The presence of arabinogalactan in the samples was detected by single radial gel diffusion in a 1% agarose gel containing 100 μg/ml β-glucosyl Yariv reagent, which selectively interacts with and precipitates compounds containing type II arabinogalactan structures (van Holst and Clarke, 1985). Four μl of polysaccharide samples (5 mg/ml) were loaded into the wells, and the samples were incubated at room temperature for 24 h in a humid atmosphere. A positive reaction was identified by a reddish circle around the well, and arabic gum (4 mg/ml) served as a positive control.

The homogeneity and average molecular weight of the polysaccharide fractions were determined by HP-SEC using a Shimadzu Class VP HPLC and Shodex OHpak SB-804 HQ column (8 mm × 300 mm) eluted with 50 mM sodium citrate buffer, pH 7.5, containing 0.15 M NaCl and 0.01% NaN_3 at a flow rate of 0.3 ml/min. Peaks were detected using a refractive index detector (RID-10A; Shimadzu, Torrance, CA). The molecular weights of the polysaccharide fractions were estimated by comparison with retention times of pullulan standards (P-800, 400, 200, 100, 50, 20, and 10).

Samples were tested for possible LPS contamination using a rhodamine-labeled peptide (EndoDtec-F, BioDtech Inc.) derived from the sushi 3 domain of horseshoe crab Factor C (Tan et al., 2000). This peptide binds to LPS with high affinity and specificity (Tan et al., 2000), and we showed previously that EndoDtec-F binding provided a sensitive measurement of LPS in plant-derived polysaccharide samples (Schepetkin et al., 2005). EndoDtec-F (250 ng) was mixed with 5 ng LPS or 5 μg polysaccharide fractions, and 10 μl samples were analyzed by HP-SEC as described above, but at a flow rate of 0.5 ml/min. The elution was monitored using tandem refractive index (RID-10A) and fluorescence detectors (RF-10A, Shimazdu). Fluorescence peaks were detected using excitation and emission wavelengths of 550 and 580 nm, respectively.

4.4. Monosaccharide composition analysis

Buffer salts were removed from the polysaccharide samples by repeated (6×) concentration in an Amicon concentrator (5 kDa cut-off PM5 membrane) and dilution with a 10-fold volume of distilled H_2O. The final concentrates and background blanks (distilled H_2O) were lyophilized and submitted for analysis. Monosaccharide analysis was performed by the Oklahoma Center for Glycobiology Analytical Core Lab (Oklahoma City, OK). Briefly, polysaccharide samples or background blanks were subjected to methanolysis (methanolic 2 M HCl, 16 h, 80 °C), followed by acid hydrolysis (2 M trifluoroacetic acid, 4 h, 100 °C), and the resulting monosaccharide mixtures were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex DX-600 HPAEC system equipped with an ED50 detector (Dionex Corporation, Sunnyvale, CA). The samples were separated on a Dionex CarboPac PA20 column eluted isocratically with 12 mM NaOH at a flow rate of 1 ml/min at 22 °C. For analysis of uronic acids, the column was eluted with 10 mM NaOH for 20 min, followed by a gradient of 100 mM NaOH/150 mM sodium acetate (0–100% in 45 min). Background signals were subtracted from all samples, and individual components were quantified based on electrochemical detection relative to known standards (De Ruiter et al., 1992; Hardy and Townsend, 1994).

4.5. Cell culture

Murine macrophage J774.A1 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were grown to confluence in sterile tissue culture flasks and gently detached by scraping. Cell number and viability were assessed microscopically using trypan blue exclusion.

4.6. Isolation of human and murine neutrophils

Human neutrophils were purified in accordance with a protocol approved by the Institutional Review Board at Montana State University. Briefly, these cells were isolated from peripheral blood of healthy donors using dextran sedimentation followed by Histopaque 1077 gradient separation and hypotonic lysis of erythrocytes, as described previously (Gauss et al., 2006). Isolated neutrophils were washed and resuspended in HBSS without Ca^{2+} and Mg^{2+}. Cell viability was determined by using the trypan blue exclusion test (>98%), and the purity of neutrophils was determined by light microscopy (>95%).

