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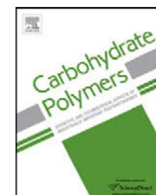

Chemical characterization and immunostimulatory effects of a polysaccharide from *Polygoni Multiflori Radix Praeparata* in cyclophosphamide-induced anemic mice

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ABSTRACT

A major polysaccharide fraction (PMPP) was obtained by purifying the crude polysaccharides extracted from *Polygoni Multiflori Radix Praeparata*. Preliminary structural characterization was conducted by physicochemical property and Fourier transform infrared spectroscopy. Monosaccharide component analysis indicated that PMPP was composed of rhamnose, arabinose, xylose and glucose in a molar ratio of 1.64:1.00:1.34:6.06. Intraperitoneal administration of PMPP could increase levels of serum IL-2 and hematological parameters (red blood cells, white blood cells and platelet counts), enhance antioxidant profiles, and promote hematopoiesis of splenocytes though up-regulating expressions of EPOR and GATA-1 proteins in cyclophosphamide (Cy) induced anemic mice. These findings indicate that PMPP played an important role in the protection against myelosuppression and immunosuppression in Cy-treated mice and may warrant further evaluation as a potential immunomodulatory agent.

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1. Introduction

Polysaccharides have recently been widely studied as a new source of additives for food and pharmaceuticals due to their unique bioactivities and chemical structures (Yang & Zhang, 2009). Particularly, botanical polysaccharides are reported to possess a wide range of pharmacological properties such as antioxidant, anti-inflammatory, anti-tumor, immunomodulatory and anti-diabetic activity (Ananthi et al., 2010; Ding, Zhu, & Gao, 2010; Schepetkin & Quinn, 2006).

Polygoni Multiflori Radix Praeparata (called Zhishouwu in Chinese) is processed from the root of *Polygonum multiflorum* Thunb. (Polygonaceae) and used as a tonic and health food in oriental counties since Tang Dynasty of China (Ogwuru & Adamczeski, 2000; Xiao, Xing, & Wang, 1993; Yu et al., 2011). This food has been reported to have antioxidant, hypolipidemic, hepatoprotective and immunomodulatory effects (Huang, Horng, Chen, & Wu, 2007; Li et al., 2005; Wang, Zhao, Wang, Mao, & Jie Yu, 2012; Wei, Zhang, Li, & Chen, 2004). It also could promote hair growth by inducing anagen (Park, Zhang, & Park, 2011) and show significant improvement in learning-memory ability for the treatment of Alzheimer's disease (Um, Choi, Aan, Kim, & Ha, 2006; Wang et al., 2007; Zhang et al., 2006). Until now, the majority of studies on this herb have focused on chemical compositions and bioactivities of anthraquinones and stilbene (Qin et al., 2011; Wang et al., 2012; Yao, Li, & Kong, 2006). However, biological activities of polysaccharides of *P. Multiflori Radix Praeparata* have not been studied previously.

Recently, several immunoceuticals composed of polysaccharides are widely used, such as lentinan, algin, krestin and tea polysaccharides (Nie & Xie, 2011; Schepetkin & Quinn, 2006; Wijesekara, Pangestuti, & Kim, 2011). Those immunomodulatory agents often act on inducing lymphocyte proliferation and cytokine production, and they have protective effects *via* the activation of hematopoietic function and immune organs (Wang et al., 2011). Furthermore, a good understanding on the structure of the polysaccharide is essential for investigating its bioactivities, especially for its possible interactions with cells or other bio-molecules *in vivo*. Therefore, this study was to investigate chemical characterization and immunomodulatory effects of a polysaccharide isolated from *P. Multiflori Radix Praeparata* (PMPP) on hematopoietic cell proliferation, cytokine productions and erythroid expansions in cyclophosphamide (Cy) induced anemic mice.

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2. Materials and methods

2.1. Reagent

The material (*Polygoni Multiflori Radix Praeparata*) was obtained from Chinese Medicine Herbal Tablets Factory, Zhejiang Province, China, and identified by Dr. Wei Cai, Zhejiang Chinese Medical University. Samples were ground and sieved using a grinder and were passed through a 40-mesh sieve. Iron dextran tablets (Lot. 091133) were purchased from Jiangxi Huatai Pharmacy Co., Ltd., China. All regents and chemicals used were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Preparation and quantitative analysis of PMPP

Pretreatment processing of raw material was carried out as reported previously (Gan, Manaf, & Latiff, 2010) with modifications. Dried sample (200 g) was defatted with petroleum ether (boiling point: 60–90 °C) and then pretreated with 80% ethanol quartic to remove colored materials, monosaccharides, oligosaccharides and small molecule materials. When the organic solvent was volatilized, the pretreated dry powders were obtained and immersed in distilled water for 1 h. The suspensions were extracted twice with 4 L of distilled water at 90 °C for 1.0 h. After centrifuged (2000 rpm, 20 min), the supernatant was precipitated by the addition of ethanol to a final concentration of 80% (v/v). Precipitates were collected by centrifugation (2000 rpm, 20 min), washed successively with ethanol, and solubilized in deionized water.

Using glucose as a standard, polysaccharide content was measured by the phenol–sulfuric acid method after a conversion factor of 0.9 was multiplied as previously described (Hou & Wei, 2008).

