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Anticancer and immunostimulating activities of a novel homogalacturonan from *Hippophae rhamnoides* L. berry

Hailiang Wang[a,1], Tingting Gao[b,c,1], Yuzhi Du[b,c], Hongxia Yang[b,c], Lixin Wei[b,c], Hongtao Bi[b,d,*], Weihua Ni[e,**]

[a] Department of Neurosurgery, The Second Hospital of Jilin University, Changchun 130033, China
[b] Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China
[c] Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China
[d] Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810000, China
[e] Department of Immunology, College of Basic Medical Science, Jilin University, Changchun 130021, China

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ABSTRACT

Our previous study isolated an anti-fatigue polysaccharide (HRWP) from the *Hippophae rhamnoides* berry. In this study, using ion-exchange chromatography and gel filtration chromatography in turn, a water-soluble homogenous polysaccharide HRWP-A was isolated from HRWP. Structural analysis determined that HRWP-A was a polysaccharide with repeating units of (1 → 4)-β-D-galactopyranosyluronic residues, of which 85.16% were esterified with methyl groups. An antitumor activity assay showed that HRWP-A could significantly inhibit the Lewis lung carcinoma (LLC) growth in tumor-bearing mice. Further experiments suggested that the antitumor effect of HRWP-A might be mediated through immunostimulating activity, as it enhances the lymphocyte proliferation, augments the macrophage activities, as well as promoting NK cell activity and CTL cytotoxicity in tumor-bearing mice. To our knowledge, this is the first report on a natural antitumor high-methoxyl homogalacturonan pectin from the *H. rhamnoides* berry—a compound that acts as a potential immunostimulant and anticancer adjuvant.

1. Introduction

Current therapies against cancer are still unsatisfactory, mainly because of their side effects. In recent years, natural polysaccharides have been described as biological response modifiers (BRMs), and their enhancement of the host defense has been recognized as a possible means of inhibiting tumor growth with no harm to the host (Leung, Liu, Koon, & Fung, 2006). The activity of polysaccharides might be caused and influenced by their glycosidic linkages, chain length, the number of branched points, molecular size and tertiary structure (Raveendran Nair et al., 2004). The unique structure diversities and physiochemical properties can be utilized successfully in various medical applications, and many polysaccharides have shown promising potential as antitumor agents, such as β-glucans, amylopectin-like polysaccharides and pectins (Brown, 2006; Cao, 2013; Jin, Zhao, Huang, & Shang, 2014; Silva et al., 2012; Sun, 2011). These polysaccharides usually have low toxicity and few side effects, which make them appropriate for immunotherapy against tumors.

The *Hippophae rhamnoides* L. berry has been a traditional medicinal food of the Tibetan plateau for thousands of years that is recorded in the classic Tibetan pharmacological book "Crystal Pearl of Materia Medica" (Chinese pinyin: jingzhubencao) and has been used for relieving cough, aiding digestion, invigorating

* Corresponding author at: Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China. Tel.: +86 971 6143765; fax: +86 971 6143765.
** Corresponding author at: Department of Immunology, College of Basic Medical Science, Jilin University, Changchun 130021, China. Tel.: +86 431 85619476; fax: +86 431 85619476.
E-mail addresses: bihongtao@hotmail.com (H. Bi), 65559211@qq.com (W. Ni).
[1] These authors contributed equally to this work.

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blood circulation and alleviating pain since ancient times (Dierma, 2012; Ma et al., 2011). To date, studies on this berry have mainly focused on small molecular compounds of the *H. rhamnoides* berry, but very few studies have reported on its polysaccharide content (Dongowski, 1996; Ni et al., 2013; Wang et al., 2001). Wang et al. (2001) isolated a type of neutral heteropolysaccharide with many branches from *H. rhamnoides* by alkaline solution. Recently, our research group isolated a water-soluble polysaccharide (HRWP) from *H. rhamnoides* berry, which was identified as being a mixture of homogalacturonan and glucan with good anti-fatigue activity (Ni et al., 2013). To further elucidate the constitution, structural features and pharmaceutical values of the water-soluble polysaccharide from the *H. rhamnoides* berry, further separation, structural analysis and anti-tumor and immunological activity assays were performed in the present study, which is the first such study to do so. This and our previous work aim to effectively utilize the distinctive resource of *H. rhamnoides* berries from the Tibetan plateau.

2. Materials and methods

2.1. Materials and chemicals

The fresh berries of *Hippophae rhamnoides* L. were collected from Dongshangen of Dulan Country (N36.321, E98.111; 3100 m altitude), Haixi national municipality of Mongol and Tibetan, Qinghai, China, and were identified by Prof. Xuefeng Lu, Northwest Plateau Institute of Biology, Chinese Academy of Sciences. The herbarium sample of *H. rhamnoides* was numbered as HR20110805, which was deposited at the Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation.

DEAE-Cellulose was purchased from Shanghai Hengxin Chemicals Co. Ltd, China. Sepharose CL-6B, Sephadex G-75, DEAE-Sepharose CL-6B and E-TOXATE kit were from Sigma-Aldrich Co. LLC, USA. Cyclophosphamide (CTX) was from Jiangsu Hengrui Medicine Co., Ltd, China. All other reagents used were of analytical grade made in China.

2.2. Isolation and purification of polysaccharides

The isolation of polysaccharide HRWP from *H. rhamnoides* berries was performed as previously described by our group (Ni et al., 2013). Briefly, fresh berries were air-dried and then exhaustively extracted with 95% ethanol to remove hydrophobic compounds. After filtration through gauze, the residue was collected, dried and extracted with hot water. After filtration through filter paper, the aqueous filtrates were obtained and concentrated to a small volume. Subsequently, up to 80% of 95% ethanol was added to the aqueous filtrates to precipitate the crude polysaccharides, which were collected by centrifugation and dried in vacuum. The crude polysaccharides were purified on a DEAE-Cellulose column to isolate the polysaccharide HRWP.

