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Immunoregulatory effects of a glucogalactan from the root of *Panax quinquefolium* L.

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ABSTRACT

In this research we purified one homogeneous glucogalactan from the roots of *Panax quinquefolium*, with a molecular weight of 54 kDa estimated by high-performance gel permeation chromatography (HPGPC). The monosaccharide composition of PPQ was composed of Glc (glucose) and Gal (galactose) in a molar ratio of 2.1:1, as determined by gas chromatography (GC). In order to evaluate the anti-lung carcinoma and immunoregulatory effects of this glucogalactan in mice, we established a Lewis lung carcinoma model in C57BL/6 mouse. The results showed that PPQ not only could inhibit the growth of lung tumor, but also enhance the thymus and spleen indices, as well as the level of IFN-γ, IL-10 and IL-2, indicating PPQ could have a possible cancer therapeutic potential.

1. Introduction

Lung cancer is one of the most common cancers in the world, which is a disease characterized by uncontrolled cell growth in tissues of the lung. Worldwide, lung cancer is a leading cause of cancer death in men and women, and rates of lung cancer are rising all the time, as the legacy of smoking and pollution from decades past comes due. There are two types of lung cancer, namely small cell lung cancer and non-small cell lung cancer, with prevalence rates of 14% and 85%, respectively (Spivey et al., 2010). These types are diagnosed based on how the cells look under a microscope. Each type of lung cancer grows and spreads in different ways and is treated differently. Treatment also depends on the stage, or how advanced it is. Treatment may include chemotherapy, radiation and surgery. The lack of effective early diagnostic and treatment methods are the main causes of high mortality in patients with lung cancer. Conventional lung cancer treatments generally show poor clinical response, thus it is of almost importance to develop novel treatment strategies directed against this cancer and its metastasis (Sève, Reiman, & Dumontet, 2010). Faced with palliative care, many alternative medicines, such as natural medicinal plant or animals had become popular used in cancer therapies (Eisenberg et al., 1998; Risberg, Lund, Wist, Kaasa, & Wilsgaard, 1998; Sadava et al., 2002). Among these therapies, crude extract or purified constituents form traditional Chinese medicine to treat specific diseases including cancers is prevailing more than before as household remedy used by people (Yin, Zhou, Jie, Xing, & Zhang, 2004).

Asian ginseng (*Panax ginseng* C.A. Meyer) and American Ginseng (*Panax quinquefolium* L.) are the two major species of ginseng having been used in various traditional medicinal therapies for many years in China, which are the two most recognized ginseng botanicals around the world (Ang-Lee, Moss, & Yuan, 2001; Jia & Zhao, 2009). Both *P. ginseng* and *P. quinquefolium* are used for their tonic and stimulant and aphrodisiac properties, but they have somewhat different in bioactivities and chemical compositions (Chen et al., 1998; Ren & Chen, 1999; Yun, Lee, Kwon, & Choi, 1996). *P. ginseng* is considered to be stimulating and invigorate yang, whereas *P. quinquefolium* is considered to be calming and nourishing yin (Dharmananda, 2002). The major active components of ginseng are ginsenosides, a diverse group of triterpenoid saponin glycosides (dammarene-type saponins). The ginsenoside profile of each ginseng species is different, which may account for the variety of therapeutic activities (Yuan et al., 2001). *P. quinquefolium*, a plant native to North America, is now also cultivated and used in many countries. It belongs to the Panax genus of the Araliaceae. It has been shown that *P. quinquefolium* has many pharmacological effects similar to ginseng as observed in the central nervous, cardiovascular, endocrine, and immune systems. However compared with the long history of use and the copious amounts of research on *P. ginseng*, studies on the constituents of *P. quinquefolium* and

