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# Antitumor activity of a polysaccharide from *Pleurotus eryngii* on mice bearing renal cancer

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ABSTRACT

One water-soluble polysaccharide (PEPw), with an average molecular weight of $2.5 \times 10^4$ Da, was isolated from the fruiting bodies of *Pleurotus eryngii* and subjected to composition analysis and evaluated for the antitumor and immunomodulatory activity. PEPw was composed of arabinose, mannose and galactose in a molar ratio of 1.2:2.3:6.2 and had a backbone mainly consisting of 1,6-linked-Gal*p*, 1,2,6-linked-Gal*p* and 1,4-linked-Man*p* residues, which was occasionally terminated with terminal-Ara*f* attached to O-2 of 1,2,6-linked-Gal*p* residue. The animal experiment results showed that PEPw significantly increased relative thymus and spleen indices, promoted the spleen lymphocytes proliferation induced by ConA or LPS, elevated the activities of NK cell and CTL in spleen, and increased the serum concentration of TNF-α and IL-2 in Renca tumor-bearing mice. As a result, the tumor growth was significantly inhibited by PEPw treatment at the doses of 50, 100 and 200 mg/kg in a dose-dependent manner. These data indicated that the anti-tumor activity of PEPw may be related to the activation of the immune response in tumor-bearing mice.



## 1. Introduction

Chemotherapy is one of the most frequently used therapeutic modalities for the treatment of cancer, but most of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells, leading to multiple organ toxicity that includes hemopoetic suppression and immunotoxicity (Ehrke, 2003). Polysaccharides from natural sources are found to be effective, relatively nontoxic substance, with wide variety of biological activities, and have attracted lots of attention in the biochemical and medical areas (Ooi & Liu, 2000). Thus, discovery and evaluation of polysaccharides with antitumor and immunostimulating properties has emerged as one of ideal candidates for tumor therapy (Schepetkin & Quinn, 2006). Recently much attention has been paid to edible and medicinal mushrooms, which are known for their nutritional and medicinal value and the diversity of their bioactive components. A number of investigators have found that these mushrooms have not only nutritional effects but also profound health promoting benefits to cure various diseases and maintain good health (Chen, Ju, Li, & Yu, 2012; Chen, Mao, et al., 2012; Huang, Ding, & Fan, 2012; Li et al., 2012; Liu, Wang, Pang, Yao, & Gao, 2010; Liu, Zhou, et al., 2010; Wu, Duan, Liu, & Cen, 2010; Zhang, Xiao, He, & Sun, 2011; Zhou & Chen, 2011). Mushroom will be important potential substances for pharmacy in the future.

*Pleurotus eryngii*, commonly called the king oyster mushroom due to its remarkable flavor and nutritional value, has rapidly become a highly valued species among consumers in North Africa, Europe, and Asia (Jeong, Jeong, Gu, Islam, & Song, 2010). It contains many biologically active substances such as polysaccharides, lipids, peptide, sterols, dietary fibre, etc. (Liu, Wang, et al., 2010; Liu, Zhou, et al., 2010). The polysaccharides extracted from *P. eryngii* have been demonstrated to have multiple functions, such as antitumor (Hwang, Nam, Chang, Noh, & Kim, 2003; Kim et al., 2004), antioxidant (Kim et al., 2004), hepatoprotective (Chen, Ju, et al., 2012, Chen, Mao, et al., 2012), enhancing immunity (Kang et al., 2004) and antihyperlipidemia (Chen, Ju, et al., 2012, Chen, Mao, et al., 2012). However, the anti-tumor activity of the polysaccharides from *P. eryngii* against the growth of renal carcinoma (Renca) in mice has not been reported.

Therefore, in the present study we focused on the polysaccharide extracted from *P. eryngii* for its antitumor and

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immunomodulatory activity in Renca-bearing mice. The results will be helpful to develop novel drugs and functional foods.

## 2. Materials and methods

### 2.1. Materials and chemicals

*P. eryngii* mushrooms were purchased from a local market in Xian city of China.

### 2.2. Preparation of P. eryngii crude polysaccharides

The dried *P. eryngii* (500 g) was exhaustively extracted with 95% ethanol (3× 10 vol) at 75 °C for 5 h under reflux to remove lipids. And then the residue was immersed into distilled water (3× 15 vol) at 100 °C for 3 h each time. The extract was filtered through gauze and the filtrate was then concentrated to one-tenth of the volume with a rotary evaporator at 80 °C under vacuum. The proteins in the extract were removed using the Sevag reagent (Staub, 1965). After removal of the Sevag reagent, a four-fold volume of ethanol was added to the mixture in a beaker overnight at 4 °C to precipitate *P. eryngii* polysaccharides (CPEPs), which was finally obtained by centrifugation at 4000 × *g* for 15 min.