HRWP was fractionated by DEAE-Cellulose ion-exchange chromatography, eluted with distilled water and then 0.5 M NaCl, giving two fractions: HRWP-N and HRWP-A, respectively. HRWP-A was further purified by Sephadex G-75 gel filtration chromatography and DEAE-Sepharose Fast Flow ion-exchange chromatography in turn to generate a homogeneous HRWP-A for structural analysis and pharmaceutical activity assays.

The total carbohydrate content was determined by the phenol–H_2SO_4 method. Uronic acid content was determined by the *m*-hydroxydiphenyl colorimetric method (Blumenkrantz & Asboe Hansen, 1973). Both total carbohydrate and uronic acid content assays used galacturonic acid as the standard. All gel filtration chromatography was monitored by assaying carbohydrate and uronic acid contents. Protein content was determined by a method published by Sedmak and Grossberg (1977), with Coomassie brilliant blue reagent and bovine serum albumin as the standard. The degree of esterification (DE) was determined by the titrimetric method of Food Chemical Codex (FCC, 1981) and USP 26 NF 21 (2003). FT-IR spectrum was obtained on a Nicolet 560 FT-IR spectrometer with DTGS detector in a range of 400–4000 cm^{-1}. The sample was measured as a film on KBr disc. Contaminant endotoxin was analyzed by a gel-clot Limulus amebocyte lysate assay (Ni et al., 2013). The endotoxin level in each polysaccharide solution was less than 0.005 EU (endotoxin units)/mg.

2.3. Determination of homogeneity and molecular mass

Determination of homogeneity and molecular weight was performed by HPGPC-linked gel filtration column of TSK-G3000 PW_{XL}, eluting with 0.2 M NaCl at a flow rate of 0.6 mL/min at 35.0 ± 0.1 °C. The gel filtration column was calibrated by standard dextrans (50 kDa, 25 kDa, 12 kDa, 5 kDa, 1 kDa) using linear regression. The sample concentration was 5 mg/mL, and the injection amount was 20 μL.

2.4. Determination of the monosaccharide component

The monosaccharide component analysis was performed by the HPLC method as described by Honda et al. (1989). Briefly, sample (2 mg) was first methanolyzed using anhydrous methanol (0.5 mL) containing 2 M HCl at 80 °C for 16 h. Then, the methanolyzed products were hydrolyzed with 2 M CF_3COOH (0.5 mL) at 120 °C for 1 h. The hydrolyzed monosaccharides were derivatized to 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives and then subsequently analyzed by HPLC on a Shim-pak VP-ODS column (250 mm × 4.6 mm i.d.) with a guard column on a Shimadzu HPLC system and with monitoring by UV absorbance at 245 nm.

2.5. Nuclear magnetic resonance (NMR) spectroscopy analysis

NMR spectra were recorded using a Bruker 5-mm broadband observation probe at 20 °C with a Bruker Avance 600 MHz spectrometer (Germany). Polysaccharide sample (20 mg) was dissolved in D_2O (99.9% D, 0.5 mL), centrifuged to remove the excessive sample, then freeze-dried from 99.9% D_2O and dissolved in it again. The polysaccharide sample was exchanged in D_2O twice again following the above procedure. The experiment was recorded using standard Bruker software.

2.6. Mice and tumor cells

Male C57BL/6 mice (6–8 weeks old, weighing 20.0 ± 2.0 g) were purchased from the Pharmacology Experimental Center of Jilin University (Changchun, China). The mice were housed on a 12/12-h light–dark cycle at room temperature and allowed free access to standard rodent food and water during the experiments. Animal handling procedures were conducted under National Institutes of Health animal care and use guidelines. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

The mouse Lewis lung carcinoma cell line LLC1 cells, purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) and supplemented with 25 mM HEPES, 10% heat-inactivated fetal calf serum (FCS), 1×10^5 IU/L penicillin G and 100 mM streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.7. Assay of antitumor activity

LLC1 cells (2×10^6) were implanted subcutaneously into the right axilla of the mice (8 mice/group). One day after inoculation, different doses of HRWP or HRWP-A (50, 100 and 200 mg/kg) were administered intragastrically each day for 14 consecutive days. The Normal group was uninoculation of tumor and treated with saline, whereas the Model group was treated inoculation of tumor and treated with saline. The positive control was given 25 mg/kg of CTX. The dose volume was 0.2 mL.

Tumor volume was measured every two days, and tumor volumes were calculated as follows: (longest diameter) × (shortest diameter)^2/2 (You et al., 2001).

2.8. Preparation of splenocytes

Spleens from the tumor-bearing mouse were collected under aseptic conditions and minced in D-Hanks solution using a pair of scissors and passed through fine steel meshes to obtain homogeneous cell suspensions. Splenocytes were isolated by density gradient centrifugation for 20 min at 850 × *g* using mouse lymphocyte separation medium (Dakewe Biotech Co., China), washed twice with PBS and resuspending in complete RPMI 1640 medium. The viability of the splenocytes was >95%, as assessed by the trypan blue dye exclusion method. The cells were used to assay lymphocyte proliferation, cytotoxic T lymphocyte (CTL) and NK cytotoxicity.