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its biological activities are much less extensive, especially regarding polysaccharide and cancer chemoprevention, in spite of some promising advances in recent years (Qi, Wang, & Yuan, 2010). Ma et al. (1998) isolated and identified one purified polysaccharide from the root of *P. quinquefolium* in 1998, which showed significant lymphocyte stimulation and interleukin induction activities and was composed of arabinose and galactose in a molar ratio of 1:1.1. Assinewe, Amason, Aubry, Mullin, and Lemaire (2002) found that significant TNF stimulating activity was found in the extractable polysaccharide fraction from the root of *P. quinquefolium*, which was hydrolyzed and found to contain glucose, galactose, arabinose, rhamnose, and mannose. In 2004, anti-hyperglycemic effect of the polysaccharides fraction from the berry extract of *P. quinquefolium* in *ob/ob* mice was investigated by Xie, Wu, Mehendale, Aung, and Yuan (2004).The data suggested that the polysaccharides fraction from American ginseng berry extract has a potential clinical utility in treating diabetic patients. Based on the above references, we can see that the research on the physicochemical properties and bioactive aspect of polysaccharide from *P. quinquefolium* was very limited. There are not much data published on their preventive and anti-tumor effects on lung cancer. Therefore, in this research we intend to make a preliminary report on the physicochemical characteristic of one homogeneous polysaccharide and evaluated its anti-Lewis lung carcinoma and immunoregulatory effects in tumor-bearing mice.

2. Materials and methods

2.1. Materials and chemicals

The dry roots of *P. quinquefolium* were commercially obtained from local market of Fusong, Jilin, China, and were crushed to coarse powder. Sephacryl™ S-200 High Resolution and DEAE Sepharose™ Fast Flow were purchased from Amersham Biosciences. Standard dextrans T-2000, T-500, T-70, T-40 and T-10 were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

2.2. The fraction procedure for PPQ

The pre-degreased powdered roots (100 g) of *P. quinquefolium* were extracted three times by using water solution for 90 min at 100 °C. The filtrate was combined and concentrated to 200 mL using a rotary evaporator at 60 °C. The protein was removed by the Sevag method (Alam & Gupta, 1986; Sun, Li, Yang, Liu, & Kennedy, 2010). The concentrated mixture was precipitated by using 3 volumes of ethanol overnight after removal of the Sevag reagent by exhaustive dialysis with water for 48 h. The crude polysaccharide (4.34 g) was obtained by centrifugation at 1700 × g for 10 min and washed with absolute ethanol, acetone and ether, alternately for three times.

The crude polysaccharide was purified on the auto liquid chromatographic fractionation apparatus (MF99-3) made in shanghai city of China, by applying a DEAE Sepharose™ Fast Flow column (30 cm × 3 cm) that was eluted with NaCl aqueous solution (0, 0.2, 0.4 and 0.6 M) stepwise at 4 mL/min. The yielded fractions were concentrated and combined according to the phenol–sulfuric acid method using an automated fraction collector. The solution eluted by distilled water was desalted through dialysis against water for 48 h, and concentrated in a rotary evaporator and applied to a Sephacryl™ S-200 High Resolution column (100 cm × 3 cm) with 0.15 mol/L NaCl at a flow rate of 1 mL/min to yield only one fraction, termed as PPQ.

2.3. Homogeneity and molecular weight of PPQ by HPGPC

The homogeneity and molecular weight of PPQ were determined by HPGPC analysis (Sun et al., 2008). The sample was analysed by a TSK-G3000PW$_{XL}$ column (7.8 mm ID × 300 mm) and a SHIMADZU RID-10A detector. The mobile phase was 0.1 M NaCl and the flow rate was 0.5 ml/min. The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular mass (T-2000, T-500, T-70, T-40 and T-10).

2.4. Measurement of monosaccharide composition, carbohydrate and protein contents

Gas chromatography (GC) was used for identification and quantification of the monosaccharide compositions. 10 mg sample was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 4 h. The hydrolyzed product was converted into the alditol acetates as described by Lehrfeld (1985) and analysed by GC. The mixture was further analysed by GC on an Varian 3400 instrument (Hewlett–Packard Component, USA) equipped with flame-ionization detector (FID) and HP-5MS column (0.25 mm × 30 m × 0.25 μm), and at temperatures programmed from 120 °C (maintained for 2 min) to 260 °C (kept for 40 min) at a rate of 15 °C/min. The concentration of polysaccharide was measured by the phenol–sulfuric acid method using D-glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein was measured by the Bradford's method (1976) using BSA as the standard.

