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Characterization of a water-soluble polysaccharide from *Boletus edulis* and its antitumor and immunomodulatory activities on renal cancer in mice

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ABSTRACT

A polysaccharide (BEP, Mw = 113,432 Da) was purified from *Boletus edulis*, which had a backbone consisting of (1→6)-linked-α-D-glucopyranosyl, (1→2,6)-linked-α-D-galactopyranosyl, (1→6)-linked-α-D-galactopyranosyl, and (1→3)-linked-α-D-rhamnopyranosyl residues, which were branched at O-2 position of (1→2,6)-linked-α-D-galactopyranosyl residue with a single terminal (1→)-linked-α-L-arabinofuranosyl residue. After 32 days' BEP administration to Renca tumor bearing mice, the tumor mass of Renca transplanted in mice was significantly repressed. Furthermore, BEP could significantly increase the spleen and thymus indices, stimulate splenocytes proliferation, augment NK cell and CTL activities in spleen, and promote the secretion of the cytokines IL-2 and TNF-α in Renca tumor bearing mice. Meanwhile oral administration of BEP (100 and 400 mg/kg) restored all the altered hematological and biochemical parameters of tumor-bearing mice to normal levels. Thus, these data demonstrate that BEP possesses potential immunomodulatory activity and might be employed as effective therapeutic agents for the prevention of renal caner.

1. Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (Jemal et al., 2011). Chemotherapy is one of the most frequently used therapeutic modalities for the treatment of cancer, but it does not achieve a satisfactory therapeutic result if it is employed alone (Li et al., 2010). Polysaccharides from natural sources are found to be effective, non-toxic substances with wide variety of biological activities, and have attracted lots of attention in the biochemical and medical areas (Ooi & Liu, 2000). In recent decades, many edible mushrooms with unique and subtle flavor have become attractive as source of physiologically functional foods and drug precursors due to their tonic and medicinal attributes (Zheng, Jie, Hanchuan, & Moucheng, 2005; Wasser, 2002). Particularly more evidences have been reported that many bioactive biopolymers, such as polysaccharides and polysaccharide–protein complexes isolated from mushrooms, have been used as a source of therapeutic agents for cancer through activation of various immune responses in the host (Wang et al., 1997; Wasser, 2002). They cause no harm and place no additional stress on the body, but help the body to adapt to environmental and biological stress (Mizuno et al., 2000; Schepetkin & Quinn, 2006). In particular, lentinan and schizophyllan have been used as immunotherapeutic agents to treat cancer in humans and in experimental tumor systems (Chihara, Hamuro, Meada, Arai, & Fukuoka, 1970; Smith, Rowan, & Sullivan, 2002). Immunomodulators are well known for their antitumor activity and polysaccharides are very proficient immunomodulators. Therefore, discovery and evaluation of polysaccharides with antitumor and immunostimulating properties from the various medicinal mushrooms has emerged as one of important research fields in chemistry and biology (Zhang & Huang, 2005).

Boletus edulis Bull., one of the most well-known delicious mushroom, distributes in many regions of China, such as Heilongjiang, Henan, Sichuan, Zhejiang, Yunnan, and so on (Luo, Luo, Huang, & Fan, 2012). Modern pharmacological studies demonstrated that polysaccharides extracted from *B. edulis* have been reported to have many biological functions such as anticancer, antioxidant and anti-inflammatory effects (Cengiz, Bektas, & Mustafa, 2008; Dentinger, Ammirati, & Both, 2010; Lemieszek et al., 2013; Luo et al., 2012; Zhang, Xiao, He, & Sun, 2011). In spite of these biological activities, the antitumor active biopolymers from *B. edulis* and related mechanisms have not been exactly defined and studied

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so far. The sufficient investigation on the physicochemical properties and antitumor activity of biopolymers from *B. edulis* was particularly necessary to broaden its applications in pharmaceutical industries and functional foods. Therefore, the purpose of the present investigation was to isolate and characterize one antitumor polysaccharide from *B. edulis*. Specifically, we used a renal cell carcinoma transplanted tumor model to evaluate its effect on antitumor activity based on the immune response.

2. Materials and methods

2.1. Materials and chemicals

B. edulis was obtained from Yabuli Co. Ltd. (Heilongjiang, China). DEAE-Sepharose CL-6B and Sepharose CL-6B were purchased from Pharmacia Chemical Co. Concanavalin A (Con A), lipopolysaccharide (LPS), the monosaccharide standards, trifluoroacetic acid (TFA) and bovine serum albumin (BSA) were purchased from Sigma Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco Invitrogen Co. (Grand Island, NY, USA). All the other chemicals used were of analytical grade.

2.2. Extraction, isolation and purification of BEP

The fruiting bodies of *B. edulis* (1.0 kg) were extracted with 95% EtOH (5 L) at 100 °C for 1.5 h to remove lipophilic compounds, and then successively extracted with 20 vol of distilled water at 100 °C for 3 times and 2 h for each time. After centrifugation (1700 × *g* for 10 min, at 20 °C), the supernatant was concentrated 10-fold and precipitated with ethanol (1:4, v/v) at 4 °C overnight. The precipitate collected by centrifugation was suspended in distilled water to remove the protein by the Sevage method (Staub, 1965), followed by exhaustive dialysis with water for 48 h to remove the low molecular weight materials, and freeze dried, giving 7.32 g of crude polysaccharide (CBEP).

The crude polysaccharide CBEP was fractionated on a DEAE-Sepharose CL-6B column (Cl^- form, 50 cm × 2 cm), eluting with distilled water and stepwise gradient of NaCl aqueous solutions (0.5 and 1 M) at a flow rate of 2.0 ml/min. After dialysis and lyophilization, the fraction BEPw eluted with distilled water was purified on a Sepharose CL-6B column (100 cm × 2 cm), eluting with 0.15 M NaCl at a flow rate of 1.0 ml/min to yield one completely separated fractions. This main fraction was collected, dialyzed and lyophilized to get a white purified polysaccharide (BEP). All the fractions were collected by the automated fraction collector, and monitored with the phenol–sulfuric acid method at 490 nm absorbance for polysaccharides.

2.3. Physicochemical characteristics, molecular weight and monosaccharide composition of BEP

The total carbohydrate content was determined by the phenol–H_2SO_4 method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content was quantified according to the Bradford's method (Bradford, 1976). Total uronic acid contents were measured by *m*-hydroxydiphenyl method using glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991).

