Carbohydrate Polymers 102 (2014) 543–549

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Antitumor and immunomodulatory effects of a water-soluble polysaccharide from *Lilii Bulbus* in mice

Xin Sun[a], Rui-Lan Gao[b], Yao-Kang Xiong[c], Qing-Cheng Huang[c], Min Xu[d,*]

[a] *Oncology Department, Zhejiang Provincial People's Hospital, Hangzhou 310024, China*
[b] *Institute of Hematology, Zhejiang Provincial Hospital of TCM, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, China*
[c] *Zhejiang Chinese Medical University, Hangzhou 310053, China*
[d] *Pharmacy Department, Zhejiang Provincial Hospital of TCM, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, China*

ARTICLE INFO

Article history:
Received 8 October 2013
Received in revised form
30 November 2013
Accepted 2 December 2013
Available online 8 December 2013

Keywords:
Characterization
Antitumor
Immunomodulatory
Lung carcinoma

ABSTRACT

Lilii Bulbus is a popular folk medicine in the worldwide and has attracted great attention due to its bioactivity against respiratory system diseases (include lung cancers). This study was the first report providing *in vivo* evidences of antitumor potential of the bioactive polysaccharide from *Lilii Bulbus*. One major fraction (LBP-1) was obtained by purifying the crude polysaccharides extracted from *Lilii Bulbus*. Chemical characterization analysis indicated that LBP-1 was only a glucan, whose average molecular weight was 30.5 kDa. Intraperitoneal administration of LBP-1 at the doses of 50–200 mg/kg significantly inhibited the growth of Lewis lung carcinoma. Moreover, it could also obviously increase macrophage phagocytosis, splenocytes proliferation and cytokine (TNF-α, IL-2, IL-6 and IL-12) production to participate in the antitumor effects. LBP-1 could act as antitumor agent with immunomodulatory activity.

1. Introduction

Lung carcinoma is the leading cause of cancer-related death in the worldwide. The overall five year survival rate of patients with lung cancer remains less than 16% and has not improved substantially over the past 30 years (Yatabe, Borczuk, & Powell, 2011). In spite of their high antitumor efficacy, the use of chemotherapeutic agents, such as DDP, fluorouracil and cyclophosphamide, are limited due to cumulative toxicities including nervous and gastrointestinal injuries (Mitchell, 2006; Perazella & Moeckel, 2010; Shahab, Haider, & Doll, 2006). Natural herbal products are used in the prevention or treatment of cancer in many countries. Although less antitumor effect than chemotherapy drugs, it can produce more immune enhancement and less toxicity (Jeong, Koh, Kim, & Kim, 2011; Kim, Moon, Choi, Kim, & Lee, 2013; Li et al., 2012; Wang et al., 2012). Recently, great majority of polysaccharides isolated from various natural sources are believed that have superior antitumor and immunomodulatory activities with no significant side effects and low toxicity could be ideal candidates for the treatment of tumor (Liu et al., 2004; Miao et al., 2013; Yu et al., 2013).

Lilii Bulbus, the root of *Lilium brownii* F. E. Brown var. *viridulium* Baker, which belongs to the *Lilium* genus, *Liliaceae* family, is widely distributed and cultivated in Eastern Asia, Europe and North America. It is known as "Lily" and widely used as a tonic and dessert in China, Japan, Korea and other Asian countries for a long history (Hong, Luo, Guo, & Kong, 2012; Munafo, Ramanathan, Jimenez, & Gianfagna, 2010). This food is used in herbal cuisine, conjee, as well as ice shavings, and it also is used as a folk medicine for treatment of cure bronchitis, pneumonia, cough, tuberculosis, pertussis, and chronic gastritis. Increasing attention has been given to its anti-inflammatory, antifungal, anti-oxidative activities (Kwon et al., 2010; Luo, Li, & Kong, 2012; Wang & Ng, 2002). Several low-molecular chemical constituents from *Lilii Bulbus* have been well investigated (Hong, Luo, Guo, et al., 2012; Hong, Luo, & Kong, 2012; Wang & Ng, 2002), but the high-molecular components such as polysaccharides are still poorly defined. To the best of our knowledge, there are no researches evaluating biological activities of polysaccharide of *Lilii Bulbus* (LBP) have been conducted. Therefore, the present study aimed to investigate the chemical characterization of LBP and its anti-tumor activity against melanoma B16 and Lewis lung cancer cells.

