Carbohydrate Polymers 78 (2009) 738–742

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Extraction, characterization of *Astragalus* polysaccharides and its immune modulating activities in rats with gastric cancer

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ARTICLE INFO

Article history:
Received 5 May 2009
Received in revised form 3 June 2009
Accepted 9 June 2009
Available online 13 June 2009

Keywords:
Gastric cancer
Polysaccharides of *Astragalus*
Rat
HPLC
Immunity

ABSTRACT

One major polysaccharide fractions, glucose, were isolated from the polysaccharides extract of *Astragalus* (AP), a valuable traditional Chinese medicine, using thin-layer chromatography (TLC) and Sephadex G-100 chromatography. HPLC and IR methods were used for a qualitative and quantitative determination of from polysaccharides of *Astragalus*. The HPLC method was validated for linearity, precision and accuracy. The results indicated that polysaccharides of *Astragalus* is an α-(1 → 4)-d-glucan with α-(1 → 6)-linked branches attached to the O-6 of branch points. Bioactivity tests showed that polysaccharides of *Astragalus* is active for spleen lymphocytes proliferation. The polysaccharides also presented anti-inflammatory activities. These data together suggest that polysaccharides of *Astragalus* presents significant immune modulating activity, thus supporting the popular use of the polysaccharides in the treatment of gastric cancer diseases.

1. Introduction

A wide variety of biological activities from medicinal plants have recently been reported, in addition to their traditional medicinal effects. Herbal medicines have attracted considerable interest as alternative cancer remedies because of their low toxicity and costs. The roots of *Astragalus* provide a traditional Chinese medicine, which is described in the 2005 version of the "Chinese Pharmacopoeia". Many reports on the biological activities of *Astragalus* indicate that it shows protective effects against hepatic injury, anti-peroxidation of lipids, inhibition of platelet aggregation, reduction of serum lipids, anti-inflammatory effects and immune enhancement through specific and non-specific immunity (Grover, Yadav, & Vats, 2002; Mao, Xie, & Gu, 2002).

Gastric cancer is the second commonest cause of death from malignant disease worldwide (Neugut, Hayek, & Howe, 1996). Although it is the worldwide trend that cancer-related deaths have decreased during the recent two decades, gastric cancer still accounts for about 23.2% of all cancer deaths (Brown & Devesa, 2002; Crew & Neugut, 2006; Sun et al., 2004). Nowadays, surgery and chemotherapy are the mainstream therapeutic methods for gastric cancer in China, but the existing chemotherapeutic drugs do not have ideal curative effects and meanwhile have many undesirable side effects. Consequently, combination of multi-therapeutic methods may effectively improve the treatment of gastric cancer patients, minimize their symptoms and prolong their survival time (Kodera, Fujiwara, Koike, & Nakao, 2006).

The aim of this study is therefore, to investigate the potential immune modulating activities of AP extract in rats with gastric cancer and its chemical constituents.

2. Material and method

2.1. Extraction of polysaccharides from Astragalus neutral

Fresh commercially obtained *Astragalus* roots were used for isolation of *Astragalus* polysaccharide.

The fresh plant material (300 g) was extracted twice with water (2 l) for 2.5 h at 100 °C. The combined extracts were concentrated to 250 ml using a rotary evaporator (BC-R203, Shanghai Biochemical Equipment Co., Shanghai, China) at 65 °C under vacuum. The proteins in the extract were removed by Sevag reagent (Niea, Xie, Fu, Wan, & Yan, 2008). After removal of the Sevag reagent, 100 ml of anhydrate ethanol was added before the mixture was maintained overnight at 4 °C to precipitate polysaccharides. The crude polysaccharides (25 g) was obtained by centrifugation at 3860*g* for 15 min.

Abbreviations: AP, *Astragalus* polysaccharides; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy; LgA, immune globulin A; LgM, immune globulin M; LgG, immune globulin G; NK, natural killer; IL-2, interleukin-2.

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doi:10.1016/j.carbpol.2009.06.005

2.2. TLC analysis

Two-dimensional thin-layer chromatography (TLC) analysis was performed with Merck DC-Alufolien cellulose plates ($20 \times 20 \times 0.1$ mm); first dimension: upper phase of the mixture methyl isobutylketone–formic acid–H_2O (3:1:2; v/v/v); second dimension: formic acid–H_2O (6:94; v/v). Polysaccharides compounds separated on the plates were visualized under UV light in the presence of liquid nitrogen and after fuming with ammonia.

