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

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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

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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

developed by adding 100 μL of chromogen TMB reagent for 30 min in a dark environment, then the reaction was stopped by adding 100 μL of H_2SO_4 and the absorbance was read at 450 nm in an automatic ELISA plate reader (model 680; Bio-Rad, US). The amount of TNF-α was calculated using standard curve.

2.8. Splenocyte proliferation assay

Splenocyte proliferation was also prepared as described previously (Tang et al., 2012). Spleens collected from tumor-bearing mice and normal control mice under aseptic conditions were chopped into small pieces and passed through a fine steel mesh to obtain a homogeneous cell suspension. Recovered spleen cells were resuspended in lysis buffer (0.15 M NH_4Cl, pH 7.4) for 5 min to remove erythrocytes. Then splenocytes were harvested and resuspended in RPMI 1640 complete medium. Cell numbers and viability (over 95%) were assessed microscopically using trypan blue dye exclusion technique. The splenocytes (100 μL) were added in 96-well plates (1×10^6 cells/well) and cultured with 100 μL RPMI 1640 medium containing (ConA, 5 μg/mL) or lipopolysaccharide (LPS, 20 μg/mL) and different doses of LBP-1 (50, 100, 200 μg/mL) at 37 °C in a humidified 5% CO_2 incubator for 72 h. Twenty microliters of MTT (5 mg/mL) were added to each well and incubated for another 4 h. After the incubation, the cell suspensions were centrifuged at 800 rpm for 10 min and the supernatants were removed, then 200 μL of dimethyl sulfoxide (DMSO) working solution was added. The absorbance was measured in an automatic ELISA plate reader (model 680; Bio-Rad, US) at 570 nm after 15 min. Each experiment was performed in triplicate.

2.9. Macrophage phagocytosis assay

The mice were randomly divided into five groups (8 mice in each group). The different doses of LBP-1 (50, 100, 200 mg/kg) were administered intragastrically each day for 7 consecutive days. The normal control and model control groups were given physiologic saline. Except for the mice in the normal control group, others received cyclophosphamide (Cy, 40 mg/kg) on the 5th day and the 6th day, respectively. Two hours after the final treatment on the 7th day, all mice were injected with 0.5 mL of chicken red blood cells (CRBCs) suspension (5%, v/v, with normal saline). Two hours after following stimulation by intraperitoneally injection of 2 mL of Hanks solution, the mice were euthanized by cervical dislocation. Peritoneal exudates (0.2 mL) were spread onto glass slides and then incubated at 37 °C for 30 min in a humidified incubator. After incubation, the supernatants were removed with 0.9% NaCl solution and the cells fixed in methanol for 15 min. It was stained with Giemsa dye for 30 min. The number of macrophage-ingesting CRBCs was calculated by direct visual counting under a light microscopy. The phagocytic rate (PR) was calculated by the following formula: PR (%) = (number of macrophage-ingesting CRBCs)/total number of macrophages × 100.

2.10. Acute toxicity assay

Acute toxicity was evaluated as previously described (Hwang, Ha, & Ma, 2013; Xu, Liu, Yuan, & Guan, 2012). Briefly, each group of mice weighing 18–22 g was given oral administration of LBP-1 (100, 200, 400, 800, 1200 mg/kg) in 0.2 mL PBS. Normal mice received PBS as control. Each group has 10 mice. All external morphological, behavioral, neurologic, autonomic changes, toxic effects were recorded continuously for 48 h.

2.11. Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed with Student's *t* test to evaluate the significance of difference between groups. Values of $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Characterization analysis

The crude polysaccharides were obtained from *Lilii Bulbus* by hot water extraction, ethanol precipitation, deproteinization, and lyophilization. The extracts were purified with DEAE-cellulose and Sephadex G-100 columns. One main fraction, named LBP-1, was collected for further structural characterization and bioactivity assay. It showed negative reactions to Fehling's reagent and iodine-potassium iodide, which indicated the absence of reducing sugars and starch-type polysaccharides. It also exhibited negative responses to Bradford test. No absorption occurred at 280 and 260 nm in the ultraviolet spectrum, which indicated the absence of protein and nucleic acid. The HPLC profile presented a single eluted peak. The average molecular weight of LBP-1 was 30.5 kDa. The quantitative result of monosaccharides analyzed by GC indicated that LBP-1 was only composed of glucose (Fig. 1).