### 2.3. Purification of the polysaccharide

The polysaccharides (50 mg) were loaded on anion exchange chromatography of a DEAE-cellulose column (2.0 × 40 cm) and eluted with 0.15 M NaCl. Each fraction of 6 mL was collected at a flow rate of 60 mL/h and monitored by the phenol–sulfuric acid method at 490 nm. The collected fractions were dialyzed, lyophilized, and then subjected to gel filtration on a column (1.5 cm × 100 cm) of Sephacryl S-300HR with 0.15 M NaCl at a flow rate of 60 mL/h. The relevant fractions were collected, concentrated, dialyzed and lyophilized to give white power, PEPw, which was subjected to the subsequent analysis.

### 2.4. Analysis of physicochemical properties of the polysaccharide

#### 2.4.1. Molecular weight determination

Measurement was done by high-performance gel-permeation chromatography (HPGPC) on a Waters High Performance Liquid Chromatography (HPLC) equipped with a TSK-GEL G3000 SWXL column (7.8 mm × 300 mm), eluted with triple-distilled water (1.0 mL/min) and detected by a Waters 2414 Refractive Index Detector. The column was calibrated using T-series Dextran standards of known molecular weight (200,000, 70,000, 40,000, 10,000, 5000 Da). The molecular weight of PEPw was estimated by reference to the calibration curve made above.

#### 2.4.2. Monosaccharide component analysis

The monosaccharide of PEPw was analyzed by the RP-HPLC method of precolumn derivation with 1-phenyl-3-5-pyrazolone (PMP) (Zhang et al., 2010). Briefly, the sample (2 mg) was first methanolyzed using 0.5 mL anhydrous methanol containing 2 M HCl at 80 °C for 16 h and then were hydrolyzed with 0.5 mL 2 M $CF_3COOH$ at 120 °C for 1 h. The hydrolysisproduct monosaccharides were derivatized to be PMP derivatives and subsequently analyzed by HPLC on DIKMA Inertsil ODS-3 column (150 × 4.6 mm i.d.) connected to a Shimadzu HPLC system and monitored by UV absorbance at 245 nm.

#### 2.4.3. Analysis of carbohydrate, protein and uronic acid concentrations of polysaccharides

Total neutral sugar content was determined by the reaction with phenol in the presence of sulfuric acid at 486 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using Glc as standard. Total uronic acid content was determined by photometry with m-hydroxybiphenyl at 523 nm (Blumenkrantz & Asboe-Hansen, 1973), using GalA as standard. The protein content of protein-bound polysaccharide was measured (Lowry, Rosebrough, Farr, & Randall, 1951), using BSA as standard. The purified polysaccharide was mixed with distilled water, ethanol, methanol, acetone, ethyl acetate and chloroform, respectively, to detect its solubility in different solvent. Phenolic hydroxyls substance was assayed by the ferric chloride method (Zhou, 1978). Starchy polysaccharide was carried out by iodine reaction (Lee, Yu, & Chen, 1994).

#### 2.4.4. Methylation analysis

The sample (20 mg) was methylated three times according to Needs and Selvendran (1993). The complete methylated products were hydrolyzed, then reduced and acetylated as described by Sweet, Shapiro, and Albersheim (1975). The partially methylated alditol acetates were analyzed by a gas chromatography/mass (GC/MS) spectrometer (GCMS-QP 2010, Shimadzu, Kyoto, Japan) fitted with a HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm). The temperature program was set as follows: the initial temperature of column was 150 °C, increased to 260 °C at 10 °C/min and maintained for 5 min at 260 °C; injection temperature: 260 °C. The nitrogen flow rate was 1.0 mL/min and the ion-source temperature was set at 250 °C.

### 2.5. Animals and treatment

Female specific pathogen-free (SPF) BALB/c mice (weighing 18–20 g) were obtained from Experimental Animal Center, the Fourth Military Medical University, Xi'an, China. The mice were acclimatized for at least 1 week before the study. Rodent laboratory chow and tap water were provided ad libitum and maintained in an air-conditioned SPF room 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 Institute for Experimental Animals of the Fourth Military Medical University and were approved by the University Committee for animal experiments.