2.3. Sephadex G-100 chromatography and IR

The concentrated preparation was applied to a 1.5×120-cm column of Sephadex G-100 (Pharmacia, Sweden) equilibrated with a pH 6.0 phosphate buffer containing 20 mM Na/K and 0.15 M NaCl. Elution was done with the same buffer at 15 ml h^{-1}. Each fraction with 1 ml of eluate was collected. Fractions (3.5 ml each) were collected and combined in accord with the results of analysis by the anthrone–sulfuric acid procedure (Morris, 1948). The chromatography profile was drawn by Microsoft Excel 2000 (Microsoft, Seattle, WA). The peak with the highest polysaccharide content was collected and then freeze-dried.

Infrared spectra (IR) were also used to identify the polysaccharides compounds. The infrared spectra (450–4000 cm^{-1}) of all the subfractions (EA1–EA7) were recorded in potassium bromide (KBr) disks with a Fourier transform IR spectrophotometer (Bio-Rad FTS-135). One milligram of dry sample was mixed with 100 mg of dry KBr, and the mixture was pressed into a disk for spectrum recording.

2.4. HPLC analysis

AP were hydrolyzed with 2 M H_2SO_4 for 5–6 h at 121 °C in sealed glass test tube. After complete hydrolysis, content was neutralized with $BeCO_3$ and filtered. Monosaccharide composition of the hydrolysate was determined by HPLC (Waters Alliance, 2996-seperation module) using Supelco gel 610H column (30 cm × 7.8 mm) and RI (2414) detector with flow rate 0.4 ml/min at temperature 30 °C and mobile phase, 0.17% H_3PO_4 in water (Meisen, Wingender, & Telgheder, 2008). The relative proportion of the peak area was calculated to estimate the monomer composition.

2.5. Animals

Male wistar rats 6–7 weeks old, weighing 200–250 g were purchased from the National Institute of Nutrition, Suzhou university, China, and maintained in the Central Animal House, Suzhou University. The animals were housed in groups of four or five in polypropylene cages and provided standard pellet diet and water ad libitum and maintained under controlled conditions of temperature and humidity, with a 12 h light/dark cycle. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Suzhou University in accordance with the China National Law on animal care and use.

2.6. Experimental design

The Institutional animal ethical committee, Suzhou University, China, approved the experimental design. A total of 40 male wistar rats were divided into five groups of eight each. Stomach carcinogenesis was developed in rats (group II–V) according to the method of Li and Xue (2006). Group III mice were orally administered AP (100 mg/kg b.w in 2 ml distilled water) once daily for 5 weeks. Group IV animals were orally administered AP (200 mg/kg b.w in 2 ml distilled water) once daily for 5 weeks. Group V animals were orally administered AP (300 mg/kg b.w in 2 ml distilled water) once daily for 5 weeks. Group I and II animals orally received an equal volume of saline. All animals were allowing to free access to water and fed with standard commercial pelleted rat chaw. At the end of the experimental period all the animals were sacrificed by cervical dislocation.

2.7. Blood and tissue collection

Blood and spleen were obtained for further analysis. Blood was allowed to clot and the serum was separated by centrifugation at 3000 rpm for 10 min at 4 °C, and then stored at −80 °C. Serum was used to analyze immunity activity and spleen was used to analyze proliferation rate.

2.8. Biochemical analysis

Spleen Lymphoproliferation rate was measured according to the method of Girón-Pérez, Zaitseva, Casas-Solis, and Santerre (2008). Blood LgA, LgM and LgG levels were measured with a commercially available ELISA kit. Natural killer (NK) cells activity was measured according to the method of Li, Yuan, & Farzana Rashid (2009). Spleen CD^{4+}/CD^{8+} was measured according to the method of Salem et al. (2009).

2.9. Statistical analysis

Data were analyzed using the SPSS 14.0 software. One-way ANOVA followed by Duncan's multiple range test was used to compare the parameters among the different groups.

3. Result and discussion

3.1. TLC separation

The extracts were pre-purified using a method described below and modified as described previously (Vidanarachchi, Iji, Mikkelsen, Sims, & Choct, 2009). The method involves a two-stage separation on a pre-coated silica gel 60 F254 TLC plate. The TLC run did not separates polysaccharides sample. The TLC analyses revealed glucose as the predominant compound in the extract (Fig. 1).