2.5. Assay for anti-tumor and immunoregulatory activities

2.5.1. Experimental animal and design

Male C57BL/6 mice (6–8 weeks old and weighing approximately 18–20 g) were used for the study. All the procedures were carried out in strict accordance with the "Principles of Laboratory Animal Care". All mice were housed under normal laboratory conditions (room temperature of 22 ± 1 °C, humidity of 50 ± 10%, and a constant 12-h light and dark cycle) with free access to standard rodent chow and water.

After Lewis cell was recovered, it was inoculated to in the right anterior limb of mice. When the transplanted tumor grow to the diameter of 2 cm, the mice was sacrificed by decapitation, sterilized, carved and the tumor tissue was taken out for preparation of cell suspension in PBS solution (1×10^7 cells/ml). Each male C57BL/6 mice was induced by subcutaneous (s.c.) injection of Lewis cells (1×10^6 cells) in the right anterior limb to form high transplanted tumor animal model.

The tumor bearing mice were divided into five groups (ten mice each group)

Group 1: Negative control group (NC).
Group 2: Positive control group (PC).
Group 3: PPQ (100 mg/kg) administered group (PL).
Group 4: PPQ (200 mg/kg) administered group (PM).
Group 5: PPQ (400 mg/kg) administered group (PH).

The tumor-bearing mice were respectively intragastrically administered with PPQ in three doses of 100, 200 and 400 mg/kg body weight/day (BW/D) for two weeks. Positive control mice were injected with cyclophosphamide (CTX 30 mg/kg), while blank control mice received 0.9% sodium chloride in the same vehicle. The body weight of experimental mice was recorded on the balance before they were sacrificed. Blood was collected by heart puncture and the tumor, thymus and spleen were gently extracted. The

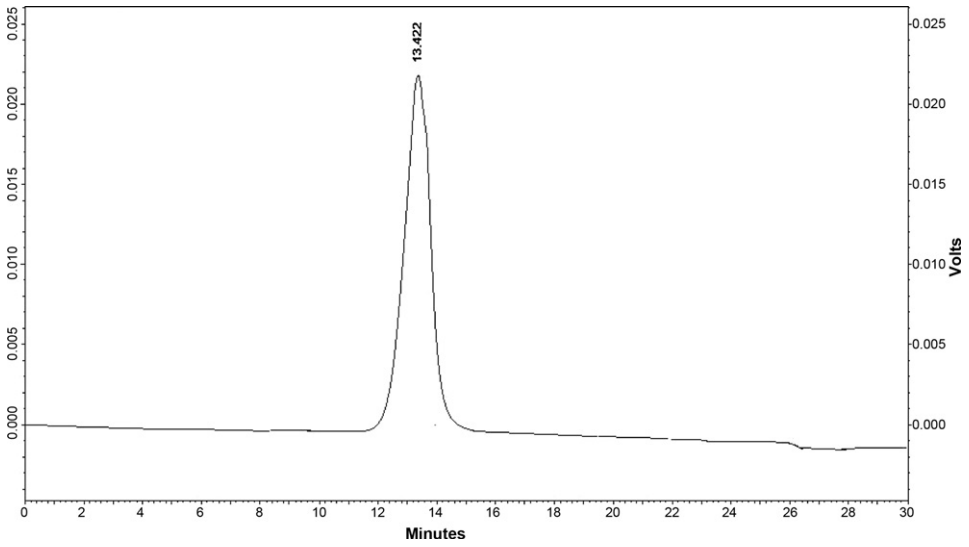

Fig. 1. The profile of PPQ in high-performance gel permeation chromatography (HPGPC).

tumor inhibition rate, thymus and spleen indices were then calculated. Tissue samples were stored at −20 °C for future study.

2.5.2. Detection of effect of PPQ on tumor growth in mice

After the body weight of mice in different group was recorded, the tumor growth inhibiting ratio was calculated as following formula:

Inhibiting ratio (%) = $[(C - T)/C] \times 100\%$ (C: average weight of tumor in model control group; T: average weight of tumor in drug administered group).