2.9. Lymphocyte proliferation

Spleen cells (5×10^6 cells/mL) were seeded in a 96-well plate and cultured with mitogen ConA (5 μg/mL; Sigma, St. Louis, USA) or LPS (10 μg/mL; Sigma, St. Louis, USA). The cells were cultured at 37 °C, 5% CO_2 for 44 h. MTS (20 μL; Promega Co., USA) was added to each well and incubated for an additional 4 h. The absorbance at 490 nm was measured using a Bio-Rad microplate reader (Model 550, USA). All determinations were conducted in triplicate.

2.10. NK cytotoxicity assay

The splenocytes were obtained as describe above. YAC-1 cells (ATCC, Rockville, MD, USA) were used as target cells to measure the NK cell activity. Briefly, splenocytes (1×10^6 cells/well) and YAC-1 cells (5×10^4 cells/well; an initial effector: target cell ratio of 20:1) were incubated in 96-well plates. The mixtures were incubated at 37 °C for 6 h in a CO_2 incubator. After centrifugation (2000 rpm for 10 min), the culture supernatants were admixed with LDH solution (Ni et al., 2009). The absorbance at 490 nm was measured using a microplate reader. The percentage of NK cell cytotoxicity was calculated from the following formula:

$$\text{Cytotoxicity}\ (\%) = \left[\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}\right] \times 100$$

2.11. Cytotoxic T lymphocyte (CTL) assay

The CTL activity was analyzed using the LDH method as described above. LLC cells (1×10^4cells/well) and splenocytes were used as target cells and effector cells, respectively, at an effector-to-target (E:T) ratio of 25:1. The percentage of CTL cytotoxicity was calculated from the following formula:

$$\text{Cytotoxicity}\ (\%) = \left[\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}\right] \times 100$$

2.12. Macrophage activity

Cells from the peritoneal exudate were collected from the tumor-bearing mouse by peritoneal lavage with serum-free RPMI 1640 medium (5 mL; Sigma, St. Louis, USA). The collected cells were washed with D-Hank's and cultured in complete RPMI 1640 medium. The cells were placed in a flat-bottom culture plate and then incubated for 2 h at 37 °C and 5% CO_2. After removal of the non-adherent cells, the monolayer of macrophages was collected.

2.12.1. Macrophage phagocytosis assay

Peritoneal macrophages (4×10^5 cells/well) were added into each well of 96-well microwell plates. After 3 h incubation to allow the cells to attach to the plate bottom, the supernatant was discarded and 0.075% of neutral red dye was added to each well (100 μL/well). The plates were incubated for another 1 h. Then, the plates were washed three times with pre-warmed D-Hanks solution (pH 7.2) and were patted gently on tissues to let it to drain. Finally, 100 μL of lysis solution (0.1 M acetic acid and ethanol in the ratio of 1:1) was pipetted into each well. The mixtures were blended completely and evaluated at a wavelength of 540 nm on a Bio-Rad microplate reader.

2.12.2. NO assay

Peritoneal macrophages were cultured for 48 h at 37 °C. At the end of the incubation, culture supernatant was collected. The isolated supernatants were mixed with an equal volume of Griess regent and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, the concentration of nitrite was measured by absorbance reading at 540 nm (Ruan, Su, Dai, &Wu, 2005).

2.12.3. TNF-α assay

The culture supernatants were assayed for TNF-α with a commercial ELISA kit according to the manufacturer's protocol (eBioscience, San Diego, California, USA). Each data point is an average of the values obtained from 6 mice. The absorbance at 450 nm in each well was measured with an automated microplate reader.

2.12.4. Macrophage-mediated cytotoxicity assay

The macrophage-mediated cytotoxicity was determined by modification of the technique described previously (Ni et al., 2009). Peritoneal macrophages (1×10^5 cells/well) prepared as described above were co-incubated with B16 melanoma cells (ATCC, Rockville, MD, USA) (1×10^4 cells/well; an initial effector: target cell ratio of 10:1) at 37 °C in a 5% CO_2 incubator for 16 h. Cell density was then assessed by incubating the cells with MTS (20 μg/mL) for another 4 h. The absorbance at 490 nm was measured using a microplate reader. Cytolytic activity is expressed as the percentage of tumor cytotoxicity, where

$$\%\ \text{Cytotoxicity} = \left\{1 - \text{O.D. of}\ \frac{[(\text{target} + \text{macrophages}) - \text{macrophages}]}{\text{O.D. of target (nontreated)}}\right\} \times 100$$

2.13. Direct cytotoxicity assay

The direct cytotoxicity of HRWP-A on LLC1 cells was detected using a MTS assay. Briefly, LLC1 cells (2×10^4 cells/well) were cultured in a 96-well plate and stimulated with HRWP-A (0, 5, 10, 50, 100, 200 and 500 μg/mL). After incubation for 48 h, the MTS assay was performed. The absorbance was recorded at 490 nm with a microplate reader.