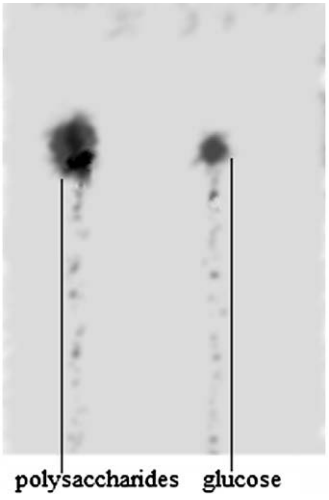

Fig. 1. TLC analysis.

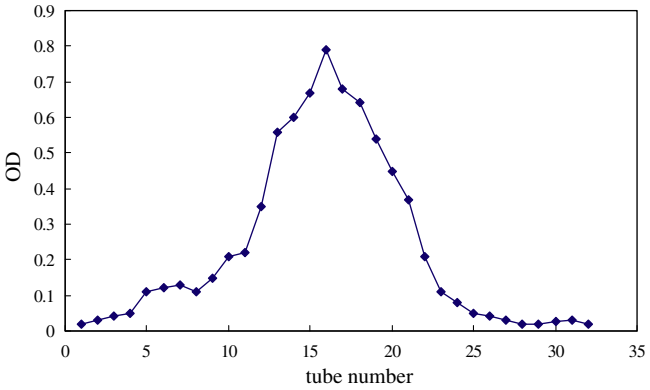

Fig. 2. Purification of AP by Sephadex G-100 chromatography.

3.2. Isolation of active compounds

The polysaccharides extract on further purification using silica gel column chromatographic technique, finally yielded one fraction (Fig. 2).

3.3. Preparative HPLC separation

The HPLC analyses of the polysaccharides showed one main peak component, detected with an ELSD system (Fig. 3a). The preparative HPLC separations of standard samples finally yielded five compounds corresponding to peaks 1, 2, 3, 4 and 5 (Fig. 3b). From the comparison of retention time of the six standard compounds in HPLC analyses, we found that the polysaccharides were composed of glucose. That means that glucan appears as the major compound in the polysaccharides extract.

In a previous study (Li & Zhang, 2009), elemental analysis found to be free of nitrogen, indicating it was a neutral polysaccharide. The GPC profile showed a single and symmetrically sharp peak, indicating that AP was a homogeneous polysaccharide, with a weight-average molecular weight of 3.6×10^4 Da. AP was composed of only glucose monomers. These finds are agreement with our experimental results.

3.4. IR spectrum

Fig. 4 shows the IR spectrum of the polysaccharides extract. The absorption band at 2360.7 and 2337.6 cm^{-1} is assigned to the hydroxyl (OH) group. The absorption bands at 1639 and