2.5.3. Detection of effect of PPQ on the thymus and spleen indices

Spleen and thymus were excised from the animal and weighed immediately after the mice were killed. Thymus index was expressed as the thymus weight relative to body weight. Spleen index was expressed as the thymus weight relative to body weight (Dai et al., 2008).

2.5.4. Detection of IL-2, IL-10 and IFN-γ concentrations

After last drug administration, blood was collected by heart puncture, centrifuged at 2000 g for 10 min and the serum was collected for the detection of IL-2, IL-10 and IFN-γ level using commercial ELISA kit according to the instructions of kits. The absorbance was measured at 450 nm in an ELISA reader (Bio-Rad, USA). Cytokine quantities in the samples were calculated from standard curves of recombinant cytokines using a regression linear method (Wang et al., 2007).

2.5.5. Statistical analysis

The data was expressed as means ± S.D. The difference between tested groups and control was analysed by Student's t-test. $P < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Isolation, purification and characteristics of PPQ

By using ethanol precipitation, the crude polysaccharide was enriched and purified from the extract of the roots of *P. quinquefolium*, having a yield of ~4.3% of the total weight. This crude polysaccharide was further fractionated on a DEAE Sepharose™ Fast Flow column, and fractions at different salt gradient were collected. Moreover the water-eluted fraction loaded on Sephacryl™ S-200 High Resolution column was purified with 0.15 mol/L NaCl buffer (flow rate of 1 mL/min) to yield only one fraction, termed as PPQ. PPQ contained ~96.3% carbohydrate and no protein, determined by Dubois and Bradford's methods, respectively. It had no absorption detected by the UV spectrum at either 280 or 260 nm indicated the absence of protein and nucleic acid. By using a size exclusion column in HPLC analysis with an RID-10A detector, PPQ was shown to be with a molecular weight of ~54 kDa. The HPGPC profile (Fig. 1) also demonstrated that PPQ had a single and symmetrically sharp peak revealing that PPSB was a homogeneous polysaccharide. Analysis by GC indicated that PPQ was composed of Glc and Gal with a relative molar ratio of 2.1:1 (Fig. 2).

3.2. Inhibiting effect of PPQ on tumor growth in mice

The inhibitory rate of PPQ on the tumor growth in mice was assessed by the equation, as seen in Table 1. The mean value of tumor in NC group arrived at about 2.94 g, whereas a significant tumor weight loss was observed in mice treated by CTX at 30 mg/kg BW in PC group. In addition to the low dose (100 mg/kg, BW) of PPQ administrated group, the other two group (PM and PH) showed significant tumor inhibiting effects in Lewis tumor-bearing C57BL/6 mice, compared with the NC group ($P < 0.01$), especially at the high

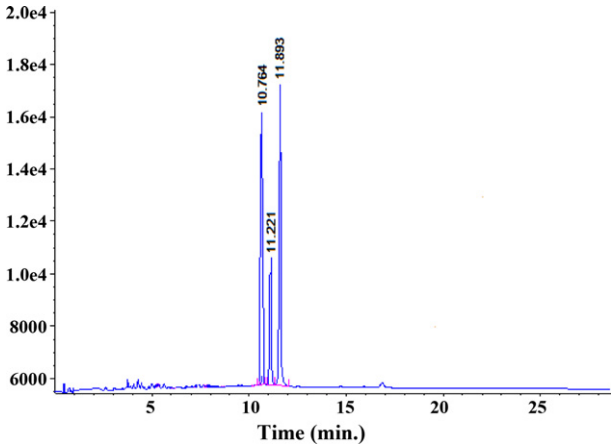

Fig. 2. The monosaccharide composition of PPQ detected by gas chromatography (GC). Peaks from left to right: Glc, Gal and Inositol.

Table 1
Comparison of tumor weight and inhibitory rate after administration in each group.