The FTIR spectra in Fig. 2 showed the structural characterization of LBP-1. The attributions of the main absorptions were characteristic of glycosidic structures and were related to OH stretching vibration (3000–3500 cm^{-1}), C—H stretching vibration (2900–3000 cm^{-1}), OH flexural vibration (1620–1680 cm^{-1}), CO and C—O—C stretching vibration (1000–1200 cm^{-1}). On the other hand, LBP-1 had the absorption band centered at 850.38 cm^{-1} due to the α-type glycosidic bond. The prominent absorptions at 1158.10, 1081.34 and 1029.83 cm^{-1} also indicated the pyranose form of the glucosyl residue. The peak at 944.83 cm^{-1} displayed the non-symmetric ring stretching vibration of D-glucopyranosyl.

3.2. Antitumor effect of LBP-1 in vivo

Lilii Bulbus has been used for treating respiratory system diseases (include lung cancers) in the Chinese folk medicine, and were proved to have antitumor and immunopotentiating activities (Wu et al., 2008). Therefore, we investigated antitumor effects of polysaccharides, the main component of the herb, in Lewis lung carcinoma bearing mice. A significant reduction in the tumor weights was observed in the LBP-1-treated mice. As shown in Table 1, LBP-1 inhibited the growth of the transplanted tumor dose-dependently, with the inhibitory rates of 23.9%, 37.2%, and 45.7% at the respective dose of 50, 100, 200 mg/kg. Furthermore, mice in the LBP-1-treated groups, as well as the normal control group, were vigorous with shiny fur and normal body weight increases. Also, LBP-1 at doses up to 1200 mg/kg had no significantly body weight (results not shown), suggesting that LBP-1 was of low toxicity. As expected, DDP, a conventional chemotherapeutic drug, showed the highest tumor inhibition rate of 49.5%. But the toxicity in the DDP-treated group was also serious, manifested by anorexia, listlessness and emaciation, as well as had significant decrease in body mass compared with that of the model control group or the normal control group ($P < 0.01$).

3.3. Effects of LBP-1 on serum cytokine levels in tumor-bearing mice

The immune system plays an important role in antitumor defense. Many reports had suggested the high molecular weight

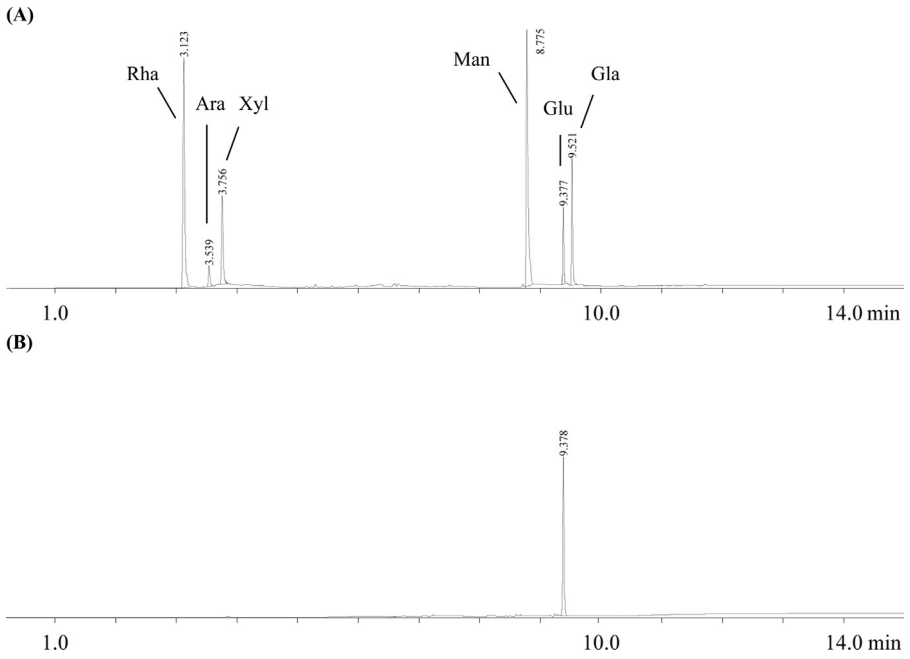

Fig. 1. Gas chromatograms of acetylated monosaccharides: (A) different standard monosaccharides and (B) LBP-1.