For assessment of in vivo antitumor effect of PEPw, Renca tumor cell lines ($2 \times 10^5$ cells) were injected subcutaneously (s.c.) in the right flank of syngenic BALB/c mice on day 0. When the tumor appeared as small nodules at the sites of injection (100–150 $mm^3$) 10 days after tumor inoculation, forty animals were randomly assigned to the control group and PEPw treatment groups (10 per group). The PEPw dissolved in phosphate buffered saline (PBS) was orally administered to the tumor-bearing mice at 50, 100 and 200 mg/kg once daily for continued 20 days. The same volume of vehicle was orally administered to the control mice. Animals were observed and graded daily for activity, thinness, appearance of skin, hair, appetite and irritability until death. Twenty four hours after last tested administration, all the tumor-bearing mice were weighed and sacrificed by cervical dislocation on day 31. The spleen and thymus were immediately dissected and weighed. The spleen and thymus indices were calculated according to the following formula: Spleen index (mg/10 g) = spleen weight/body weight, Thymus index (mg/10 g) = thymus weight/body weight. Tumor size was measured daily using a ruler, and plotted against time to measure tumor growth velocity. The size of the tumors was measured twice a week, and calculated by the following formula: tumor size ($mm^3$) = $0.5 \times L \times W^2$, wherein $L$ and $W$ are the largest and smallest diameters as described previously (Cai et al., 2008).

### 2.6. Measurement of serum cytokines

Blood were collected in polystyrene tubes without the anticoagulant and serum samples were immediately separated by centrifugation at 3000 rpm at room temperature for 10 min and preserved at −80 °C until assayed. The concentration of TNF-α and IL-2 in serum of the treated mice was measured by enzyme-linked immunosorbent assay (ELISA) at 450 nm according to the manufacturer's instructions.

### 2.7. Splenocyte proliferation assay

The spleens collected from the above treated groups under aseptic conditions were immediately minced into small pieces and filtered over a fine nylon mesh to obtain single-cell suspensions. The cells were washed and lymphocytes were separated from red blood cells by lysis buffer (0.15 M $NH_4Cl$, pH 7.2). Cell viability (over 95%) was assessed microscopically using trypan blue dye exclusion technique. Splenocytes were seeded in 96-well plates ($1 \times 10^7$ cells/well) with or without Con A (2.5 μg/mL) or LPS (5.0 μg/mL), and incubated in a humidified atmosphere with 5% $CO_2$ at 37 °C. After 44 h cultivation, 50 μL of 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (2.0 mg/mL) were added to each well and incubated for another 4 h. The medium was discarded and 100 μL dimethylsulfoxide (DMSO) was then added. The absorbance of each well was measured by an ELISA reader using a test wavelength of 570 nm after 15 min. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

### 2.8. Assay of natural killer (NK) cell activity

The activity of NK cell was measured using the standard $^{51}Cr$-release assay as previously described with minor modification (Zeytin et al., 2008). Briefly, Yac-1 cells as the target cells in the logarithmic phase of growth were labeled with 250 μCi of $Na_2{}^{51}CrO_4$ for 30 min at 37 °C, washed, and resuspended at a concentration of $1 \times 10^4$ cells/per well in 96-well U-bottomed culture plates. The NK cells (100 μL) as effector cells from control or PEPw-treated groups were mixed with $Na_2{}^{51}CrO_4$ labelled Yac-1 cells (100 μL) at a ratio of 50:1 in 96-well round-bottom plates. After 4 h incubation at 37 °C in 5% $CO_2$ atmosphere, the cell-free supernatants were harvested and the radioactivity was counted by a Beckman LS 6500 gamma counter. Specific cellular cytotoxicity was determined by the following formula: percentage of specific lysis = (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100. Spontaneous cpm was the radioactivity from wells with target cells alone, and maximum cpm was determined by addition of 1% Triton X-100 to the target cells in the absence of effector cells. All assays were done in triplicate.

### 2.9. Assay of cytotoxic T lymphocytes (CTL) activity

CTLs were harvested from splenocytes of mice in each group and co-cultured with $Na_2{}^{51}CrO_4$ labelled Renca cells ($1 \times 10^4$ cells/well) at effector:target (E:T) ratio of 100:1 for 5 h in 96-well round-bottom plates in the presence of IL-2 (50 ng/mL),. Target cell killing was determined by a Beckman LS 6500 gamma counter and percent cytotoxicity was calculated as described above.

**Table 1**
The results of methylation analysis of PEPw.

| Peak | Partially methylated alditol acetate | Molar ratios | Linkage type |
|---|---|---|---|
| Residue A | 2,3,4-$Me_4$-Gal | 5.1 | 1,6-Linked-Gal*p* |
| Residue B | 3,4-$Me_3$-Gal | 1.1 | 1,2,6-Linked-Gal*p* |
| Residue C | 2,3,6-$Me_3$-Man | 2.3 | 1,4-Linked-Man*p* |
| Residue D | 2,3,5-$Me_3$-Ara | 1.2 | Terminal-Ara*f* |

### 2.10. Statistical analysis

All results were presented as mean ± S.D. Data were analyzed by one-way ANOVA using SPSS and Dunnett's test. *P*-values of less than 0.05 were considered to be statistically significant.