Groups	*n*	Tumor weight (g)	Inhibitory rate (%)
NC	10	2.94 ± 0.23	
PC	10	1.12 ± 0.14**	61.9**
PL	10	2.64 ± 0.19	10.2
PM	10	1.54 ± 0.14**	47.6**
PH	10	1.25 ± 0.13**	57.5**

NC: Negative control group; PC: positive control group; PL: PPQ (100 mg/kg, BW) administered group; PM: PPQ (200 mg/kg, BW) administered group; PH: PPQ (400 mg/kg, BW) administered group. All values represent the mean ± standard deviation ($n = 10$).
** Significant differences with NC group were designated as $P < 0.01$.

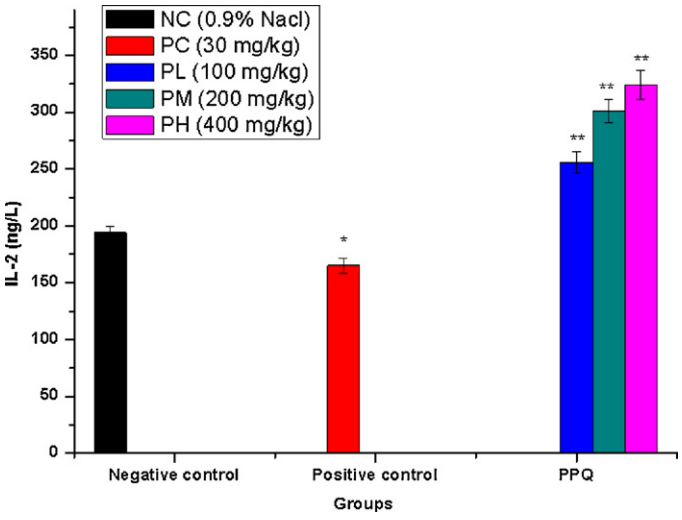

Fig. 3. The effect of PPQ on the level of IL-2 in mice.

dose of 400 mg/kg BW. The above results agreed closely with the observation for weight of tumor in each group.

3.3. Effect of PPQ on the thymus and spleen indices in mice

As shown in Table 2, PPQ obviously improved the weight of the immune organ in Lewis-bearing mice compared with the NC group. A great weight loss of thymus and spleen in PC group could be seen, which account for the immunosuppressive side effect by CTX during the therapy for cancer patients. The thymus and spleen indices in the CTX-treated were lower than those of the control. The thymus index ($^{*}P < 0.05$ and $^{**}P < 0.01$) in PL, PM and PH group was markedly elevated along with the increase of dose as compared to NC group. The same effect to the spleen index happened except for the PL group.

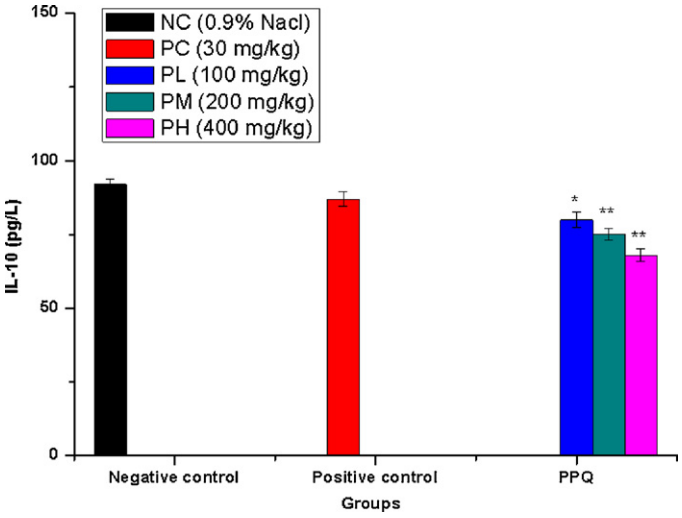

Fig. 4. The effect of PPQ on the level of IL-10 in mice.