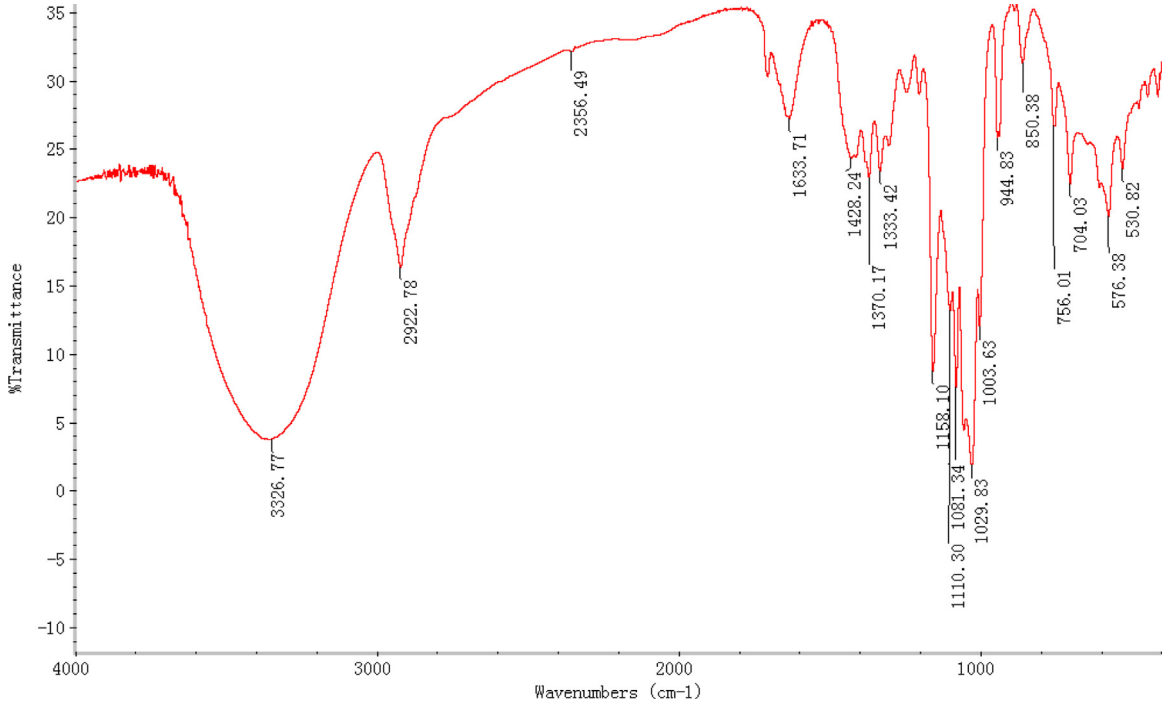

Fig. 2. The infrared spectrum of LBP-1.

Table 1
Tumor inhibition of LBP-1 in Lewis tumor-bearing mice.

Group	Dose (mg/kg)	Tumor weight (g)	Inhibitory rate (%)	Body weight (g)
Normal control	–		–	33.3 ± 3.7
Model control	–	1.88 ± 0.25	–	34.2 ± 2.3
DDP	2	0.92 ± 0.16[b]	49.5	23.2 ± 1.0[b]
LBP-1	50	1.43 ± 0.22[a]	23.9	32.9 ± 2.8
LBP-1	100	1.18 ± 0.20[b]	37.2	32.9 ± 2.6
LBP-1	200	1.02 ± 0.18[b]	45.7	34.0 ± 2.6

Data were expressed as mean ± SD ($n = 8$).
[a] Compared with the model group, $P < 0.05$.
[b] Compared with the model group, $P < 0.01$.

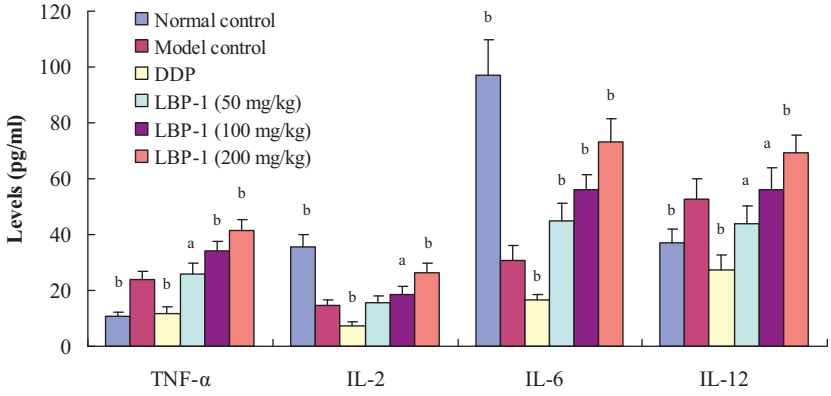

Fig. 3. Levels of serum cytokines in Lewis tumor-bearing mice. Data denoted were means ± SD ($n = 8$). Compared with model control, $^{a}P < 0.05$, $^{b}P < 0.01$.