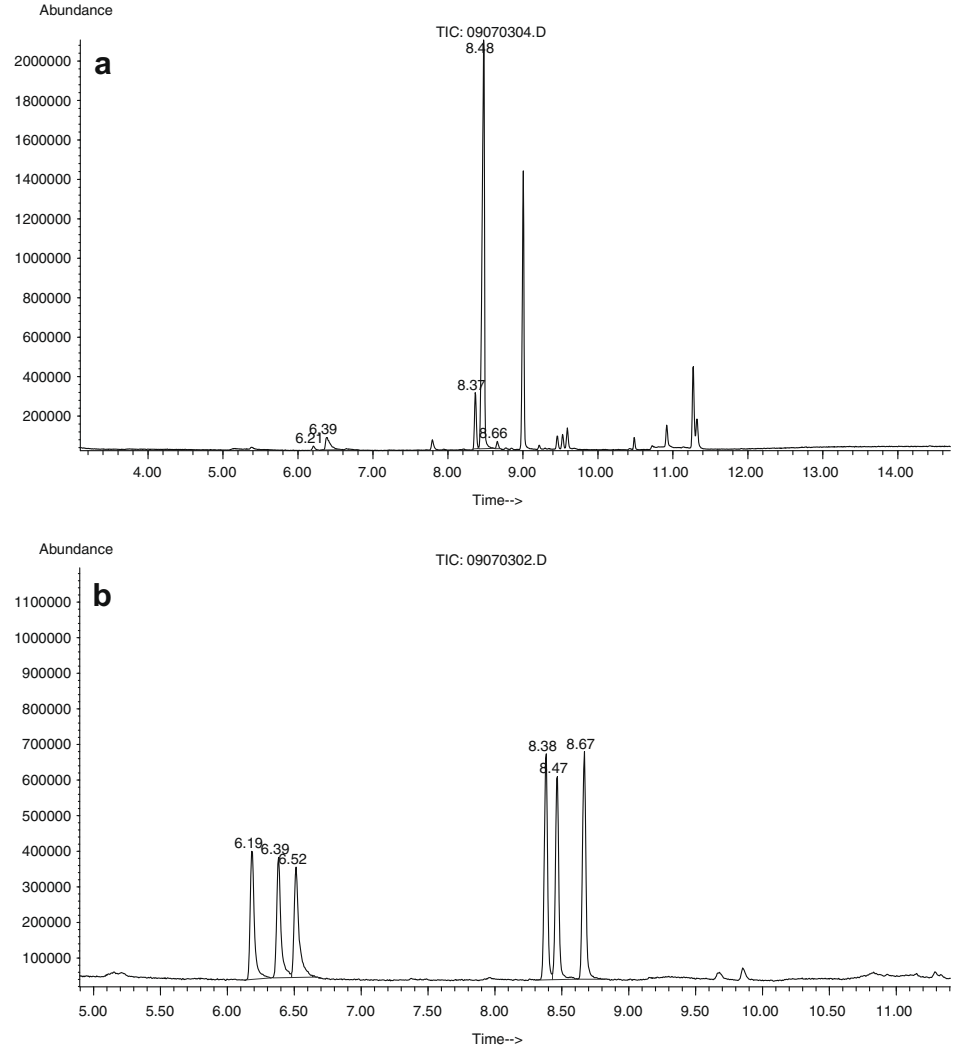

Fig. 3. (a) HPLC of AP; (b) HPLC of standard sample.

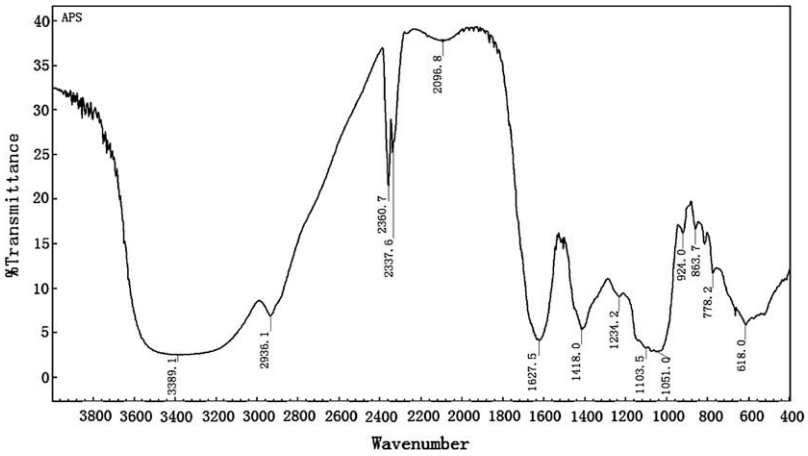

Fig. 4. Infrared spectroscopy of AP.

1538 cm^{-1} are attributed to the stretching vibration of the C–O bond of carboxyl group. The absorption band at 1035 and 956 cm^{-1} suggests that the extract contains pyrene monomer in its structure.

The band at 850.81 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide (Barker, Bourne, Stacey, & Whiffen, 1954). The bands at 850.81 and 915.56 cm^{-1} were characteristic of (1 → 4)-α-glucan (Li et al., 2008).

Table 1
Effect of AP on spleen lymphocytes proliferation and IL-2, NK activity.

Group	Proliferation rate (%)	IL-2 (OD)	NK (%)
Control (I)	0.505 ± 0.031	4.4732 ± 0.0231	21.5481 ± 1.0972
Model (II)	0.311 ± 0.013[a]	2.8952 ± 0.0211[a]	7.9494 ± 0.2769[a]
ACP (III)	0.394 ± 0.021[b]	3.2411 ± 0.0234[b]	12.6841 ± 0.9573[b]
ACP (IV)	0.463 ± 0.023[b]	3.7832 ± 0.0274[b]	17.3525 ± 1.0043[b]
ACP (V)	0.513 ± 0.028[b]	4.2852 ± 0.0507[b]	20.4825 ± 1.7404[b]

[a] $P < 0.01$, vs control group (I).
[b] $P < 0.01$, vs model group (II).