## 3. Results and discussion

### 3.1. Isolation and purification of the polysaccharide

The crude polysaccharide from the fruiting bodies of *P. eryngii* was separated and sequentially purified through DEAE-cellulose and Sephacryl S-300HR gel-permeation chromatography, leading to the isolation of a water-soluble purified polysaccharide PEPw, with its yield being 1.8% of the dry material. The fractionations containing polysaccharide were enriched based on carbohydrate content detection by the phenol–sulfuric acid assay.

### 3.2. Chemical characterization of the polysaccharide

PEPw appear to be primrose yellow powder, which is insoluble in a majority of organic reagents such as ethanol, methanol, acetone, ethyl acetate and chloroform, but easily dissolved in water, especially in hot water. The ferric chloride method showed that they did not contain phenolic hydroxyls substance. Iodine reaction had no obvious phenomenon, demonstrating that there is no starch in the polysaccharide. As determined by meta-hydroxydiphenyl colorimetric method, PEPw contains no uronic acid. No absorption at 280 or 260 nm in the UV spectrum indicated absence of protein and nucleic acid, supporting the fact that ninhydrin reaction was not obvious. The total carbohydrate content of PEPw was determined to be 94.5% using the phenol–sulfuric method.

PEPw was further eluted as a single and symmetrical sharp peak on HPGPC, indicating that it is a homogeneous polysaccharide. Based on the calibration with Dextran T-series standard samples of known molecular weights, the average molecular weight was $2.5 \times 10^4$ Da. Sugar composition analysis by HPLC indicated that PEPw was determined to be composed of arabinose, mannose and galactose with the molar ratio of 1.2:2.3:6.2.

In order to determine the linkage types, PEPw was subjected to methylation analysis. Methylation analysis of fractions PEPw showed the presence of four linked types (Table 1), namely 1,6-linked-Gal*p* (Residue A), 1,2,6-linked-Gal*p* (Residue B), 1,4-linked-Man*p* (Residue C) and terminal-Ara*f* (Residue D) in molar ratio of 5.1:1.1:2.3:1.2. This showed a good correlation between terminal and branched residues. In addition, these molar ratios agree with the overall monosaccharide composition of PEPw described above. On the basis of the aforementioned results and the previous structure information regarding the polysaccharide on this plant or genus (Carbonero et al., 2006, 2008; Rosado et al., 2002, 2003; Smiderle et al., 2008), it was possible to conclude that the repeating unit of PEPw was mainly composed of Residue A, B and C as the backbone, and terminated with Residue D at O-2 position of Residue B.

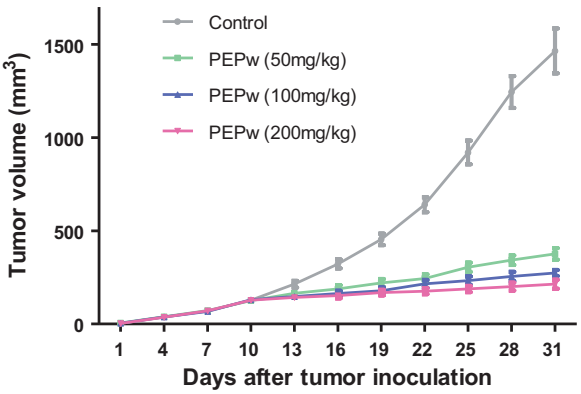


**Fig. 1.** Effect of PEPw on tumor growth in Renca-bearing mice. Data are presented as mean ± SD values based on 10 mice in each group.

### 3.3. Effect of PEPw on tumor growth in Renca-bearing mice

To evaluate the antitumor activity of PEPw in vivo, murine renal cancer allograft model were established by s.c. injection of Renca cell ($2 \times 10^5$ cells, 0.1 mL of PBS) on the right flank of syngenic BALB/c mice. After tumor nodules grow for 10 days, mice received either PBS, or low-dose (50 mg/kg), medium-dose (100 mg/kg) and high-dose (200 mg/kg) of PEPw via oral administration for additional 20 days. The tumor volume was significantly suppressed at three doses in a dose-dependent manner, whereas the tumors growth did not exhibit any delay in the control group (Fig. 1). Meanwhile, the appetite, activity and coat luster of each animal in PEPw-treated groups were better than the tumor-bearing controls.

### 3.4. Effect of PEPw on serum cytokine production in Renca-bearing mice

Since cytokines play a prominent role in the development of immune response, we investigated the effect of PEPw on the production of serum cytokines IL-2 and TNF-α in Renca-bearing mice by ELISA. The serum levels of IL-2 and TNF-α were markedly increased in Renca-bearing mice administrated with PEPw at three doses in a dose-dependent manner, compared with those in Renca-bearing control mice administrated with PBS (Fig. 2).