The homogeneity and average molecular weight of BEP were performed on high performance size-exclusion chromatography (HPSEC) using a Waters HPLC system, including TSK-G3000 PW_{XL} column (7.8 mm ID × 30.0 cm), a Waters 2410 differential refractive index detector and an on-line degaser. The polysaccharide was diluted to a concentration of approximately 2 mg/ml by adding 0.1 M Na_2SO_4 solution and centrifuged (10,000 rpm, 3 min), and 20 μL of supernatant was injected for each run and eluted with 0.1 M Na_2SO_4 solution at a flow rate of 0.8 ml/min at 25 °C. The molecular weights of the polysaccharide fractions were determined by comparison with retention times of Dextran T-series standards (T-10, T-40, T-70, T-500, T-2000 and blue dextran T-2000).

Gas chromatography (GC) was used for identification and quantification of the monosaccharides as described by Xie et al. (2010). Briefly, BEP (10 mg) was hydrolyzed with 2 M TFA at 100 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates as described previously (Johnes & Albersheim, 1972; Oades, 1967) and were analyzed by GC on an Agilent 6280 instrument fitted with FID and equipped with a HP-5MS column (0.25 mm × 30 m × 0.25 μm).

2.4. Methylation analysis

The sample (20 mg) was methylated three times according to Needs and Selvendran (1993). Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated products were hydrolyzed, then reduced and acetylated as described by Sweet, Shapiro, and Albersheim (1975). The partially methylated alditol acetates were analyzed by GC/MS as described by Sun and Liu (2008).

2.5. UV, IR and NMR analysis

UV–vis absorption spectra were recorded with a Shimadzu MPS-2000 spectrophotometer between 190 and 290 nm. The FTIR spectra (KBr pellets) were recorded on SPECORD in a range of 400–4000 cm^{-1}. For NMR measurements POP was dried in a vacuum over P_2O_5 for several days, and then exchanged with deuterium (Dueñas-Chasco et al., 1997) by lyophilizing with D_2O for several times. The deuterium-exchanged polysaccharide (50 mg) was put in a 5-mm NMR tube and dissolved in 0.6 mL 99.98% D_2O, followed by recording with a Bruker AV-400 spectrometer at 55 °C.

2.6. Cell lines

The mouse renal cancer cell line Renca was obtained from Shaihai Fuxiang Biological Technology Co., Ltd. (Shanghai, China). They were maintained in the logarithmic phase of growth in RPMI 1640 medium supplemented with 2 mM L-glutamine (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% FBS at 37 °C under humidified air with 5% CO_2.

2.7. Experimental animals

Female BALB/c mice (5 weeks old) weighing 18–22 g were purchased from Experimental Animal Center of Fujian Medical University and acclimatized for 1 week before use. Rodent laboratory chow and tap water were provided ad libitum and maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of 50 ± 10%, and a 12/12 h light/dark cycle. All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals and with the guidelines established by Fuzhou General Hospital and approved by the Animal Care Review Committee, Fuzhou General Hospital.

2.8. In vivo antitumor activity

Under sterile condition, 0.1 ml of Renca cells suspension (2×10^6 cells/ml) was implanted subcutaneously into the rump of mice on day 0. Forty tumor-bearing BALB/c mice were divided into four groups ($n = 10$): two BEP-treated groups at two doses of 100 and 400 mg/kg, Renca-bearing group (model control group) and 5-FU positive control group. A normal control group ($n = 10$) without tumor inoculation and drug administration was also used

in this experiment. BEP dissolved in normal saline was administered intragastrically once daily for 32 consecutive days, starting 24 h after tumor inoculation. The mice in the model control and normal control group were orally administrated with an equal volume of normal saline (0.2 ml/10 g b.w.) instead of the test solution. The positive control was given 5-FU (20 mg/kg) via intraperitoneal injection. 24 h after last drug administration, all the mice were weighed and sacrificed by cervical dislocation to measure antitumor and hematological parameters. The spleen, thymus and tumor were immediately dissected and weighed. The spleen and thymus indices were calculated according to the following formula: spleen and thymus index (mg/10 g) = (weight of spleen or thymus/body weight). Tumor mass in the mice was measured every 3 days from day 0 to day 32. Tumor volume was assessed by measuring two perpendicular dimensions (long and short) using a caliper and calculated using the formula $(a \times b^2)/2$, where a is the larger and b is the smaller dimension of the tumor (Zhang et al., 2003).

2.9. Hematological and biochemical parameters assay

Blood samples were obtained on day 32 by heart puncture from all animals for the estimation of hematological and biochemical parameters and were collected with the anticoagulant in polystyrene tubes. Serum samples were separated by centrifugation at 3000 rpm at room temperature for 10 min and stored at −80 °C until assayed. Hemoglobin (Hb) content, red blood cell count (RBC), white blood cell count (WBC), total leukocytes count and lymphocyte count were determined by standard procedures. Likewise, the serum aspartate transanimase (AST), alanine transaminase (ALT), urea nitrogen (BUN), and creatinine levels were also determined by the enzymatic method using commercial reagent kits (Lanji, Shanghai, China), according to the manufacturer's instructions.

2.10. Evaluation of immunomodulatory properties of BEP in tumor-bearing mice

2.10.1. Spleen lymphocyte proliferation assay

Spleens collected from sacrificed mice under aseptic condition were chopped into small pieces and passed through a fine steel mesh to obtain a homogeneous cell suspension. The cells were washed and lymphocytes were separated from red blood cells by lysis buffer (0.15 M NH_4Cl, pH 7.4). After centrifugation ($380 \times g$ at 4 °C for 10 min), the pelleted cells were washed three times in PBS and resuspended in RPMI 1640 complete medium. Cell viability exceeded 95% were determined with a haemocytometer by trypan blue dye exclusion technique. Splenocyte proliferation was assayed as previously described (Tu, Sun, & Ye, 2008). The splenic cells (100 μl) were seeded in 96-well plates (2×10^6 cells/well) together with Con A (2.5 μg/ml), LPS (5.0 μg/ml) or RPMI1640 medium giving a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2. After 44 h, 20 μl MTT (5 mg/ml) were added to each well and incubated for another 4 h. The cell suspensions were centrifuged at $1400 \times g$ for 10 min and the supernatants were removed. Then 150 μl of dimethyl sulfoxide (DMSO) working solution (180 μl DMSO with 20 μl 1NHCl) was added. The absorbance was evaluated in an ELISA reader (Bio-Rad, USA) at 570 nm with a 630 nm reference after 15 min.