2. Materials and methods

2.1. Materials and regent

The raw material of *Lilii Bulbus* were purchased from Chinese Medicine Herbal Factory, Zhejiang Province, China, and

* Corresponding author. Tel.: +86 0571 87072976; fax: +86 0571 87077785.
E-mail address: zjszyyxumin@163.com (M. Xu).

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http://dx.doi.org/10.1016/j.carbpol.2013.12.002

identified by Professor Jian-Ping Wang, Zhejiang provincial Hospital of TCM. Samples were dried at 60 °C for 24 h, and then ground and passed through a 12-mesh sieve. Cisplatin (*i.e.*, cis-dichlorodiamineplatinum (II), DDP) injections were purchased from Dezhou Deyao pharmaceutical Co., Ltd., Dezhou, China. Other reagents in this study were of analytical grade and were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Preparation, separation and purification of LBP

The polysaccharide fraction of *Lilii Bulbus* was prepared, analyzed, isolated and purified as the previous report with little modification (Yu et al., 2013; Zhao et al., 2013). One kilogram of herbal powders was extracted three times with the boiling distilled water (1:10, w/v) for 3 h. After centrifugation (3500 rpm, 20 min) and concentration, the supernatant (200 mL) was precipitated by adding 4 volumes of 95% ethanol (v/v). And then the precipitates were collected and dissolved in some distilled water, deproteined with the Sevag method, and followed by exhaustive dialysis with water for 48 h. Finally, the precipitates were successively washed by diethyl ether and dried to give crude polysaccharide (LBP). The yield of the crude polysaccharide was 5.1%.

The obtained polysaccharide was redissolved in distilled water, then was applied to a diethylaminoethyl (DEAE)-cellulose column (5 cm × 60 cm) pre-equilibrated with water and eluted in NaCl gradient solution (0–3 M) at a flow rate of 1 mL/min (10 mL/tube). Each elution fraction was collected and monitored for carbohydrate content through the phenol–sulfuric acid method (using D-glucose as a standard), as previously described (Chen, Zhang, et al., 2012). Finally, the same carbohydrate-positive fractions were pooled together, dialyzed, and lyophilized to afford two fractions. The products were further purified on a Sephadex G-100 column (2.6 cm × 60 cm) with water and lyophilized to afford one major polysaccharide, named as LBP-1.

2.3. Monosaccharide composition analysis

Gas chromatography–mass spectrometry (GC–MS) was employed for the identification and quantification of monosaccharides (Chen et al., 2013; Ding et al., 2010; Wu et al., 2010). Fifty milligrams of LBP-1 were hydrolyzed with 5 mL of 1 M sulfuric acid at 100 °C for 6 h. The hydrolyzed polysaccharide was mixed with BaOH for pH up to neutrality and was then evaporated continuously using a rotary evaporator at 45 °C. The hydrolyzed products were acetylated with 8 mg of hydroxylamine hydrochloride and 0.5 mL of pyridine at 90 °C for 30 min. Then acetic anhydride (0.5 mL) was added after cooling, and the tube was sealed and incubated at 90 °C for 30 min. The corresponding alditol acetates were analyzed by gas chromatography on an Agilent 6890 gas chromatograph/5973 mass selective detector with HP-5 capillary column (30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The oven conditions included an initial temperature of 50 °C and an initial time of 2 min, 30 °C/min to 150 °C, 3 °C/min to 220 °C, and finally 30 °C/min to 300 °C for a 10-min bakeout. The inlet temperature was kept constant at 250 °C, and the MS transfer line was set at 300 °C. MS acquisition parameters included scanning from m/z 50 to 550 in the electron impact (EI) mode for routine analysis.