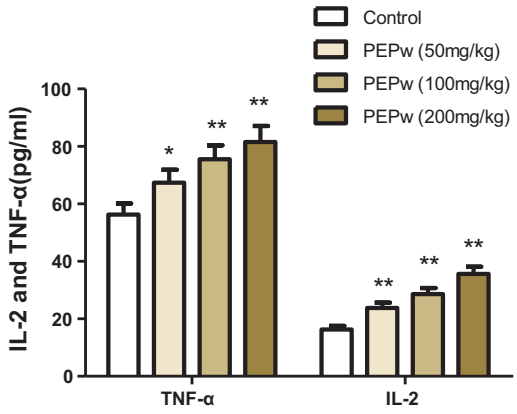


**Fig. 2.** Effect of PEPw on the production of serum cytokines IL-2 and TNF-α in Renca-bearing mice. Data are presented as mean ± SD values based on 10 mice in each group. Significant differences compared to model control group are designated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

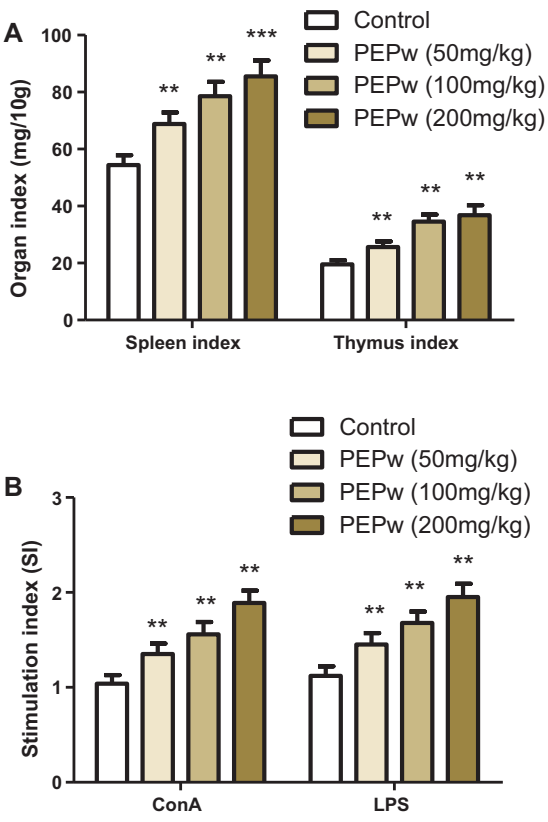


**Fig. 3.** Effect of PEPw on (A) the relative thymus and spleen indices; and (B) Con A- and LPS-stimulated splenocyte proliferation in Renca-bearing mice. Data are presented as mean ± SD values based on 10 mice in each group. Significant differences compared to model control group are designated as $^{**}P < 0.01$ and $^{***}P < 0.001$.

### 3.5. Effect of PEPw on organ indices and splenocyte proliferation in Renca-bearing mice

Extensive experimentation demonstrated that many polysaccharides from mushrooms produce their antitumor effects by activating different immune responses in the host (Ooi & Liu, 2000). To investigate whether PEPw enhance humoral immunity or cell-mediated immunity, we measured the effect of PEPw on organ indices and mitogen-stimulated splenocyte proliferation in tumor-bearing mice. As shown in Fig. 3A, PEPw increased the relative spleen and thymus indices in Renca-bearing mice in the increasing order of 50, 100 and 200 mg/kg. At the same time, Con A- and LPS-induced splenocyte proliferation was significantly increased in PEPw treated mice compared with that in control mice, which was significant ($P < 0.01$) (Fig. 3B).

### 3.6. Effect of PEPw on NK cells and CTL activities in Renca-bearing mice

CTLs play a major role as effector cells in cancer immunity. In addition, NK cells are effector cells that attack tumor cells (Sunila, Hamsa, & Kuttan, 2011). Therefore we measured the effects of PEPw on the cytotoxic activities of NK cells and CTL, and the results were shown in Fig. 4. To investigate NK cell activity, the cytotoxic activity of splenic NK cells from PBS or PEPw-treated group against YAC-1 cells was analyzed. NK cell-mediated cytotoxicity in mice treated with PEPw was significantly higher compared to control mice at the 50:1 E:T ratio ($P < 0.05$ or $P < 0.01$) in a dose-dependent manner. We further examined the effect of PEPw on the induction of tumor-specific CTL against $Na_2{}^{51}CrO_4$ labelled Renca cells in the presence of IL-2 (50 ng/mL). PEPw treatment dose-dependently induced CTL