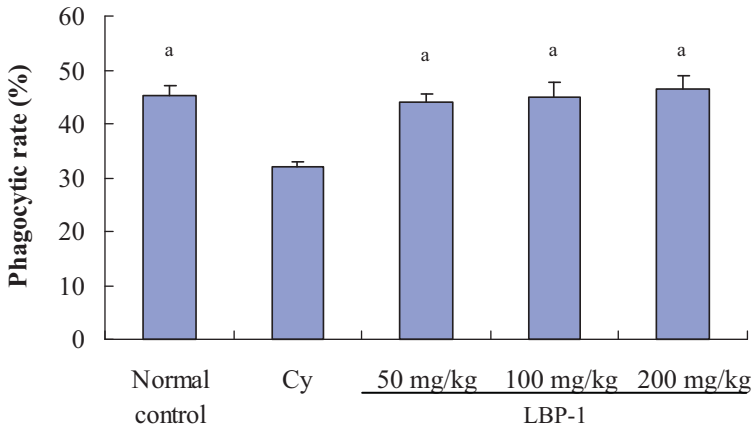

Fig. 4. Effect of LBP-1 on macrophage phagocytosis in mice. Mice were randomly divided into five groups. Different doses of LBP-1 were administered intragastrically each day for 7 days. All mice except for the normal received cyclophosphamide (Cy, i.p.) twice and then injected with 5% chicken red blood cell (CRBC) suspension. The peritoneal exudates were prepared to calculate the phagocytic rates. Each value represents mean ± SD ($n = 8$). Compared with the normal control group, $^{a}P < 0.01$.

polysaccharides are considered to be a consequence of stimulation of the immune response in the host, rather than direct killing of tumor cells (Bao, Yuan, Wang, Liu, & Lan, 2013; Ding, Zhu, & Gao, 2012; Liu et al., 2013; Tang et al., 2012; Xu et al., 2009). To assess cytokine secretion associated with the antitumor immunity induced by LBP-1 in Lewis tumor-bearing mice, the serum cytokines levels were measured. It is well known that cytokines play a pivotal role in fighting against the tumor growth. TNF-α and IL-6 can cause apoptosis of tumor, resulting in tumor necrosis (Mariño & Cardier, 2003; Wagley et al., 2007; Yun, Park, Jo, Kim, & Shin, 2012). They also play pivotal roles in host defense and induce the expression of a number of other immunoregulatory and inflammatory mediators (Derin et al., 2008; Kastamoulas et al., 2013). IL-2 has many immunopotentiating effects, such as proliferation of T cells, B cells, NK cells and monocytes, augmentation of cytotoxicities of T cells and NK cells and *in vivo* generation of lymphokine-activated killer (LAK) cells, which exhibit high cytolytic activities against autologous tumor cells. IL-2 modulates IL-12 signaling through JAK-STAT pathway leading to the development of Th1 response (Kisseleva, Bhattacharya, Braunstein, & Schindler, 2002; Liu, Gaffen, & Goldsmith, 1998; Watanabe & Arai, 1996). As shown in Fig. 3, the serum levels of TNF-α and IL-12 in the model control mice were significantly increased while IL-2 and IL-6 were significantly decreased owing to the transplanted tumor ($P < 0.01$). These cytokine levels in serum of the DDP-treated group were significantly lower than those of the model control ($P < 0.01$). However, LBP-1, especially treated at the dose of 200 mg/kg, significantly augmented the secretion of serum TNF-α, IL-2, IL-6 and IL-12 compared with those in the model control ($P < 0.05$). It demonstrated that LBP-1 could enhance the immune function by promoting cytokine levels in Lewis lung carcinoma-bearing mice.