Murine neutrophils were isolated and purified from bone marrow following previously described methods (Schepetkin et al., 2007). All animal use was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Montana State University. Briefly, leukocytes were flushed from tibias and femurs of BALB/c mice with HBSS containing 0.1% bovine serum albumin and 1% glucose, filtered through a 70 μm nylon cell strainer (Becton Dickson, Franklin lakes, NJ) to remove cell clumps and bone particles, and pelleted by centrifugation. The bone marrow leukocyte pellets were resuspended in 3 ml of 45% Percoll solution and layered on top of a Percoll gradient consisting of 2 ml each of 50, 55, 62, and 81% Percoll solutions in a conical 15-ml polypropylene tube, and the gradient was centrifuged at 1600*g* for 30 min at 10 °C. The neutrophil band located between 61 and 81% Percoll was collected, washed, layered on top

of Histopaque 1119, and centrifuged at 1600*g* for 30 min at 10 °C to remove contaminating erythrocytes. The purified neutrophils were collected, washed, and resuspended in HBSS without Ca^{2+} and Mg^{2+}.

4.7. Measurement of NO production

J774.A1 cells were plated at a density of 1.5×10^5 cells/well in a final volume of 200 μl in 96-well flat-bottomed tissue culture plates and incubated in medium alone or medium containing various concentrations of polysaccharide fractions or *E. coli* LPS as a positive control. Cells were incubated at 37 °C in the presence of 5% CO_2 for 24 h, and 100 μl of the cell culture supernatants were removed and analyzed for NO using a colorimetric method with $NaNO_2$ as a standard, as described previously (Schepetkin et al., 2005). Briefly, supernatants were mixed with an equal volume of Griess reagent, which was prepared by mixing one part of 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine with one part of 1% (w/v) sulfanilamide in 5% phosphoric acid. After 20 min, absorbance was measured at 540 nm using a SpectraMax Plus microplate reader.

4.8. Analysis of phagocyte ROS production

Macrophage ROS production was analyzed using the chemiluminescent probe, L-012, which is highly sensitive for ROS generated in biologically complex systems (Imada et al., 1999; Daiber et al., 2004). Murine J774.A1 macrophages (1.5×10^5 cells/well) were incubated with various concentrations of polysaccharide fractions or positive control LPS for 24 h. After incubation, 100 μl of culture supernatant (taken for NO or cytokine analysis) was replaced by an equal volume of HBSS supplemented with 25 μM L-012 and 5 μg/ml horseradish peroxidase, as described previously (Schepetkin et al., 2007). The reaction was monitored on a Fluoroskan Ascent FL microtiter plate reader (ThermoElectron, Milford, MA) at 37 °C. Chemiluminescence was measured every 2 min for 2 h and is expressed as the integrated response over this time (arbitrary units).

To evaluate Artemisia polysaccharide effects on neutrophil ROS production, human neutrophils (10^5 cells/well) or murine neutrophils (5×10^4 cells/well) were added to microplate wells containing the indicated concentrations of polysaccharide fractions plus 25 μM L-012, and chemiluminescence was monitored at 37 °C for 1 h, as described above. To evaluate effects of polysaccharides on intracellular ROS production, human neutrophils were added to microplate wells containing serum-opsonized zymosan particles (100 μg/ml), 25 μM L-012, and 50 U/ml SOD, and chemiluminescence was monitored at 37 °C for 1 h (Schepetkin et al., 2006). Serum-opsonized zymosan was prepared by incubating zymosan A with human serum for 45 min at 25 °C. After centrifugation (800*g* for 10 min), the zymosan particles were washed and resuspended in HBSS (without Ca^{2+} and Mg^{2+}).