2.10.2. Assay of natural killer (NK) cell activity

The activity of NK cells was measured as previously described (Tu et al., 2008). Briefly, Renca cells (target cells) and splenocytes (effector cells) were mixed in 96-well U-bottom microtiter plate in a 50:1 ratio and incubated at 37 °C in 5% CO_2 atmosphere. After 24 h incubation, 50 μl of MTT solution (2 mg/ml) was added to each well and the plate was incubated for another 4 h and subjected to MTT assay. NK cell activity was calculated as following equation: NK activity (%) = $(OD_T - (OD_S - OD_E))/OD_T \times 100\%$, where OD_T, optical density value of target cells control, OD_S, optical density value of test samples, OD_E, optical density value of effector cells control.

2.10.3. Assays of cytotoxic T lymphocyte (CTL) activity

The CTL activity was analyzed using MTT method as described above. Renca cells (target cells) and splenocytes (effector cells) were mixed in a 50:1 ratio. To determine the percentage of target cells killed, specific lysis was calculated using the following formula: percentage of specific lysis = $(OD_T - (OD_S - OD_E))/OD_T \times 100\%$, where OD_T, optical density value of target cells control, OD_S, optical density value of test samples, OD_E, optical density value of effector cells control.

2.10.4. Assay for serum cytokine levels

Serum collected from tested mice were drawn into tubes and allowed to clot for 20 min for further use. The level of IL-2 and TNF-α using commercially available kits from R&D systems, with a solid-phase enzyme-linked immunoabsorbent assay (ELISA) as described by the manufacturers.

2.11. Statistical analysis

The data were expressed as mean ± standard deviation (SD) and examined for their statistical significance of difference with ANOVA and a Tukey post hoc test. *P*-values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Purification and characterization of polysaccharide BEP from B. edulis

The crude water-soluble polysaccharide, CBEP, was obtained from the fruiting bodies of *B. edulis* by derosination, hot-water extraction, ethanol precipitation, deproteinization, and lyophilization. CBEP was further subjected to a DEAE-Sepharose CL-6B chromatography and eluted with distilled water, followed by 0.5 and 1.0 M NaCl at a flow rate of 2.0 mL/min. According to the charge difference, three fractions of CBEPA, CBEPB, and CBEPC were isolated from distilled water elute, 0.5 M and 1.0 M NaCl elute, respectively (Fig. 1A). Among them, neutral fraction CBEPA showed the relatively high content. Therefore CBEPA was repeatedly subjected to size-exclusion column chromatography on a Sepharose CL-6B gel filtration column and eluted with 0.1 M NaCl at a flow rate of 1 mL/min, whereby one major polysaccharide peak was collected and then freeze-dried to yield BEP (Fig. 1B, 3.42 g, 0.45% of starting material).

3.2. Structural characterization of polysaccharide BEP from B. edulis

BEP presented a symmetrical, narrow peak on HPSEC (Fig. 2). According to the calibration curve with standard dextrans, the average molecular weight (MW) of this homogeneous polysaccharide was approximately 113,432 Da. Negative responses to the Bradford test further indicated that BEP did not contain protein. The negative results of Fehling's and iodine-potassium iodide tests showed BEP did not contain reducing sugar and did not belong to starch-type polysaccharide (Yang, Zhao, & Lv, 2008). The total carbohydrate content of BEP was 93.4%, as determined by the phenol-sulfuric acid method and no uronic acid was detected by *m*-hydroxydiphenyl method using glucuronic

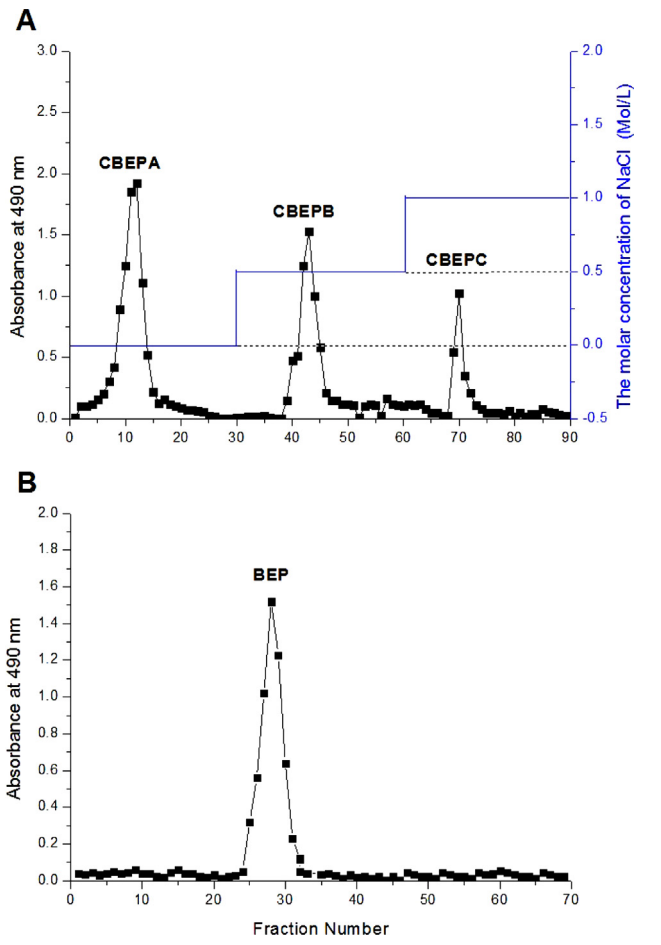

Fig. 1. (A) The profile of different polysaccharide fractions isolated from the fruiting bodies of *Boletus edulis* on a DEAE-Sepharose CL-6B column eluted with distilled water and stepwise gradient of NaCl aqueous solutions (0.5 and 1.0 M) at a flow rate of 2.0 ml/min. (B) The profile of the water-soluble polysaccharide (PTP) isolated from the fruiting bodies of *Boletus edulis* on a Sepharose CL-6B column eluted with 0.15 M NaCl aqueous solutions at a flow rate of 1.0 ml/min.

acid as the standard. GC analysis showed that BEP was composed of glucose, galactose, rhamnose and arabinose with molar ratios of 2.9:3.2:1.3:1.6, indicating BEP was a heteropolysaccharide.