3.4. *Effects of LBP-1 on immune function in tumor-bearing mice*

Many reports had demonstrated that antitumor effects of botanical or fungi polysaccharides may begin with the activation of effector cells such as lymphocytes and macrophages (Bao et al., 2013; Fan, Ding, Ai, & Deng, 2012; Xu et al., 2009). Therefore, to further analyze the underlying mechanism of LBP-1 on the cellular immunity in tumor-bearing mice, the immunologic action of LBP-1 on peritoneal macrophages and spleen lymphocyte was investigated.

Macrophages not only can initiate innate immune responses but also provide a defense mechanism against tumor cells. In the last decades, the mechanism of tumor cell killing by macrophages has been studied extensively. Activated macrophages secrete several substances that are directly involved in tumor cell killing, *i.e.* TNF-α and nitric oxide (Galdiero et al., 2013; Klimp, de Vries, Scherphof, & Daemen, 2002; Schmieder, Michel, Schönhaar, Goerdt, & Schledzewski, 2012; Sica et al., 2008). As shown in Fig. 4, the phagocytic activity of macrophages in the Cy-treated group was much lower than that of the normal control group ($P < 0.01$). However, LBP-1 could significantly improve the phagocytic activity of macrophages in immune suppressed mice. The phagocytic

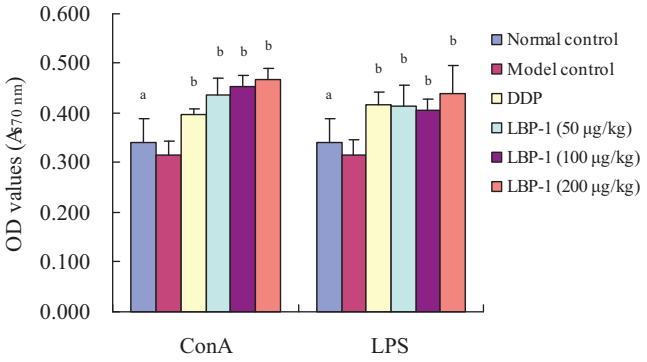

Fig. 5. Effects of LBP-1 on the proliferation of tumor-bearing mice splenocytes *in vitro*. Splenocytes were treated with different doses of LBP-1 (50, 100, 200 μg/mL) plus mitogens (A) ConA (5 μg/mL) or (B) LPS (20 μg/mL) for 72 h. After incubation, splenocyte proliferation was measured by MTT assay. Data denoted were means ± SD ($n = 8$). Compared with model control group, $^{a}P < 0.05$, $^{b}P < 0.01$.

activities of LBP-1 at the doses of 50–200 mg/kg were enhanced significantly compared to that of Cy-treated group. This implies that LBP-1 was capable of stimulating macrophage functions.

On the other hand, splenocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. T and B lymphocytes are two important classes of immunologically active cells. The former is mainly responsible for cellular immunity, and the latter is the only cell capable of producing antibodies (Chiou, Sheu, Chang, Huang, & Hong-Nerng, 2005; Eerola, Soini, & Pääkkö, 1999; Ruffell, DeNardo, Affara, & Coussens, 2010). Therefore, the co-mitogenic effects of LBP-1 on the proliferations of splenocytes in response to mitogens were investigated. It is well known that splenocyte proliferative response induced by ConA *in vitro* may be used as a conventional method to evaluate T lymphocyte activity, while that induced by LPS may be used to evaluate B lymphocyte activity. As shown in Fig. 5, splenocyte activities induced by ConA and LPS in the model control group were both lower than that in the normal control group. It indicated that immune function of tumor-bearing mice was decreased compared with that of the normal control mice. However, after ConA or LPS challenge, splenocyte of the LBP-1 treated group had stronger proliferation activity than that of the model control group ($P < 0.01$). And these proliferation activity of LBP-1 to spleen cells responded in the dose-dependent manner. The above results indicated that LBP-1 could enhance immune function of tumor-bearing mice.

4. Conclusions

In the present study, we successfully isolated and purified a polysaccharide from *Lilii Bulbus* and characterized its physicochemical properties. It was a glucan with molecular weight of 30.5 kDa. Besides, we evaluated the tumor growth inhibitory effects of the polysaccharide on Lewis lung carcinoma in mice. LBP-1 could significantly inhibit the growth of Lewis lung carcinoma in mice, but also remarkably increase the level of serum cytokines, macrophage phagocytosis and splenocytes proliferation in mice. The above results suggested that the antitumor activity of LBP-1 might be achieved by improving immune system functions, but further investigation about the relationship between immunomodulation and antitumor activity is needed.

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