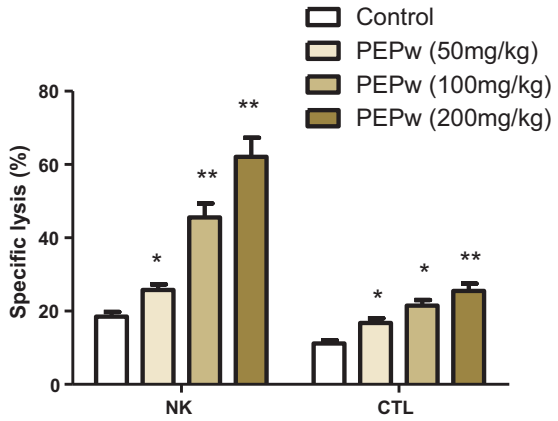


**Fig. 4.** Effect of PEPw on (A) NK cell activity; and (B) CTL activity in Renca-bearing mice. Data are presented as mean ± SD values based on 10 mice in each group. Significant differences compared to model control group are designated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

activity compared with the untreated control at an E:T ratio of 100 ($P < 0.05$ or $P < 0.01$). These results suggest that NK cells and CTLs contribute to the antitumor effects induced by PEPw.

## 4. Conclusions

In the present study, one water-soluble polysaccharide, PEPw, was successfully isolated and purified from the fruiting bodies of *P. eryngi*, with an average molecular weight of $2.5 \times 10^4$ Da, and PEPw had been proved as a heteropolysaccharide consisting of arabinose, mannose and galactose in the molar ratio of 1.2:2.3:6.2. Methylation analysis revealed that PEPw had a backbone composed of 1,6-linked-Gal*p*, 1,2,6-linked-Gal*p* and 1,4-linked-Man*p*, which was terminated with terminal-Ara*f* attached to O-2 position of 1,2,6-linked-Gal*p* along the main chain.

To investigate the anti-tumor activity of PEPw in vivo, the mouse renal cancer allograft model was created by s.c. injection of Renca cells into the right flank of syngenic BALB/c mice. The significant reduction of tumor volumes was observed in Renca-bearing mice following PEPw treatment at the dose of 50, 100 and 200 mg/kg in a dose-dependent manner. Furthermore, PEPw increased the relative spleen and thymus indices in Renca-bearing mice in the increasing order of 50, 100 and 200 mg/kg. As we know, the immune system plays an important role in antitumor defense. Many reports suggested that the antitumor activity of the polysaccharides was also mediated through augmentation of the immune response (Kim, Cho, Karnjanapratum, Shin, & You, 2011; Patra, Das, Behera, Maiti, & Islam, 2012; Xu et al., 2012; Zhang, Liu, Park, Xia, & Kim, 2012). Therefore, we further investigated the effect of PEPw on the cellular and humoral immunity in Renca tumor-bearing mice to analyze the underlying antitumor mechanism.

Splenocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. It is generally known that Con A stimulates T-cells, responsible for cellular immunity, and LPS stimulates B-cell proliferation, responsible for humoral immunity (Marciani et al., 2000). The proliferation assay showed that PEPw could significantly promote the Con A- and LPS-stimulated splenocyte proliferation in tumor-bearing mice, which indicated that PEPw could significantly increase the activation potential of T- and B-cells and enhance the cellular immunity and humoral immunity in tumor-bearing mice.

NK cells and CTL represent two major populations of cytotoxic lymphocytes (Kos & Engleman, 1996; Medzhitov & Janeway, 1997), and tumor cell elimination is believed to be mediated in part by the cytotoxic activity of NK cells and CTL (Boon, Cerottini, Van den Eynde, van der Bruggen, & Van Pel, 1994; Moretta, Bottino, Cantoni, Mingari, & Moretta, 2001). In this study, PEPw were found to significantly enhance the killing activity of NK cells and CTL from splenocytes in tumor-bearing mice, suggesting that it could enhance the specific and non-specific cytolytic activities against autologous tumor cells.

Cytokines regulate both cellular and humoral immune responses by affecting immune cell proliferation, differentiation and functions. TNF-α plays a pivotal role in host defense and can induce the expression of a number of other immunoregulatory and inflammatory mediators (Baugh & Bucala, 2001). IL-2 stimulates the proliferation of T-cells, B-cells, NK cells, CTL, lymphokine-activated killer (LAK) cells and macrophages, all of which can participate in immunological antitumor mechanisms (Asano, Kaneda, Hiragushi, Tsuchida, & Higashino, 1997; Mizuno et al., 2000). In the present study, the levels of serum IL-2 and TNF-α in Renca-bearing mice were significantly increased by PEPw treatment. The increase of IL-2 and TNF-α also explain the antitumorigenic properties of PEPw.