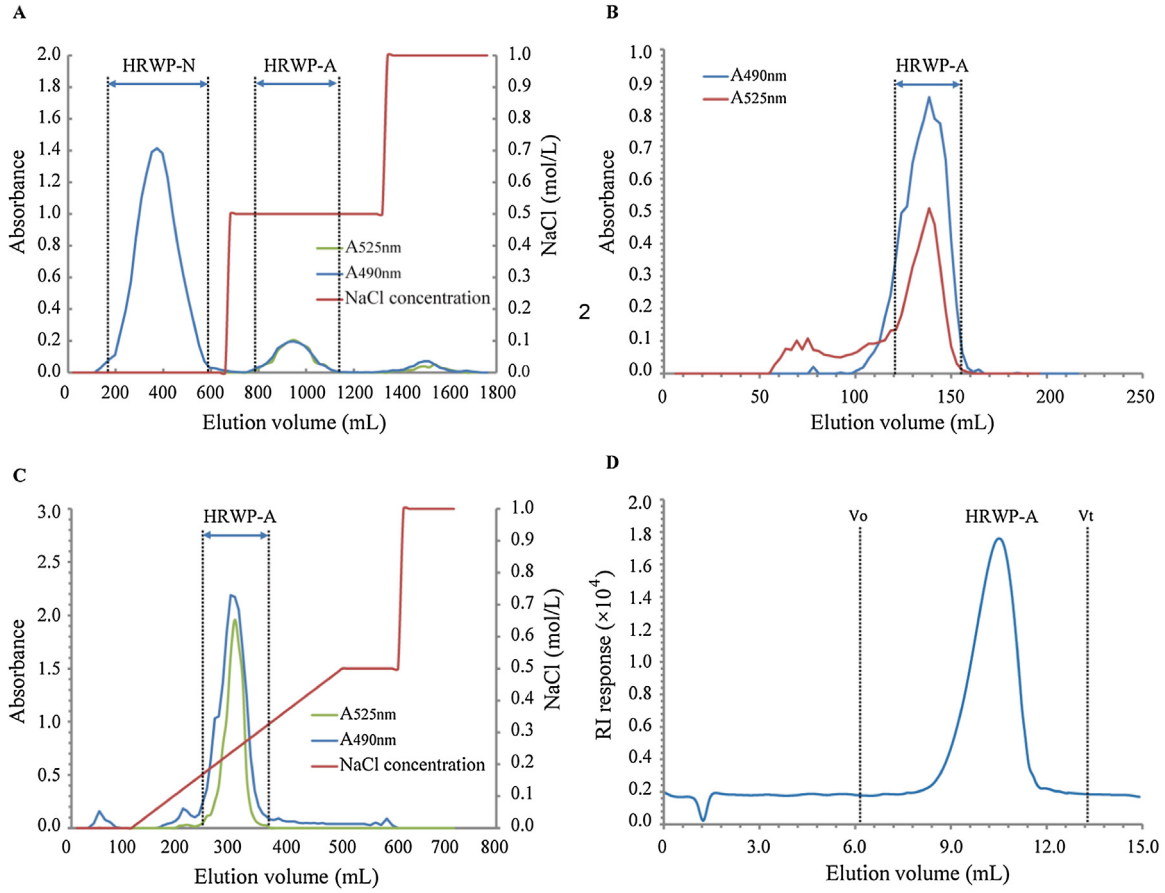

Fig. 1. Elution profiles of polysaccharides from the *Hippophae rhamnoides* L. berry.
(A) DEAE-Cellulose ion-exchange chromatography, (B) Sephadex G-75 gel filtration chromatography, (C) DEAE-Sepharose Fast Flow ion-exchange chromatography and (D) high performance gel filtration chromatography on a TSK-G3000 PWXL column.

2.14. Statistical analysis

Data were analyzed by Student's *t*-test or Mann–Whitney's *U* test (SPSS statistical software package, version 13.0, SPSS, Chicago, IL) when appropriate. A *P*-value of <0.05 was considered to be significant.

3. Results and discussion

3.1. Isolation and structure characterization of HRWP-A

H. rhamnoides berries were treated with 95% ethanol to remove hydrophobic compounds, and then extracted with hot water to obtain crude polysaccharide (4.78% yield of raw material). The crude polysaccharide was purified on a DEAE-Cellulose column with 0.5 M NaCl elution to generate the polysaccharide HRWP (83.29% yield of crude polysaccharide). HRWP exhibited a wide molecular weight distribution (from 1 kDa to 50 kDa) on Sephadex G-75 gel filtration chromatography. The monosaccharide composition analysis (supplementary data) indicated that HRWP contained mannose, rhamnose, galacturonic acid, glucose, galactose, xylose and arabinose in a molar ratio of 4.23:1.33:59.35:20.82:9.68:1.06:3.54. As shown in Fig. 1A, a further separation of HRWP by DEAE-Cellulose ion-exchange chromatography resulted in two fractions: HRWP-N (eluted with H_2O, 65.62% yield of HRWP) and HRWP-A (eluted with 0.5 M NaCl, 7.86% yield of HRWP).

HRWP-N was a heteropolysaccharide fraction consisting of mannose, rhamnose, galacturonic acid, glucose, galactose, xylose and arabinose in a molar ratio of 6.24:0.77:3.42:38.73:15.03:0.81:5.00. Its molecular weight showed a heterogeneous distribution from 1 kDa to 50 kDa. HRWP-A contained mainly galacturonic acid (94.43%), and some glucose (0.59%), galactose (4.11%) and arabinose (1.06%). HRWP-A gave one main peak on the elution profiles of Sephadex G-75 gel filtration chromatography (Fig. 1B) and DEAE-Sepharose FF ion-exchange chromatography (Fig. 1C); next, it was further purified and was determined to be a homogeneous HRWP-A, which was used for assaying the structure and pharmaceutical activity.

HRWP-A is a white powder without any protein, and its total carbohydrate and uronic acid contents were determined to be 97.52% and 96.54%, respectively. HRWP-A contained 97.46% of galacturonic acid and a trace amount of glucose (0.49%), galactose (1.22%) and arabinose (0.83%). HPGPC (Fig. 1D) indicated that the molecular weight distribution of HRWP-A was homogeneous and *Mw* was estimated to be 4992 Da (*Mw*/*Mn* = 1.3). DE of HRWP-A was 85.16% by the titrimetric method, indicating that it was a natural high-methoxyl HG-type pectin.

