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

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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

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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.