To evaluate polysaccharide effects on ROS production after polysaccharide removal, human or murine neutrophils were pre-incubated with polysaccharides fractions for 15 min at 25 °C. After centrifugation of the samples at 82*g* for 5 min to pellet the cells, the media was aspirated, and the cells were washed with HBSS. The washed cells were placed in L-012 assay buffer, and chemiluminescence was monitored at 37 °C for 1 h.

4.9. Cytokine determination

J774.A1 cells were incubated at a density of 1.5×10^5 cells/well in final volume of 200 μl for 24 h with or without Artemisia polysaccharide fractions or LPS. A cytokine cytometric bead array was used to simultaneously detect interleukins (IL)−6 and −10, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1. Standard curves (ranging from 0 to 5000 pg/ml) for the assay system were obtained from dilutions of the mixed pool of all four cytokines that was provided with the kit. The concentration for each cytokine in the cell supernatants was determined by extrapolation from the corresponding standard curve using the CBA Software (BD Biosciences Pharmigen).

4.10. Complement-fixing assay

The complement-fixing assay was performed as previously described by Diallo et al. (Diallo et al., 2001). Antibody-sensitized sheep erythrocytes were washed three times with GVB containing 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+} (GVB^{2+}) before use. The erythrocytes were resuspended in GVB^{2+} at a concentration of 2×10^8 cells/ml, and human serum was diluted with GVB^{2+} to a concentration giving about 50% hemolysis. Triplicate samples containing 50 μl of each serially-diluted polysaccharide fraction were mixed with 50 μl diluted serum and added to microplate wells and incubated at 37 °C. After 30 min, the sensitized sheep erythrocytes (50 μl) were added to each well, and the samples were incubated for an additional 30 min at 37 °C. After centrifugation (900*g* for 5 min), 50 μl of the supernatants was mixed with 200 μl distilled H_2O in flat-bottom microplates, and absorbance was measured at 405 nm. One hundred percent lysis was obtained by adding distilled H_2O to sensitized sheep erythrocytes. Samples containing GVB^{2+}, serum, and sensitized sheep erythrocytes were used as background controls ($A_{control}$), while heparin served as a positive control. Inhibition of hemolysis induced by the test sample was calculated by the formula: $[(A_{control} - A_{sample})/A_{control}] \times 100\%$ (Diallo et al., 2001).

4.11. Evaluation of ROS scavenging activity

ROS ($O_2^{-\cdot}/H_2O_2$) were generated enzymatically using a xanthine/xanthine oxidase system consisting of 500 μM xanthine, 18 mU/ml xanthine oxidase, and 0.1 M phosphate buffer (pH 7.5). This system produces $O_2^{-\cdot}$, which

can spontaneously dismutate to H_2O_2, but does not generate OH· (Britigan et al., 1990). L-012 (25 μM) was used as a probe to measure ROS in this system. ROS-scavenging activity was evaluated in the presence or absence of polysaccharide factions at 25 °C. SOD (25 U/ml) was used as a positive control for O_2^{-}·-scavenging activity.

OH· was generated enzymatically using an H_2O_2/HRP system consisting of 7 μM H_2O_2, 0.25 μg/ml HRP, 25 μM L-012, and 0.1 M phosphate buffer (pH 7.5), and OH· production was determined by monitoring L-012 chemiluminescence. Scavenging activity was evaluated by monitoring OH· in the presence or absence polysaccharide fractions at 25 °C. Mannitol (200 mM) and dextran (5 mM) were used as positive controls for OH·-scavenging activity.

4.12. Cytotoxicity assay

Cytotoxicity was analyzed with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega Inc., Madison, WI) according to the manufacturer's protocol. Briefly, J774.A1 cells were cultured at a density of 3×10^4 cells/well with the polysaccharide fractions for 24 h at 37 °C and 5% CO_2. Following treatment, the cells were allowed to equilibrate to room temperature for 30 min, substrate was added, and the samples were analyzed with a Fluoroskan Ascent FL.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.01.009.

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