The FT-IR spectrum (Fig. 2) showed the absorption at 1740.44 cm^{-1} caused by stretching vibration of methyl-esterified carboxyl groups, the absorption at 1602.07 cm^{-1} caused by the C=O stretching vibration of carboxyl groups, which suggested that HRWP-A might be a methyl-esterified acidic polysaccharide. The absorptions at 3305.85, 2937.54, and 1412.60 cm^{-1} are corresponding to the stretching from O—H, C—H, and carboxyl C—O groups, and those at 1143.10, 1096.33, and 1014.87 cm^{-1} are assigned to various in-plane C—O vibrations, respectively (Synytsya, Copikova, Matejka, & Machovic, 2003).

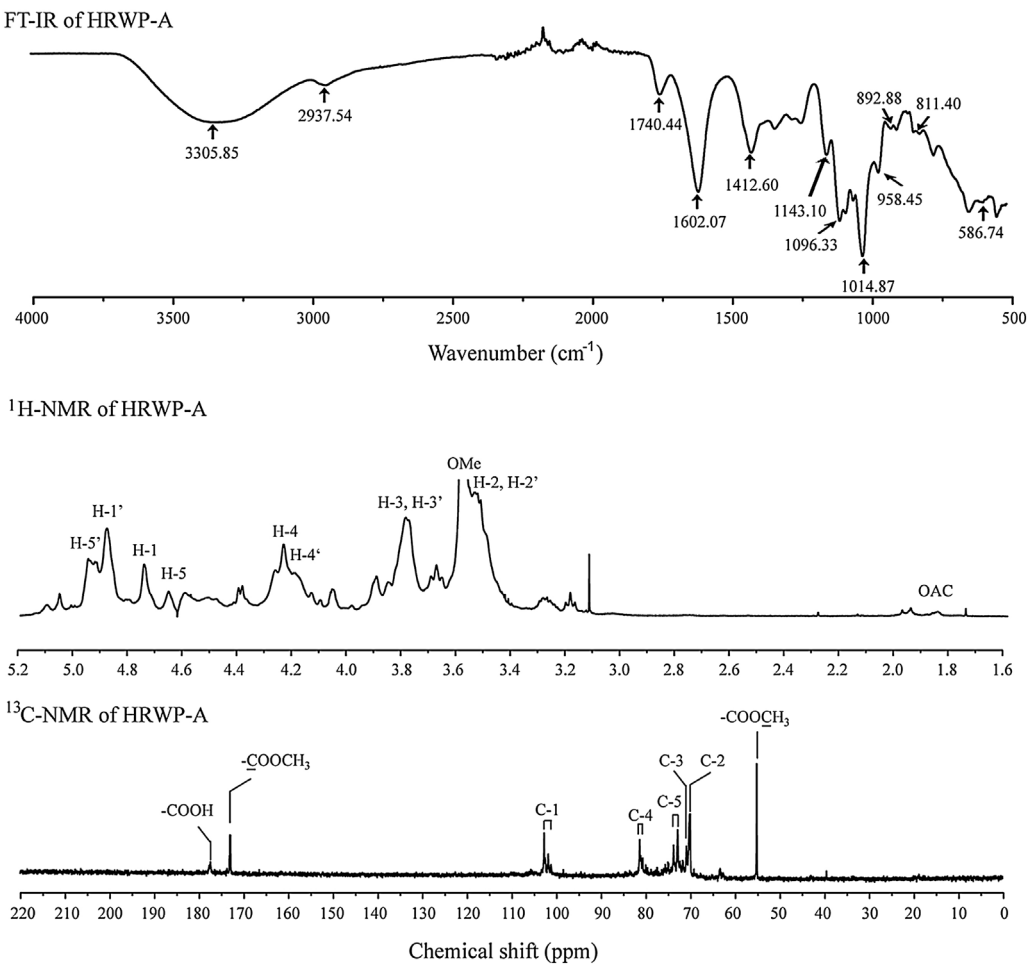

Fig. 2. FT-IR and NMR spectra of HRWP-A from the *Hippophae rhamnoides* L. berry.

^{13}C NMR spectrum of HRWP-A is shown in Fig. 2, and the data are listed in Table 1. All signals were identified with respect to those of β-(1,4)-D-galacturonans reported in the literatures (Cheng et al., 2014; Košťálová, Hromádková, & Ebringerová, 2013). The signal at 177.53 ppm was assigned to C-6 of (1,4)-linked Gal*p*A. The signals at 173.17 ppm and 55.26 ppm suggested that partial Gal*p*A residue might exist as a methyl ester. In the anomeric carbon region, the signals at 101.93–102.83 ppm indicated an α-anomeric configuration in the galacturonic acid residue units. The signals at the regions 79.98 and 80.77 ppm were assigned to methyl and nonmethyl esterified C-4, and those at 70.16–71.00 ppm to C-2 and C-3. The signals at 72.95 and 73.87 ppm were attributed to nonmethyl and methyl esterified C-5 of (1,4)-linked Gal*p*A.

The ^{1}H NMR signals of HRWP-A were assigned by literature data (Shakhmatov, Udoratina, Atukmaev, & Makarova, 2015; Wang et al., 2014; Xu, Dong, Qiu, Ma, & Ding, 2011). As shown in Fig. 2, the signal at 3.58 ppm showed the characteristics of protons of methyl ester existed in (1,4)-linked GalA. The signals at 4.74 and 4.88 ppm were assigned to nonmethyl and methyl esterified H-1 of (1,4)-linked GalA, which also indicated that the GalA residues possessed an α configuration. The signals at 3.54 and 3.79 ppm were assigned to H-2 and H-3 of (1,4)-linked GalA. The signals at 4.65 and 4.94 ppm were attributed to nonmethyl and methyl esterified H-5 of (1,4)-linked GalA.