In conclusion, all the data implied that PEPw may indirectly play the role of antitumor activity through potentiating immunologic function of tumor-bearing mice. However, further investigation on the mechanism by which PEPw induces these effects and additional clinical usefulness in therapies of cancer are needed.

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## References

Asano, Y., Kaneda, K., Hiragushi, J., Tsuchida, T., & Higashino, K. (1997). The tumor-bearing state induces augmented responses of organ-associated lymphocytes to high-dose interleukin-2 therapy in mice. *Cancer Immunology Immunotherapy*, *45*, 63–70.

Baugh, J. A., & Bucala, R. (2001). Mechanisms for modulating TNF alpha in immune and inflammatory disease. *Current Opinion in Drug Discovery & Development*, *4*, 635–650.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*, 484–489.

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.

Cai, K. X., Tse, L. Y., Leung, C., Tam, P. K., Xu, R., & Sham, M. H. (2008). Suppression of lung tumor growth and metastasis in mice by adenoassociated virus-mediated expression of vasostatin. *Clinical Cancer Research*, *14*, 939–949.

Chen, H. L., Ju, Y., Li, J. J., & Yu, M. (2012). Antioxidant activities of polysaccharides from *Lentinus edodes* and their significance for disease prevention. *International Journal of Biological Macromolecules*, *50*, 214–218.

Chen, J. J., Mao, D., Yong, Y. Y., Li, J. L., Wei, H., & Lu, L. (2012). Hepatoprotective and hypolipidemic effects of water-soluble polysaccharidic extract of *Pleurotus eryngii*. *Food Chemistry*, *130*, 687–694.

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.

Ehrke, M. J. (2003). Immunomodulation in cancer therapeutics. *International Immunopharmacology*, *3*, 1105–1119.

Carbonero, E. R., Gracher, A. H., Rosa, M. C., Torri, G., Sassaki, G. L., Gorin, P. A., et al. (2008). Unusual partially 3-O-methylated alpha-galactan from mushrooms of the genus *Pleurotus*. *Phytochemistry*, *69*, 252–257.

Carbonero, E. R., Gracher, A. H. P., Smiderle, F. R., Rosado, F. R., Sassaki, G. L., Gorin, P. A. J., et al. (2006). A b-glucan from the fruit bodies of edible mushrooms *Pleurotus eryngii* and *Pleurotus ostreatoroseus*. *Carbohydrate Polymers*, *66*, 252–257.

Huang, S. Q., Ding, S., & Fan, L. (2012). Antioxidant activities of five polysaccharides from *Inonotus obliquus*. *International Journal of Biological Macromolecules*, *50*, 1183–1187.

Hwang, Y. J., Nam, H. K., Chang, M. J., Noh, G. W., & Kim, S. H. (2003). Effect of Lentius edodes and *Pleurotus eryngii* extracts on proliferation and apoptosis in human colon cancer cell lines. *Journal of the Korean Society of Food Science and Nutrition*, *32*, 217–222.

Jeong, Y. T., Jeong, S. C., Gu, Y. A., Islam, R., & Song, C. H. (2010). Antitumor and immunomodulating activities of endo-biopolymers obtained from a submerged culture of *Pleurotus eryngii*. *Food Science and Biotechnology*, *19*, 399–404.

Kang, H. I., Kim, J. Y., Moon, K. D., Seo, K. I., Cho, Y. S., Lee, S. D., et al. (2004). Effect of the crude polysaccharide of *Pleurotus eryngii* on the activation of immune cells. *Journal of the Korean Society of Food Science and Nutrition*, *33*, 1092–1097.

Kim, J. K., Cho, M. L., Karnjanapratum, S., Shin, I. S., & You, S. G. (2011). In vitro and in vivo immunomodulatory activity of sulfated polysaccharides from *Enteromorpha prolifera*. *International Journal of Biological Macromolecules*, *49*, 1051–1058.

Kim, J. Y., Kang, H. I., Park, K. U., Moon, K. D., Lee, S. D., Cho, S. H., et al. (2004). Antioxidative and antitumor activities of crude polysaccharide fraction from *Pleurotus eryngii*. *Journal of the Korean Society of Food Science and Nutrition*, *33*, 1589–1593.

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

Lee, J. W., Yu, R. Y., & Chen, L. R. (1994). *Principles and methods of biochemistry experiment* (pp. 151–278). Beijing: Beijing University Press.

Li, H., Liu, X., Li, Y., Hua, Y., Zhi, D., & Pang, G. (2012). Effects of the polysaccharide from *Pholiota nameko* on human cytokine network in serum. *International Journal of Biological Macromolecules*, *50*, 164–170.