Table 2
Effect of AP on blood LgA, LgM and LgG levels.

Group	Lg A (g/L)	Lg M (g/L)	Lg G (g/L)
Control (I)	10.34 ± 0.78	5.32 ± 0.24	7.22 ± 0.47
Model (II)	6.78 ± 0.33[a]	3.06 ± 0.14[a]	4.26 ± 0.21[a]
ACP (III)	8.34 ± 0.42[b]	4.37 ± 0.12[b]	5.98 ± 0.11[b]
ACP (IV)	8.92 ± 0.37[b]	4.93 ± 0.19[b]	6.54 ± 0.18[b]
ACP (V)	9.71 ± 0.52[b]	5.21 ± 0.15[b]	7.14 ± 0.13[b]

[a] $P < 0.01$, vs control group (I).
[b] $P < 0.01$, vs model group (II).

Table 3
Effect of AP on CD^{4+}, CD^{8+} and CD^{4+}/CD^{8+}.

Group	CD^{4+} (%)	CD^{8+} (%)	CD^{4+}/CD^{8+}
Control (I)	64.64 ± 3.53	36.48 ± 1.32	1.75 ± 0.09
Model (II)	47.83 ± 2.77[a]	37.43 ± 1.42	1.31 ± 0.07[a]
ACP (III)	53.36 ± 3.09[b]	38.39 ± 1.11	1.42 ± 0.08[b]
ACP (IV)	58.48 ± 4.62[b]	37.27 ± 2.65	1.63 ± 0.05[b]
ACP (V)	63.52 ± 3.49[b]	39.63 ± 3.28	1.59 ± 0.08[b]

[a] $P < 0.01$, vs control group (I).
[b] $P < 0.01$, vs model group (II).

3.5. Effect of polysaccharides on immunity activities of rats with stomach cancer

As a first step towards understanding how polysaccharides conditionally promotes antibody responses in vivo, proliferation studies on purified populations of spleen lymphocytes have been performed. In this study, we found that proliferation of antigen receptor-stimulated rat peripheral blood lymphocyte cells was significantly ($P < 0.01$) inhibited in rats with stomach cancer (Table 1). The AP administration dose-dependently significantly ($P < 0.01$) increased proliferation of spleen lymphocytes. We also found that AP administration dose-dependently significantly ($P < 0.01$) increased blood IL-2 levels and NK activities (Table 1).

There was significant ($P < 0.01$) decrease in blood LgA, LgG and LgM levels of model rats (group II) as compared with the control rats (group I) (Table 2). The AP treatment dose-dependently significantly ($P < 0.01$) increased the blood LgA, LgG and LgM levels of rats with stomach cancer (group III, IV and V) (Table 2).

There was significant ($P < 0.01$) decrease in CD^{4+} and CD^{4+}/CD^{8+} of model rats (group II) as compared with the control rats (group I) (Table 3). However, there was not significant ($P > 0.01$) change in CD^{8+} between all groups. AP treatment resulted in significant ($P < 0.01$) increase in CD^{4+} and CD^{4+}/CD^{8+} when compared to model rats (group II) (Table 3).

Glucans are a basis of fungal cell wall structure. They are not found in animals, so that as carbohydrates they can be recognized by the innate immune system of vertebrates (Brown & Gordon, 2003). In vitro experiments showed that β-glucans can directly activate leukocytes, stimulating their phagocytic, cytotoxic, and antimicrobial activity. In addition, they can stimulate the production of proinflammatory mediators, such as cytokines and chemokines (Schepetkin & Quinn, 2006), and have anti-tumor (Mizuno et al., 1990), anti-oxidative (Toklu et al., 2006), anti-inflammatory (Dore et al., 2007), and immunomodulating (Zhang, Cui, Cheung, & Wang, 2007) activities. Our work showed that AP was composed of glucan. This just explains the mechanism of immune modulating activity of AP.

Together, these results indicated that the AP treatment could stimulate immunity activities in rats with stomach cancer.

Acknowledgement

The work was supported by the National Natural Science Foundation of China (No. 30872943).

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