The fully methylated product of BEP was hydrolyzed with acid, converted into alditol acetates, and analyzed by GC/MS. The GC–MS results (Table 1) indicated the liberation of 2,3,4-Me_2-Glcp (Residue A: →6)-α-Glcp-(1→), 3,4-Me_3-Gal*p* (Residue B: →2,6)-α-Gal*p*-(1→), 2,3,4-Me_3-Gal*p* (Residue C: →6)-α-Gal*p*-(1→), 2,4,-Me_3-Rhap (Residue D: →3)-α-Rhap-(1→) and 2,3,5,-Me_3-Araf (Residue E: α-Araf-(1→) in a relative molar ratio of 2.9:1.6:1.6:1.4:1.5. This ratio indicated a good correlation between terminal and branched

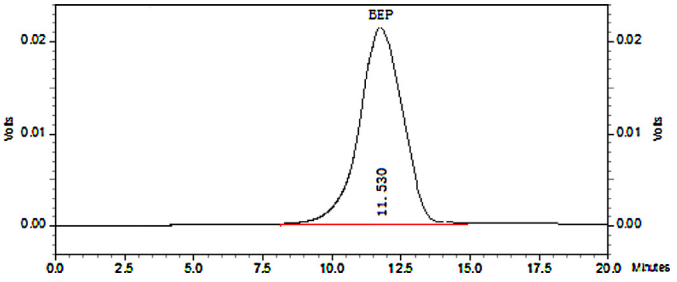

Fig. 2. The average molecular weight and homogeneity of BEP were determined by high-performance size-exclusion chromatography (HPSEC), eluting with 0.1 M Na_2SO_4 solution at a flow rate of 0.8 ml/min at 25 °C.

Table 1
The results of methylation analysis of BEP.

Peak no.	Methylated sugar	Molar ratio	Linkage type
Residue-A	2,3,4-Me_2-Glcp	2.9	→6)-α-Glcp-(1→
Residue-B	3,4-Me_3-Gal*p*	1.6	→2,6)-α-Gal*p*-(1→
Residue-C	2,3,4-Me_3-Gal*p*	1.6	→6)-α-Gal*p*-(1→
Residue-D	2,4,-Me_3-Rhap	1.4	→3)-α-Rhap-(1→
Residue-E	2,3,5,-Me_3-Araf	1.5	α-Araf-(1→

residues as evidenced by the results of the monosaccharide composition determined by GC. Residue A, B, C and D were major components of the backbone structure with single terminal of Residue E at the O-2 position of Residue B.

The data on UV, IR and NMR analysis of BEP were showed in Table 2. No absorption was detected by the UV spectra at both 260 nm and 280 nm, indicating that the nucleic acids and protein were absent in BEP. The broad bands at 3452.65 cm^{-1} were the characteristic peaks of the hydroxyl O—H stretching vibration and the signals at 2931.25 cm^{-1} indicated C—H stretching vibration. The bands at 1445.82 and 1341.24 cm^{-1} were the absorption peaks of variable angle vibration of C—H bond. A band at 1045.02 cm^{-1} caused by non-symmetric C—O—C stretching vibration was also observed. On the other hand, the absorption band at 844.32 cm^{-1} suggested the linkage of α-glycosides in the molecular structure of BEP (Yu et al., 2009). Based on published data, this analysis clarified that purified BEP had the typical absorption of polysaccharides (Barros et al., 2013; Chen et al., 2013; Yan, Yin, Zhang, Yang, & Yu, 2013). In the ^{13}C NMR spectrum of BEP, the peaks at 99.14, 100.67, 100.69, 101.42 and 105.63 ppm attributed to the anomeric carbons of Residue A, B, C, D, and E, respectively. The predominance of Residue A, together with the typical signal at 99.14 ppm, supported the high proportion of 1,6-linked-α-D-Glc*p* in BEP. The signal of substituted C-3 of Residues D had moved downfield to 74.32 ppm. Peak at 79.23 ppm in the spectrum appeared to come from C-2 resonance of Residue-B and signal 84.43 ppm appeared in low magnetic field should be attributed to C-4 of Residue E. Three signals of 69.56, 69.32 and 69.98 at high magnetic filed seemed to substituted C-6 signal of Residue A, B and C. On the contrary, peaks 58.98 and 61.43 ppm were assigned to unsubstituted C-6 of Residue D and C-5 of Residue E, respectively, because of no substitution on the C-6 for Residue D and no substitution on the C-5 position for Residue E. In ^1H NMR spectrum of BEP, the peaks at 5.34, 5.08, 5.03, 5.12 and 5.15 ppm were assigned to the H1 signals of Residue A, B, C, D and E, respectively. The special anomeric carbons and their protons chemical shift confirmed that sugar residues were linked by α-glycosidic bond, which agreed with the presence of 844.32 cm^{-1} on IR spectra (Barker, Bourne, Stacey, & Whiffen, 1954). All the NMR chemical shifts were compared with the literature values (Fan et al., 2006; Jia, Liu, Dong, & Fang, 2004; Liu, Sun, Yu, & Liu, 2012; Pramanik, Mondal, Chakraborty, Rout, & Islam, 2005; Roger, Kervarec, Ratiskol, Colliec-Jouault, & Chevolot, 2004; Sun & Liu, 2009; Sun, Liang, Zhang, Tong, & Liu, 2009; Ye et al., 2008).

Table 2
The data of UV analysis, IR analysis and NMR analysis of the purified polysaccharide BEP.

Assay	Peaks or signals at
UV analysis (nm)	260; 280
IR analysis (cm^{-1})	3452.65; 2931.25; 1445.82; 1341.24; 1045.02; 844.32
^1H NMR analysis (ppm)	5.34; 5.15; 5.12; 5.08; 5.03
^{13}C NMR analysis (ppm)	105.63; 101.42; 100.69; 100.67; 99.14; 84.43; 79.23; 74.32; 69.98; 69.56; 69.32; 61.43; 58.98

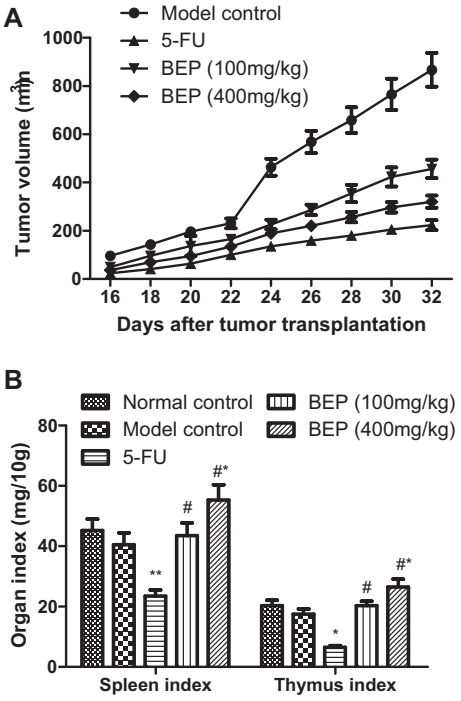

Fig. 3. (A) Effect of BEP on in vivo tumor growth; and (B) Effect of BEP on the spleen and thymus indices in Renca tumor-bearing mice. Data were expressed as mean ± SD (n = 10 mice per groups). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model control group; $^{\#}P < 0.05$ compared with 5-FU group.