3.2. HRWP-A induces antitumor effects

The antitumor activity of *H. rhamnoides* berry polysaccharides was determined by using a mouse Lewis lung carcinoma model. Tumor volume was measured every 2 days. As shown in Fig. 3A, at day 10 and 12, HRWP was unable to cause significant tumor regression as compared to the model group, even at a high dose

Table 1
^{13}C and ^{1}H NMR chemical shifts of HRWP-A.

Glycosidic linkage	Chemical shift (δ, ppm)						
	C-1 H-1	C-2 H-2	C-3 H-3	C-4 H-4	C-5 H-5	C-6 H-6	—OCH$_3$
→4)-α-D-Gal*p*A-(1→	102.83 4.74	70.16 3.54	71.00 3.79	79.98 4.23	73.87 4.65	177.53 –	– –
→4)-α-D-Gal*p*A6Me-(1→	101.93 4.88	70.16 3.64	71.00 3.79	80.77 4.19	72.95 4.94	173.17 –	55.26 3.58

Table 2
Effect of HRWP-A on body weight and organ indices in mice.

Group	Dose (mg/kg)	Body weight (g)		Immune organ index (mg/g)	
		Day 1	Day 16	Thymus	Spleen
Normal	–	21.3 ± 2.3	30.3 ± 2.1	2.12 ± 0.13	4.64 ± 0.29
Model	–	20.4 ± 2.7	27.5 ± 3.9	1.54 ± 0.22	4.15 ± 0.23
CTX	25	19.8 ± 1.7	23.4 ± 3.7	1.33 ± 0.05**	3.12 ± 0.09**
HRWP-A	50	18.6 ± 3.9	28.6 ± 3.4	1.98 ± 0.11*	4.98 ± 0.24*
	100	21.3 ± 2.3	29.2 ± 4.9	2.10 ± 0.08**	5.66 ± 0.23**
	200	19.9 ± 2.3	31.7 ± 3.7	2.85 ± 0.27**	5.73 ± 0.35**

Organ indices = weight of organ (mg)/body weight (g). Normal group = mice uninoculation of tumor and treated with saline, Model group = mice inoculation of tumor and treated with saline. Each value represents the mean ± SD ($n = 8$). Significant differences from the Model group were evaluated using Student's t test: * $P < 0.05$, ** $P < 0.01$.

(200 mg/kg). At day 14, HRWP (200 mg/kg) exhibited a significant inhibition of tumor growth. At day 16, HRWP at all testing doses exhibited a significant inhibition of tumor growth. However, HRWP-A with the low dose of 50 mg/kg was found to be associated with significant inhibition of LLC tumor growth at all testing days (Fig. 3B). The inhibition effect of HRWP-A was more prominent than that of HRWP. These results implied that the high-methoxyl HG-type pectin contributed to the antitumor activity in HRWP.

From day 10 to 16, tumor growth in the HRWP-A group was slower than that in the model group. Tumor volumes were significantly decreased by HRWP-A in a dose-dependent manner ($P < 0.05$ or $P < 0.01$). At the same time, as a positive control, CTX significantly inhibited tumor growth. At day 16, the tumor volume in mice treated at 200 mg/kg of HRWP-A was comparable to that in CTX.

Subsequently, the research was focused on the activity of HG-type pectin HRWP-A. Body weight was recorded before the experiment (initial) and after 16 days (final), and weight gain

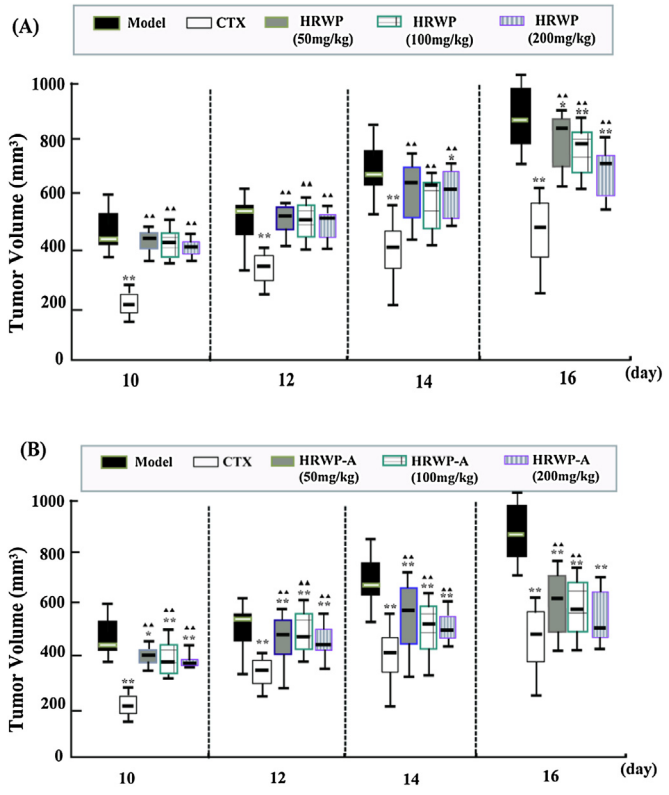

Fig. 3. *Inhibition of Lewis lung carcinoma tumor growth in mice by polysaccharides.* Normal group = mice uninoculation of tumor and treated with saline, Model group = mice inoculation of tumor and treated with saline. Each bar and box indicates the median and interquartile range, respectively ($n = 8$). * $P < 0.05$ or ** $P < 0.01$, significantly different from the Model group by the Mann–Whitney's U test.

was computed (Table 2). HRWP-A had no significant effect on the body weight as compared to the model group. Spleen and thymus as immune function-related organs were further evaluated. As expected, CTX obviously decreased the spleen weight as compared to the model group. However, HRWP-A significantly increased the relative spleen and thymus weights as compared to the model groups. Consistent with antitumor activity, these two indices increased to a maximum at the dose of 200 mg/kg. These findings suggested that HRWP-A inhibited tumor growth accompanied with an improvement of immune organs.