Liu, W., Wang, H. Y., Pang, X. B., Yao, W. B., & Gao, X. D. (2010). Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, *46*, 451–457.

Liu, X. N., Zhou, B., Lin, R. S., Jia, L., Deng, P., Fan, K. M., et al. (2010). Extraction and antioxidant activities of intracellular polysaccharide from *Pleurotus* sp. mycelium. *International Journal of Biological Macromolecules*, *47*, 116–119.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biochemistry*, *193*, 265–275.

Marciani, D. J., Press, J. B., Reynolds, R. C., Pathak, A. K., Pathak, V., Gundy, L. E., et al. (2000). Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. *Vaccine*, *18*, 3141–3151.

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

Mizuno, H., Yanoma, S., Nishimura, G., Hattori, S., Ito, T., Okudera, K., et al. (2000). Therapeutic efficiency of IL-2 gene transduced tumor vaccine for head and neck carcinoma. *Cancer Letters*, *152*, 175–185.

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.

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

Rosado, F. R., Carbonero, E. R., Kemmelmeier, C., Tischer, C. A., Gorin, P. A., & Iacomini, M. (2002). A partially 3-O-methylated (1 → 4)-linked alpha-D-galactan and alpha-D-mannan from *Pleurotus ostreatoroseus* Sing. *FEMS Microbiology Letters*, *212*, 261–265.

Rosado, F. R., Carbonero, E. R., Claudino, R. F., Tischer, C. A., Kemmelmeier, C., & Iacomini, M. (2003). The presence of partially 3-O-methylated mannogalactan from the fruit bodies of edible basidiomycetes *Pleurotus ostreatus* 'florida' Berk. and *Pleurotus ostreatoroseus* Sing. *FEMS Microbiology Letters*, *221*, 119–124.

Smiderle, F. R., Olsen, L. M., Carbonero, E. R., Marcon, R., Baggio, C. H., Freitas, C. S., et al. (2008). A 3-O-methylated mannogalactan from *Pleurotus pulmonarius*: Structure and antinociceptive effect. *Phytochemistry*, *69*, 2731–2736.

Patra, P., Das, D., Behera, B., Maiti, T. K., & Islam, S. S. (2012). Structure elucidation of an immunoenhancing pectic polysaccharide isolated from aqueous extract of pods of green bean (*Phaseolus vulgaris* L.). *Carbohydrate Polymers*, *87*, 2169–2175.

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

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

Sunila, E. S., Hamsa, T. P., & Kuttan, G. (2011). Effect of *Thuja occidentalis* and its polysaccharide on cell-mediated immune responses and cytokine levels of metastatic tumor-bearing animals. *Pharmaceutical Biology*, *49*, 1065–1073.

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.

Wu, D. M., Duan, W. Q., Liu, Y., & Cen, Y. (2010). Anti-inflammatory effect of the polysaccharides of Golden needle mushroom in burned rats. *International Journal of Biological Macromolecules*, *46*, 100–103.

Xu, C. L., Li, Y. H., Dong, M., Wu, X., Wang, X. C., & Xiao, Y. S. (2012). Inhibitory effect of *Schisandra chinensis* leaf polysaccharide against L5178Y lymphoma. *Carbohydrate Polymers*, *88*, 21–25.

Zeytin, H., Reali, E., Zaharoff, D. A., Rogers, C. J., Schlom, J., & Greiner, J. W. (2008). Targeted delivery of murine interferon-c using a recombinant fowlpox virus: NK cell recruitment to regional lymph nodes and priming of tumor-specific host immunity. *Journal of Interferon & Cytokine Research*, *28*, 73–87.

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

Zhang, J., Liu, Y. J., Park, H. S., Xia, Y. M., & Kim, G. S. (2012). Antitumor activity of sulfated extracellular polysaccharides of *Ganoderma lucidum* from the submerged fermentation broth. *Carbohydrate Polymers*, *87*, 1539–1544.

Zhang, X., Yu, L., Bi, H. B., Li, X. H., Ni, W. H., Han, H., et al. (2010). Total fractionation and characterization of the water-soluble polysaccharides isolated from *Panax ginseng* C. A. Meyer. *Carbohydrate Polymers*, *77*, 544–552.

Zhou, L. B., & Chen, B. (2011). Bioactivities of water-soluble polysaccharides from Jisongrong mushroom: Anti-breast carcinoma cell and antioxidant potential. *International Journal of Biological Macromolecules*, *48*, 1–4.

Zhou, K. Y. (1978). *Organic chemistry experiment* (p. 272). Beijing: Higher Education Press.