3.3. Effect of BEP on tumor growth and immune organ indices in tumor-bearing mice

To investigate whether BEP had inhibitory effects on tumor growth in vivo, Renca cells suspension (2×10^6 cells/ml) was implanted subcutaneously into the rump of BALB/c mice. Mice were then treated by daily oral administration of 100 and 400 mg/kg of BEP or 5-FU for 32 days. Oral administration of BEP (100 and 400 mg/kg) decreased the volume of the tumor mass, whereas the tumors growth did not exhibit any delay in the control group (Fig. 3A). Especially at the high dose of 400 mg/kg, BEP exhibited an obvious repression effect on Renca transplanted tumor growth, comparable to 5-FU.

The result of the immune organ indices in tumor-bearing mice was shown in Fig. 3B. As expected, tumor transplanted in mice induced significant attenuation of the relative spleen and thymus weight, and two indices further rapidly decreased after 5-FU treatment than those in normal mice ($P < 0.05$ or $P < 0.01$), supporting the fact that tumor itself or 5-FU often resulted in immunological suppression. Consistent with the antitumor activity, treatments with BEP could restore the spleen and thymus indices in tumor-bearing mice to normal level and even surpass the normal values at the dose of 400 mg/kg ($P < 0.05$).

3.4. Effect of BEP on hematological and biochemical parameters in tumor-bearing mice

Hematological parameters in Renca tumor-bearing mice on day 32 were found to be significantly altered when compared to the normal group, except for WBC count. The total RBC count and Hb content were found to be significantly decreased in the untreated tumor-bearing animals. An increase in the total leukocytes was observed, but the percentage of lymphocyte (%) had a significant decrease as compared to that of normal mice ($P < 0.05$). Administration of BEP (100 and 400 mg/kg) to tumor-bearing mice restored all the altered hematological parameters to almost near to normal values (Table 3). Hematological markers like total leukocytes count and lymphocyte count were remarkably increased in 100 and 400 mg/kg BEP-treated mice, which supported the immunomodulatory property of the compound. Additionally, the WBC count of Renca tumor-bearing mice was obviously decreased in the 5-FU-treated group, while it showed no significant difference in the BEP-treated groups compared to that in normal mice ($P > 0.05$), suggesting that BEP caused no bone marrow suppression. Usually in cancer chemotherapy the major problem is anemia due to reduction in RBC. Present study indicated that BEP might significantly enhance the total RBC count and Hb level when compared to that of model control.

ALT and AST are considered to be sensitive indicators of hepatocellular damage and the values provide a quantitative evaluation of the degree of damage to the liver (Al-Habori, Al-Aghbari, Al-Mamary, & Baker, 2002). BUN and creatinine levels are biomarkers or indicators of renal injury, and its high content often indicates the malfunctions of kidney (Fu et al., 2012). In Table 4, an obvious increase in renal (creatinine) or liver (ALT and AST) parameters were seen in Renca tumor transplanted mice as compared to those of normal control group ($P < 0.05$), except for BUN ($P > 0.05$). As suspected, 5-FU administration to tumor-bearing mice resulted in their rise, supporting the fact that 5-FU had an adverse effect on liver and kidney function. The levels of AST, ALT, BUN and creatinine were significantly decreased by BEP treatment, and almost restored to the normal standard.

These results indicated that BEP treatment exhibited no toxic effect on hematological system and liver/kidney organ of mice, and was superior to 5-FU therapy.

3.5. Effect of BEP on splenocyte proliferation in tumor-bearing mice

The immune system plays an important role in antitumor defense (Aravind, Joseph, Varghese, Balaram, & Sreelekha, 2012). Splenocyte proliferation is a crucial event in the activation cascade

Table 3
Effects of BEP on hematological parameters in Renca tumor-bearing mice. Data were expressed as mean ± SD (n = 10 mice per groups).

Drug	Dose	RBC (10^6 cells/μl)	WBC (10^3 cells/μl)	Hb (g/dl)	Total leukocytes (10^3 cells/μl)	Lymphocyte (%)
Normal control saline	0.2 ml/10 g	4.38 ± 0.32	5.32 ± 0.65	9.54 ± 0.66	5.36 ± 0.45	53.6
Model control saline	0.2 ml/10 g	3.17 ± 0.24[a]	5.56 ± 0.51	6.22 ± 0.70[a]	7.57 ± 0.64[a]	42.5[a]
5-FU	20 mg/kg	4.66 ± 0.45[b]	4.08 ± 0.47[a,b]	9.32 ± 0.81[b]	3.13 ± 0.31[b]	43.6[a]
BEP	100 mg/kg	4.24 ± 0.45[b]	4.89 ± 0.38[b,c]	8.90 ± 0.65[b]	9.82 ± 0.76[aa,b,cc]	53.5[b,c]
BEP	400 mg/kg	4.55 ± 0.34[b]	5.14 ± 0.46[c]	9.11 ± 0.74[b]	11.53 ± 0.89[aa,bb,cc]	58.5[b,c]

[a] $P < 0.05$ compared with normal control group.
[aa] $P < 0.01$ compared with normal control group.
[b] $P < 0.05$ compared with model control group.
[bb] $P < 0.01$ compared with model control group.
[c] $P < 0.05$ compared with 5-FU group.
[cc] $P < 0.01$ compared with 5-FU group.

Table 4
Effects of BEP on liver and kidney biochemical parameters in Renca tumor-bearing mice. Data were expressed as mean ± SD (n = 10 mice per groups).