2.4. Molecular weight determination

LBP-1 (5 mg), was swelled with 0.1 mol/L NaCl solution, and loaded onto a Sephadex G-100 column (15 mm × 900 mm). It was eluted with 0.1 mol/L NaCl solution at a flow rate of 0.1 mL/min and collected as 1 mL each tube. Polysaccharide content was detected by phenol–sulfate method. Dextran T-series standard of known molecular weight (T-500, T-110, T-70, T-40, and T-10) were loaded on the column and their elution volume V_e were obtained, respectively. And void volume V_o was known by loading Dextran blue on the column as well. Relationship between $\log M$ and V_e/V_o can be calculated as a calibration curve (Tian, Che, Ha, Wei, & Zheng, 2012; Yu et al., 2013). The average molecular weight was finally calculated by the calibration equation.

2.5. Infrared spectroscopy analysis

The infrared spectrum of LBP-1 was recorded with a SPECORD spectrometer in a range 400–4000 cm^{-1}. The samples were analyzed as KBr pellets.

2.6. Animals and experimental design

Male specific pathogen-free C57BL/6 mice weighing 23–26 g were purchased from Siper-BK Co. Ltd., Shanghai, China. Experiments were carried out in accordance with local guidelines for the care of laboratory animals of Zhejiang Academy of Medical Sciences, and were approved by the ethics committee for research on laboratory animal use of the institution. The mice were acclimatized for a period of 2–3 days prior to the experiment. During the experiment the mice were fed under controlled environmental conditions and temperature (24 ± 1 °C) with a normal day/night cycle and humidity (55–60%). The mice were provided with basal diet and free access to drinking water.

Lewis lung cancer cells were maintained in DMEN (Dulbeco's Modified Eagle's Medium) supplemented with 10% fetal calf serum and antibiotics. The cell viability was determined by trypan blue dye exclusion. A subcutaneous injection of 1×10^6 cells/mL Lewis lung cancer cells suspended in 0.1 mL phosphate buffered saline (PBS) was administered in the right axilla of each mouse. After tumor cell injection, the mice were randomly divided into the following six groups (8 mice in each group): the model control group; the LBP-1 treated group (50, 100 and 200 mg/kg); the positive group (DDP, 1 mg/kg); the normal control group. Treatment began when tumors were palpable and no less than 100 mm^3 in volume calculated from caliper measurements. DDP or LBP-1 was intraperitoneally administered to experimental groups daily for 14 days, while the mice in the normal control group and the model control group were only given saline at the same intervals. The chosen doses of the drug were based on previous studies (Chen, Xu, et al., 2012; Tu, Sun, & Ye, 2008). On the 15th day, diets were removed from the cages 12 h before the mice were sacrificed by cervical dislocation and tumors were peeled off and weighed after washing with PBS. The inhibitory rates against the growth of tumors were calculated using the following formula:

$$\text{Inhibitory rate } (\%) = \frac{C - T}{C} \times 100\%$$

where C is the average tumor weight of the negative control group while T is the average tumor weight of treated groups.

2.7. Cytokine assay

Blood samples were collected and centrifuged at 3000 × g for 20 min to obtain serum. The levels of tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-6 and IL-12 in serum were determined by ELISA using commercially available kits (Biovol Technologies Co., Ltd., Shanghai, China). Briefly, 50 μL of cell supernatant was plated in a 96-well and incubated for 2 h at room temperature. After incubation, the plate was washed using the provided washing buffer and incubated with 50 μL of biotinylated anti-mouse TNF-α (IL-2, IL-6, or IL-12) for 1 h. Then, the plate was washed and incubated with 100 μL of streptavidin-HRP for 30 min. After washing, the color was