3.3. HRWP-A enhanced lymphocyte proliferation

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. Stimulating proliferation of lymphocytes results in an increase in cytokine release, potentially accounting for the antitumor activity of the polysaccharides isolated from medicinal plants (Zeng, Ju, Shen, Zhou, & Huang, 2013). The effects of HRWP-A on the proliferation of spleen lymphocytes from tumor-bearing mice were evaluated (Fig. 4A). The effects of HRWP-A on ConA- and LPS-induced lymphocyte proliferation were tested by the MTS method. Lymphocytes induced by ConA may be used as a method to evaluate T lymphocyte activity, while those lymphocytes induced by LPS may be used to evaluate B lymphocyte activity (Ni et al., 2009; Sun & Liu, 2008). In the presence of ConA or LPS, HRWP-A could significantly increase lymphocyte proliferation in all three testing doses ($P < 0.05$). The increase in T cell proliferation was demonstrated a dose-dependent manner. However, the increase in B cell proliferation peaked at 100 mg/kg. These results showed that HRWP-A was able to activate both T and B cells, suggesting that it might be a potent T/B-cell stimulator.

3.4. HRWP-A enhances NK cell and CTL cytotoxicity in mice

Tumor cell elimination can be partially mediated by the NK cells and CTL cytotoxicity (Liu et al., 2014). In this paper, the effects of HRWP-A on the NK and CTL activities from tumor bearing mice were evaluated. Results showed that HRWP-A markedly enhanced the NK cell cytotoxicity and CTL response compared with those in the model group ($P < 0.05$ or $P < 0.01$) (Table 3). From the dosages of 50 mg/kg to 200 mg/kg, the effects increased to a maximum. In CTX-treated mice, it is clear that NK and CTL cell activities were suppressed, which resulted from the side effects of CTX ($P < 0.05$). Thus, the antitumor activity of HRWP-A is partially due to the improvement of NK and CTL responses in LLC tumor-bearing mice.

3.5. HRWP-A augments macrophage activity

Simulating macrophages is an important way to enhance immunological activity (Gordon & Martinez, 2010). Many

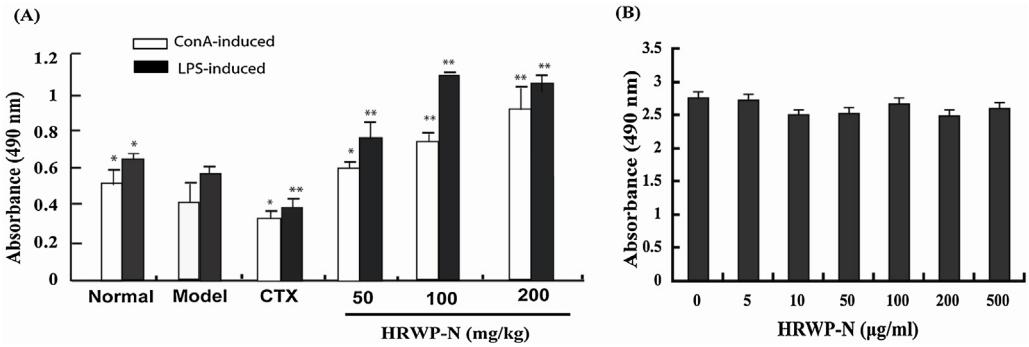

Fig. 4. *Effect of HRWP-A on ConA- or LPS-induced lymphocyte proliferation activity* (A). Normal group = mice uninoculation of tumor and treated with saline, Model group = mice inoculation of tumor and treated with saline. Each value represents the mean ± SD ($n = 8$). Significant differences from the Model group were evaluated using Student's *t* test: * $P < 0.05$, ** $P < 0.01$. *Direct cytotoxicity of HRWP-A on LLC1 cells in vitro* (B). LLC1 cells (2×10^4 cells/well) were added to wells of a 96-well plate in triplicate, treated with the indicated dose of HRWP-A (0, 5, 10, 50, 100, 200 and 500 μg/mL), and cultured in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h. After performing an MTS assay, the absorbance was recorded at 490 nm with a microplate reader. Each value represents the mean ± SD ($n = 6$). Significantly different from the Model group by Student's *t* test.

Table 3
Effects of HRWP-A on cytotoxicity of NK cell and CTL from LLC tumor-bearing mice.

Group	Dose (mg/kg)	NK cytotoxicity (%)	CTL cytotoxicity (%)
Normal	–	22.39 ± 1.27*	23.54 ± 2.12
Model	–	28.11 ± 0.99	25.14 ± 3.31
CTX	25	19.74 ± 1.21**	16.77 ± 1.12**
HRWP-A	50	33.50 ± 2.57**	33.42 ± 1.98*
	100	38.77 ± 2.36*	37.46 ± 2.73**
	200	43.26 ± 4.76**	35.66 ± 3.01**

Normal group = mice uninoculation of tumor and treated with saline, Model group = mice inoculation of tumor and treated with saline. Each value represents the mean ± SD ($n = 8$). Significant differences from the Model group were evaluated using Student's *t* test: * $P < 0.05$, ** $P < 0.01$.

polysaccharides activate macrophages by binding to the receptors on the surface of immune cells. Botanical polysaccharides and/or glycoproteins interact with macrophages *via* dectin-1, Toll-like receptor 4 (TLR_4), complement receptor 3 and the mannose receptor (Ni et al., 2009; Schepetkin & Quinn, 2006). Activation of these receptors leads to intracellular signaling cascades, resulting in transcriptional activation and the production of pro-inflammatory cytokines. In our experiment, as shown in Table 4, HRWP-A augmented the macrophage phagocytic activity, increased the macrophage secretion of NO and TNF-α, and enhanced macrophage cytotoxic activity against tumor cells. By contrast, CTX partially inhibited macrophage activity because of its cytotoxicity. Phagocytosis is one of the primary functions of macrophages. TNF-α and NO are important mediators in the destruction of tumor cells. Macrophage cytotoxic activity is sensitive to either TNF-α or NO, and these can directly eliminate the tumor cells. Altogether, these results indicated that the activation of macrophage by HRWP-A led to the inhibition of tumor regression (Gordon & Martinez, 2010).