Drug	Dose	AST (U/L)	ALT (U/L)	BUN (mmol/L)	Creatinine (mmol/L)
Normal control saline	0.2 ml/10 g	42.55 ± 3.52	33.65 ± 3.03	5.65 ± 0.35	22.14 ± 1.37
Model control saline	0.2 ml/10 g	58.45 ± 3.65[a]	41.12 ± 3.65[a]	6.24 ± 0.65	32.13 ± 2.31[a]
5-FU	20 mg/kg	85.72 ± 5.65[aa,b]	63.65 ± 3.98[aa,b]	10.32 ± 0.75[a,b]	53.31 ± 3.58[aa,b]
BEP	100 mg/kg	46.54 ± 3.87[b,c]	43.43 ± 3.21[a,c]	6.03 ± 0.35[c]	47.25 ± 2.42[b,c]
BEP	400 mg/kg	43.08 ± 3.05[b,cc]	37.43 ± 3.28[c]	5.89 ± 0.31[c]	39.63 ± 2.37[c]

[a] $P < 0.05$ compared with normal control group.
[aa] $P < 0.01$ compared with normal control group.
[b] $P < 0.05$ compared with model control group.
[c] $P < 0.05$ compared with 5-FU group.
[cc] $P < 0.01$ compared with 5-FU group.

of both cellular and humoral immune responses. T lymphocytes are mainly responsible for cellular immunity and B lymphocytes are the only cell capable of producing humoral immune responses (Fan, Ding, Ai, & Deng, 2012). Extensive documents demonstrated that many natural biological macromolecules from mushrooms produced their antitumor effects by activating different immune responses in the host (Ooi & Liu, 2000). As a first step toward understanding the mechanism of the antitumor activity of BEP, we investigated its effect on lymphocyte proliferation in tumor-bearing mice. As shown in Fig. 4A, Con A- and LPS-induced splenocyte proliferation in the Renca tumor-bearing mice was significantly enhanced by BEP at the doses of 100 and 400 mg/kg ($P < 0.01$) compared to those of model control mice. However, Con A- and LPS-stimulated splenocyte proliferations in the 5-FU-treated group decreased significantly ($P < 0.05$) as compared with model control. Moreover, BEP itself could act as an independent mitogen similarly to Con A and LPS and thus showed a direct mitogenic activity for mouse splenocytes. Our experiment results also proved that immune function of tumor-bearing mice was inferior to normal mice, which was not significant ($P > 0.05$). These results indicated that BEP combined with Con A or LPS, or itself could significantly increase the activation potential of T- and B-cells and enhance the humoral immunity and cell-mediated immunity in tumor-bearing mice.

3.6. Effect of BEP on NK cells and CTL activities in tumor-bearing mice

NK cells and CTL represent two major populations of cytotoxic lymphocytes (Kos & Engleman, 1996; Medzhitov & Janeway, 1997), and play a major role as effector cells in the defense against tumors and viruses (Boon, Cerottini, Van den Eynde, van der Bruggen, & Van Pel, 1994; Moretta, Bottino, Cantoni, Mingari, & Moretta, 2001). Therefore we measured the effects of BEP on the cytotoxic activities of NK cells and CTL, and the results were shown in Fig. 4B. Splenic NK and CTL cytotoxic activity in model group was lower than normal control, while BEP (100 and 400 mg/kg) administrated intragastrically increased the splenic NK and CTL cytotoxic activities of Renca tumor-bearing mice as compared to those of the model control rats ($P < 0.01$). However, NK cell and CTL activities in the 5-FU-treated mice were significantly lower than those of the model control ($P < 0.05$). These results suggested that NK cells and CTL contributed to the antitumor effects induced by BEP.

3.7. Effect of BEP on serum cytokines levels in tumor-bearing mice

On the basis of the activation of BEP on splenic lymphocytes, NK cells and CTL, the production of serum cytokines by BEP treatment in Renca tumor-bearing mice was detected and the results were shown in Fig. 4C. BEP could markedly augmented the secretion of IL-2 and TNF-α in Renca tumor-bearing mice at 100 and 400 mg/kg doses ($P < 0.05$ or $P < 0.01$) compared with model control group. The production of IL-2 was significantly decreased in 5-FU-treated mice as compared with model control ($P < 0.05$). Conversely, there was no significant difference on the concentrations of TNF-a between the 5-FU-treated group and the model control group ($P > 0.05$). An

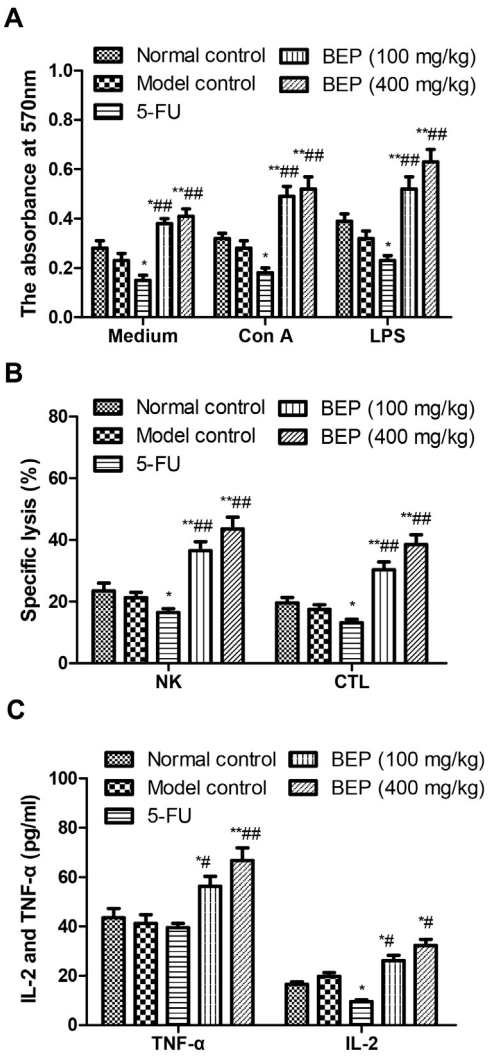

Fig. 4. (A) The effect of BEP on lymphocyte proliferation in Renca tumor-bearing mice. Data were expressed as mean ± SD (n = 10 mice per groups). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model control group; $^{\#\#}P < 0.01$ compared with 5-FU group. (B) Effect of BEP on Splenic NK and CTL cytotoxic activity in Renca tumor-bearing mice. Data were expressed as mean ± SD (n = 10 mice per groups). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model control group; $^{\#\#}P < 0.01$ compared with 5-FU group. (C) Effect of BEP on serum cytokines L-2 and TNF-α levels in Renca tumor-bearing mice. Data were expressed as mean ± SD (n = 10 mice per groups). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model control group; $^{\#\#}P < 0.01$ compared with 5-FU group.

increase in serum cytokines IL-2 and TNF-α level may also explain the antitumor properties of this polysaccharide.