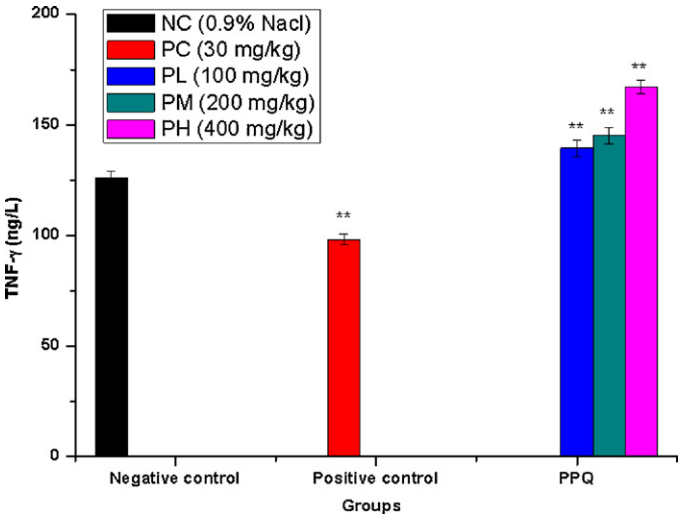

Fig. 5. The effect of PPQ on the level of IFN-γ in mice.

3.4. Effect of PPQ on the level of serum cytokines in mice

As shown in Figs. 3–5, the level of IL-2 and IFN-γ in PC group had a significant decrease ($^{*}P < 0.05$ and $^{**}P < 0.01$) as compared with the NC group, whereas there was no difference on the IL-10 expression between PC and NC group. The immune system was suppressed by CTX, which support its immunosuppression effect in tumor-bearing animals. However the level of IL-2 and IFN-γ was restored and enhanced after administered with PPQ in mice in a dose-dependent manner. With respect of IL-10 concentrations in serum of mice, obvious attenuation was observed accompanied

Table 2
Effect of PPQ on the thymus and spleen indices in mice.

Group	Thymus (mg)	Spleen (mg)	Weight (g)	Thymus index (mg/g)	Spleen index (mg/g)
NC	36.04 ± 5.87	148.54 ± 15.55	22.15 ± 1.34	1.63 ± 0.25	6.71 ± 0.76
PC	32.67 ± 6.38	100.09 ± 16.28	20.43 ± 1.88	1.60 ± 0.19	4.90 ± 0.40
PL	39.23 ± 7.07	160.87 ± 19.64	19.89 ± 2.09	1.97 ± 0.23*	8.09 ± 0.66
PM	46.76 ± 7.11	188.65 ± 21.54	20.74 ± 2.14	2.25 ± 0.24**	9.10 ± 0.71**
PH	49.90 ± 8.23	199.67 ± 23.99	21.48 ± 2.57	2.32 ± 0.28**	9.30 ± 0.83**

NC: Negative control group; PC: positive control group; PL: PPQ (100 mg/kg, BW) administered group; PM: PPQ (200 mg/kg, BW) administered group; PH: PPQ (400 mg/kg, BW) administered group. All values represent the mean ± standard deviation ($n = 10$).
* Significant differences with NC group were designated as $P < 0.05$.
** Significant differences with NC group were designated as $P < 0.01$.

with the increase of PPQ administration. All the data implied that PPQ can activate T cells by up-regulating Th-1 response and that Th-1 cells might be the main target cells of the PPQ.

4. Conclusion

In this research, we successfully purified one homogeneous glucogalactan from the root of *P. quinquefolium* obtained by DEAE Sepharose™ Fast Flow and Sephacryl™ S-200 High Resolution chromatography. Its physical and chemical property was reported for the first time. The purified PPQ was a netural water-soluble species, with a molecular weight of 54 kDa, and comprised of Glc and Gal in the proportion of 2.1:1. Therapeutic efficacy of the polysaccharide PPQ against Lewis lung cancer was evaluated in vivo. The results indicated that PPQ could effectively inhibit the tumor weight and increase the thymus index and spleen index in tumor-bearing mice when the mice was administrated with PPQ, especially at the high dose of 400 mg/kg. In addition, PPQ remarkably enhanced the IL-2 and IFN-γ production and reduce the IL-10 expression. We can conclude that the antitumor activity might be achieved by immunity stimulation through increasing the expression of IL-2 and IFN-γ and decreasing the expression of IL-10, which results in modulation of secreting Th1/Th2 cytokine for enhancement of immunity (Chen et al., 2010). Taken together, the present results suggested that PPQ would be expected as potential antitumor drugs with immunomodulatory activity.

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