3.6. HRWP-A has no direct cytotoxicity on LLC1 cells

Direct cytotoxic effect of HRWP-A on LLC1 cells was observed. LLC1 cells (2×10^4 cells/well) were treated with different concentrations of HRWP-A (0, 5, 10, 50, 100, 200 and 500 μg/mL) for 48 h. As shown in Fig. 4B, at all dosages, HRWP-A has no direct cytotoxicity against LLC1 cells.

The antitumor efficacy of polysaccharides was first recognized by Nauts et al. in 1946 when it was found that certain polysaccharides could induce complete remission in patients with cancer (Zhang, Cui, Cheung, & Wang, 2007). Since then, various type of polysacchairdes have shown promising potential as antitumor agents (Jin et al., 2014; Silva et al., 2012; Sun, 2011). Pectin, structurally and functionally the most complex polysaccharide in plant cell walls, is a family of galacturonic acid-rich polysaccharides including homogalacturonan (HG), rhamnogalacturonan I (RG-I), and the substituted galacturonans, such as rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA) (Mohnen, 2008). Pectic polysaccharides are the primary components of plant cell walls with intriguing structural diversity (Caffall & Mohnen, 2009). Several studies have reported that pectic polysaccharides possess antitumor and a diverse immunomodulating activity that can mediate both phagocytosis and antibody production (Popov, Popova, Ovodova, Bushneva, & Ovodov, 1999; Inngjerdingen et al., 2005; Ye & Lim, 2010). The wide structural diversity of plant cell wall polysaccharides reflects the different mechanisms exerted on the immune systems.

In our study, a natural high-methoxyl homogalacturonan pectin HRWP-A from the *H. rhamnoides* berry. As far as we know, there has been no report on the antitumor and immunostimulating activities of polysaccharides from the *H. rhamnoides* berry to date. Similar polysaccharides had been isolated from other species, and the differences lie in the molecular weight and esterification degree (Makarova, Patova, Shakhmatov, Kuznetsov, &

Table 4
Effects of HRWP-A on macrophage-mediated phagocytosis, production of TNF-α and NO (as Nitrite), as well as macrophage cytotoxicity from LLC bearing-mice.

Group	Dose (mg/kg)	Phagocytosis (A 540 nm)	Nitrite (μM)	TNF-α (pg/ml)	Macrophage cytotoxicity (%)
Normal	–	0.33 ± 0.06	21.0 ± 1.31	41.81 ± 2.60	19.65 ± 1.17
Model	–	0.38 ± 0.19	21.7 ± 2.01	46.39 ± 2.34	21.64 ± 1.98
CTX	25	0.21 ± 0.08**	14.3 ± 2.03**	30.73 ± 0.91**	14.87 ± 0.76**
HRWP-A	50	0.48 ± 0.03*	33.0 ± 2.34**	49.21 ± 1.68	39.16 ± 2.13**
	100	0.52 ± 0.16**	42.7 ± 4.63**	65.57 ± 3.62**	42.23 ± 2.87**
	200	0.54 ± 0.17**	51.0 ± 3.21**	82.76 ± 2.01**	48.67 ± 3.33**

Normal group = mice uninoculation of tumor and treated with saline, Model group = mice inoculation of tumor and treated with saline. Each value represents the mean ± SD ($n = 8$). Significant differences from the Model group were evaluated using Student's *t* test: * $P < 0.05$, ** $P < 0.01$.

Ovodov, 2013; Shang et al., 2012; Xu, Dong, Qiu, Ma, & Ding, 2011). However, the activity study of these polysaccharides focused mainly on anti-angiogenesis and cell proliferation. Research on this structure may contribute to understanding the structure-activity relationship (SAR) of polysaccharides. The relationships between structure and activity of these polysaccharides need to be further discussed.

4. Conclusion

In this study, a natural high-methoxyl homogalacturonan pectin, HRWP-A were successfully separated from the *H. rhamnoides* berry by using DEAE-Cellulose ion-exchange chromatography, Sephadex G-75 gel filtration chromatography and DEAE-Sepharose Fast Flow ion-exchange chromatography. Structural analysis showed HRWP-A is a pectic polysaccharide with repeating units of (1 → 4)-β-D-galactopyranosyluronic residues (of which 85.16% were methyl esterified). Notably, HRWP-A significantly inhibited the tumor growth by intragastric administration (i.g.) in a mouse LLC model. Moreover, the results of immunological activity assays suggested that HRWP-A-induced antitumor effects might be mediated through immunological activities, given its effect on increasing the proliferation of lymphocytes, augmenting the phagocytosis and cytotoxicity of macrophages, promoting levels of NO and TNF-α, and increasing NK cell activity and CTL cytotoxicity in LLC tumor-bearing mice. Altogether, HRWP-A induced an enhancement of innate immune responses, and these results might benefit the understanding of the immunostimulating effect of HRWP-A and the underlying molecular mechanisms of its antitumor effect.

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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.carbpol.2015.06.021

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