4. Conclusions

In the present study, one water-soluble polysaccharide, BEP, was successfully isolated and purified from the fruiting bodies of *B. edulis*, with an average molecular weight of 113,432 Da, and BEP contained no protein and uronic acid, but has a high carbohydrate content of 93.4%. Monosaccharide component analysis indicated that BEP was composed of glucose, galactose, rhamnose and arabinose with molar ratios of 2.9:3.2:1.3:1.6. The data obtained from GC/MS, IR and NMR (^{1}H and ^{13}C) analysis revealed that BEP had a backbone consisting of (1→6)-linked-α-D-glucopyranosyl, (1→2,6)-linked-α-D-galactopyranosyl, (1→6)-linked-α-D-galactopyranosyl, (1→3)-linked-α-D-rhamnopyranosyl residues that terminated with a single terminal (1→)-linked-α-L-arabinofuranosyl residue at the O-2 position of (1→2,6)-linked-α-D-galactopyranosyl residue along the main chain in the ratio of 2.9:1.6:1.6:1.4:1.5. In vivo antitumor experiment, BEP could significantly inhibit the growth of Renca transplanted in mice, and had no cytotoxicity to hematological system and liver/kidney function of tumor-bearing mice at test doses. Acute toxicity was also investigated by oral administration of serial BEP dilution (from 500 mg/kg to 2000 mg/kg body weight) in 200 μL PBS to the normal mice. Acute toxicity results proved that there was no toxicity up to a concentration of 2000 mg/kg body weight in BALB/c mice as evidenced by absence of significant behavioral changes and animal death (data now shown). Furthermore, BEP could significantly stimulate splenocytes proliferation, increase the spleen and thymus indices, elevate the activities of NK cell and CTL in spleen, and promote the secretion of the cytokines IL-2 and TNF-α in Renca tumor-bearing mice. The above results suggested that the BEP had indirect anti-tumor activity achieved by improving immune response. This is only a preliminary study to prove the antitumor effect of BEP on renal cancer. Further researches about elucidating detailed mechanism of its anticancer actions are in process.

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References

Al-Habori, M., Al-Aghbari, A., Al-Mamary, M., & Baker, M. (2002). Toxicological evaluation of *Catha edulis* leaves: A long-term feeding experiment in animals. *Journal of Ethnopharmacology*, *83*, 209–217.

Aravind, S. R., Joseph, M. M., Varghese, S., Balaram, P., & Sreelekha, T. T. (2012). Antitumor and immunopotentiating activity of polysaccharide PST001 isolated from the seed kernel of *Tamarindus indica*: An in vivo study in mice. *Scientific World Journal*, *2012*, 361382.

Barker, S. A., Bourne, E. J., Stacey, M., & Whiffen, D. H. (1954). Infra-red spectra of carbohydrates. Part I. Some derivatives of D-glucopyranose. *Journal of the Chemical Society*, *0*, 171–176.

Barros, F. C., da Silva, D. C., Sombra, V. G., Maciel, J. S., Feitosa, J. P., Freitas, A. L., et al. (2013). Structural characterization of polysaccharide obtained from red seaweed *Gracilaria caudata* (J Agardh). *Carbohydrate Polymers*, *92*, 598–603.

Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P., & Van Pel, A. (1994). Tumor antigens recognized by T lymphocytes. *Annual Review of Immunology*, *12*, 337–365.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein binding. *Analytical Biochemistry*, *72*, 248–254.

Cengiz, S., Bektas, T., & Mustafa, Y. (2008). Evaluation of the antioxidant activity of four edible mushrooms from the Central Anatolia, Eskisehir – Turkey: *Lactarius deterrimus*, *Suillus collitinus*, *Boletus edulis*, *Xerocomus chrysenteron*. *Bioresource Technology*, *99*, 6651–6655.

Chen, Y., Mao, W., Gao, Y., Teng, X., Zhu, W., Chen, Y., et al. (2013). Structural elucidation of an extracellular polysaccharide produced by the marine fungus *Aspergillus versicolor*. *Carbohydrate Polymers*, *93*, 478–483.

Chihara, G., Hamuro, J., Meada, Y., Arai, Y., & Fukuoka, F. (1970). Fractionation and purification of polysaccharides with marked antitumour activity, especially Lentinan from *Lentinus edodes* (Bark) sing, an edible mushroom. *Cancer Research*, *30*, 2776–2781.

Dentinger, B. T. M., Ammirati, J. F., & Both, E. E. (2010). Molecular phylogenetics of porcini mushrooms (*Boletus* section). *Molecular Phylogenetics and Evolution*, *57*, 1276–1292.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Dueñas-Chasco, M. T., Rodríguez-Carvajal, M. A., Mateo, P. T., Franco-Rodríguez, G., Espartero, J., Irastorza-Iribas, A., et al. (1997). Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus*. *Carbohydrate Research*, *303*, 453–458.

Fan, J. M., Zhang, J. S., Tang, Q. J., Liu, Y. F., Zhang, A. Q., & Pan, Y. J. (2006). Structural elucidation of a neutral fucogalactan from the mycelium of *Coprinus comatus*. *Carbohydrate Research*, *341*, 1130–1134.

Fan, L., Ding, S., Ai, L., & Deng, K. (2012). Antitumor and immunomodulatory activity of water-soluble polysaccharide from *Inonotus obliquus*. *Carbohydrate Polymers*, *90*, 870–874.

Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*, 157–162.

Fu, J., Fu, J., Yuan, J., Zhang, N., Gao, B., Fu, G., et al. (2012). Anti-diabetic activities of *Acanthopanax senticosus* polysaccharide (ASP) in combination with metformin. *International Journal of Biological Macromolecules*, *5*, 619–623.

Jia, L. M., Liu, L., Dong, Q., & Fang, J. N. (2004). Structural investigation of a novel rhamnoglucogalactan isolated from the fruiting bodies of the fungus *Hericium erinaceus*. *Carbohydrate Research*, *339*, 2667–2671.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians*, *61*, 69–90.

Johnes, T. M., & Albersheim, P. (1972). A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharide. *Plant Physiology*, *49*, 926–936.

Kos, F. J., & Engleman, E. G. (1996). Immune regulation: A critical link between NK cells and CTLs. *Immunology Today*, *17*, 174–176.

Lemieszek, M. K., Cardoso, C., Ferreira Milheiro Nunes, F. H., Ramos Novo Amorim de Barros, A. I., Marques, G., Pożarowski, P., et al. (2013). *Boletus edulis* biologically active biopolymers induce cell cycle arrest in human colon adenocarcinoma cells. *Food & Function*, *25*, 575–585.

Li, J., Li, Q. W., Peng, Y., Zhao, R., Han, Z. S., & Gao, D. W. (2010). Protective effects of fraction 1a of polysaccharides isolated from *Solanum nigrum* Linne on thymus in tumor-bearing mice. *Journal of Ethnopharmacology*, *129*, 350–356.

Liu, J., Sun, Y., Yu, C. L., & Liu, L. (2012). Chemical structure of one low molecular weight and water-soluble polysaccharide (EFP-W1) from the roots of *Euphorbia fischeriana*. *Carbohydrate Polymers*, *87*, 1236–1240.

Luo, A., Luo, A., Huang, J., & Fan, Y. (2012). Purification, characterization and antioxidant activities in vitro and in vivo of the polysaccharides from *Boletus edulis* Bull. *Molecules*, *17*, 8079–8090.

Medzhitov, R., & Janeway, C. A., Jr. (1997). Innate immunity: Impact on the adaptive immune response. *Current Opinion in Immunology*, *9*, 4–9.

Mizuno, M., Shiomi, Y., Minato, K., Kawakami, S., Ashida, H., & Tsuchida, H. (2000). Fucogalactan isolated from *Sarcodon aspratus* elicits release of tumor necrosis factor-α and nitric oxide from murine macrophages. *Immunopharmacology*, *46*, 113–121.

Moretta, L., Bottino, C., Cantoni, C., Mingari, M. C., & Moretta, A. (2001). Human natural killer cell function and receptors. *Current Opinion in Pharmacology*, *1*, 387–391.

Needs, P. W., & Selvendran, R. R. (1993). Avoiding oxidative degradation during sodium hydroxide/methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide. *Carbohydrate Research*, *245*, 1–10.

Oades, J. M. (1967). Gas–liquid chromatography of alditol acetates and its application to the analysis of sugars in complex hydrolysates. *Journal of Chromatography*, *28*, 246–252.

Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry*, *7*, 715–729.

Pramanik, M., Mondal, S., Chakraborty, I., Rout, D., & Islam, S. S. (2005). Structural investigation of a polysaccharide (Fr. II) isolated from the aqueous extract of an edible mushroom, *Pleurotus sajor-caju*. *Carbohydrate Research*, *340*, 629–636.

Roger, O., Kervarec, N., Ratiskol, J., Colliec-Jouault, S., & Chevolot, L. (2004). Structural studies of the main exopolysaccharide produced by the deep-sea bacterium *Alteromonas infernus*. *Carbohydrate Research*, *339*, 2371–2380.

Schepetkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*, 317–333.

Smith, J. E., Rowan, N. J., & Sullivan, R. (2002). Medicinal mushrooms: A rapidly developing area of biotechnology for cancer therapy and other bioactivities. *Biotechnology Letters*, *24*, 1839–1845.

Staub, A. M. (1965). Removal of protein – Sevag method. *Methods in Carbohydrate Chemistry*, *5*, 5–6.

Sun, Y., Liang, H., Zhang, X., Tong, H., & Liu, J. (2009). Structural elucidation and immunological activity of a polysaccharide from the fruiting body of *Armillaria mellea*. *Bioresource Technology*, *100*, 1860–1863.

Sun, Y. X., & Liu, J. C. (2008). Structural characterization of a water-soluble polysaccharide from the roots of *Codonopsis pilosula* and its immunity activity. *International Journal of Biological Macromolecules*, *43*, 279–282.

Sun, Y., & Liu, J. (2009). Purification, structure and immunobiological activity of a water-soluble polysaccharide from the fruiting body of *Pleurotus ostreatus*. *Bioresource Technology*, *100*, 983–986.

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative analysis by various G.L.C. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research*, *40*, 217–225.

Tu, J., Sun, H. X., & Ye, Y. P. (2008). Immunomodulatory and antitumor activity of triterpenoid fractions from the rhizomes of *Astilbe chinensis*. *Journal of Ethnopharmacology*, *119*, 266–271.

Wang, S. Y., Hsu, M. L., Hsu, H. C., Tzeng, C. H., Lee, S. S., Shiao, M. S., et al. (1997). The antitumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. *International Journal of Cancer*, *70*, 699–705.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, *60*, 258–274.

Xie, Y., Jiang, S. P., Su, D. H., Pi, H. N., Ma, C., & Cao, P. (2010). Composition analysis and anti-hypoxia activity of polysaccharide from *Brassica rapa* L. *International Journal of Biological Macromolecules*, *47*, 528–533.

Yan, C., Yin, Y., Zhang, D., Yang, W., & Yu, R. (2013). Structural characterization and in vitro antitumor activity of a novel polysaccharide from *Taxus yunnanensis*. *Carbohydrate Polymers*, *96*, 389–395.

Yang, X. B., Zhao, Y., & Lv, Y. (2008). In vivo macrophage activation and physicochemical property of the different polysaccharide fractions purified from *Angelica sinensis*. *Carbohydrate Polymers*, *71*, 372–379.

Ye, L. B., Zhang, J. S., Ye, X. J., Tang, Q. J., Liu, Y. F., Gong, C. Y., et al. (2008). Structural elucidation of the polysaccharide moiety of a glycopeptide (GLPCWII) from *Ganoderma lucidum* fruiting bodies. *Carbohydrate Research*, *343*, 746–752.

Yu, R. M., Yin, Y., Yang, W., Ma, W. L., Yang, L., Chen, X. J., et al. (2009). Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured *Cordyceps militaris*. *Carbohydrate Polymers*, *75*, 166–171.

Zhang, A., Xiao, N., He, P., & Sun, P. (2011). Chemical analysis and antioxidant activity in vitro of polysaccharides extracted from *Boletus edulis*. *International Journal of Biological Macromolecules*, *49*, 1092–1095.

Zhang, C., & Huang, K. (2005). Characteristic immunostimulation by MAP, a polysaccharide isolated from the mucus of the loach, *Misgurnus anguillicaudatus*. *Carbohydrate Polymers*, *59*, 75–82.

Zhang, D. Y., Wu, J., Ye, F., Xue, L., Jiang, S., Yi, J., et al. (2003). Inhibition of cancer cell proliferation and prostaglandin E2 synthesis by *Scutellaria baicalensis*. *Cancer Research*, *63*, 4037–4043.

Zheng, R., Jie, S., Hanchuan, D., & Moucheng, W. (2005). Characterisation and immunomodulating activities of polysaccharides from *Lentinus edodes*. *International Immunopharmacology*, *5*, 811–820.