2.3. Separation and purification of PMPP

The protein was removed using the Sevage method and then followed by papain digestion. The supernatant was separated from insoluble residue with a nylon cloth (pore diameter: 38 μm) according to previous reports (Xie et al., 2010; Yan et al., 2011). The crude polysaccharides were dissolved in 0.1 M NaCl solution and filtered through 0.45 μm Millipore filter. The solution was subjected to DEAE Sephadex A-50 column (5 × 60 cm) chromatography and eluted with deionized water at a flow rate of 2.0 mL/min. The elution fraction were collected and monitored for carbohydrate content based on phenol–sulfuric acid method at 490 nm absorbance. Finally, the eluted fractions were concentrated, dialyzed and lyophilized. The products were further purified on Sephadex G-100 column (2.6 cm × 60 cm) with water at a flow rate of 0.4 mL/min and lyophilized to afford one major fraction (PMPP). HPLC was performed using an Agilent 1100 apparatus, consisting of a vacuum degasser, binary pump, and ELSD detection, controlled by Class-up station (Agilent Co. Ltd., USA). The HPLC column was a COSMOSIL Packed column (Sugar-D 250 mm × 4.6 mm ID). The distilled water as mobile phase was used to elute for 15 min, and the flow rate was kept at 0.8 mL/min. Column temperature was kept constantly at 27 °C. The injection volume was 20 μL.

2.4. Analysis of monosaccharide composition

GC–MS was used for identification and quantification of the monosaccharides (Xu, Yao, Sun, & Wu, 2009; Zhong, Jin, Lai, Lin, & Jiang, 2010). Fifty milligrams of polysaccharides were hydrolyzed with 5 mL of 1 M sulphuric acid at 100 °C for 6 h. The hydrolyzed polysaccharide was mixed with BaOH for pH up to neutrality and then evaporated continuously by a rotary evaporator at 45 °C. The hydrolysate was dissolved in 0.5 mL of pyridine with 10.0 mg hydroxylamine hydrochloride, and allowed to react at 90 °C for 30 min. After cooled, 0.5 mL of acetic anhydride was added and the tube was sealed and incubated at 90 °C for 30 min. Its corresponding alditol acetates were analyzed by gas chromatography (GC) on a Hewlett-Packard model 6890 instrument equipped with a capillary column of HP-5MS phenyl methyl siloxane (30m × 0.25 mm × 0.25 μm). The operation was performed in the following conditions: injection temperature: 100–240 °C; detector temperature: 230 °C; column temperature programmed: 160 °C holding for 2 min, then increasing to 230 °C at 10 °C/min and finally holding for 5 min at 230 °C. Nitrogen was used as the carrier gas and maintained at 1.0 mL/min.

2.5. Molecular weight determination

The molecular weight of fractions were evaluated and determined by the gel permeation chromatography with a HPLC apparatus (LC-10AD, Shimadzu Co. Ltd., Japan) equipped with an ultrahydrogel column (30 cm × 7.5 mm), a model 10-A refractive index detector (RID). The detailed operation conditions were mobile phase: 0.7% (w/v) sodium sulfate; flow rate: 0.5 mL/min; column temperature: 35 °C; injection volume: 20 μL. The calibration curve for molecular weight determination was made using a series of Dextran standards.

2.6. Infrared spectroscopy analysis

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

2.7. Animals and experimental design

SPF ICR mice weighing 16–20 g were purchased from Animal Experimental Center, Zhejiang Chinese Medical University, China. Experiments were carried out in accordance with local guidelines for the care of laboratory animals of Zhejiang Chinese Medical University, and were approved by the ethics committee for research on laboratory animal use of the institution [No. SCXK (zhe) 2008-0116].

Ten mice were selected randomly as the normal control group (NC), while the other mice were subjected daily to immunosuppression by intraperitoneal administration of Cy (40 mg/kg/d) for 5 days. Then these challenged mice were divided into model control group (MC), iron dextran-treated group (ID) and PMPP-treated groups (10 mice in each group). Mice in PMPP groups were intraperitoneally administered daily at the doses of 20, 40 and 80 mg/kg, respectively, for 7 days after first injection of Cy, while mice in iron dextran group were orally administered. Other mice in NC and MC were only given saline at the same intervals. All mice were observed daily for changes in weight and for any death.

2.8. Biochemical analysis

Twenty-four hours after the last drug administration, peripheral blood samples were collected, centrifuged at 3000 × *g* for 20 min to obtain serum and stored at 4 °C for hemopoietic function test. Then, all mice were weighted and sacrificed by cervical dislocation; spleen and thymus were immediately removed and weighted. Serum levels of superoxide dismutase (SOD), malondialdehyde (MDA) and total antioxidant capacity (T-AOC) were determined by colorimetric method, while serum levels of erythropoietin (EPO) and IL-2 were determined by ELISA using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). Red blood cells (RBC), white blood cells (WBC), platelet (PLT) counts and

hemoglobin (Hb) levels were assessed by using automatic counter System (HITACHI 7100, Tokyo, Japan).

2.9. Western blotting in spleen tissues

Spleen samples were homogenized and lysed in SDS–PAGE sample buffer, boiled, centrifuged and the supernatant recovered. Samples were run on 10% SDS polyacrylamide gels, electroblotted onto nitrocellulose membranes. Immunoblotting was assayed using anti-erythropoietin receptor (EPOR) (1:200), anti-GATA-1 (1:200) antibodies (Wuhan Boster Biological technology Ltd., Hunan, China). The detection was done using an enhanced chemiluminescence detection kit (Wuhan Boster Biological technology Ltd., Hunan, China). The bands density was quantified using Lab works (GelPro4.0, Media Cybernetics, LP) *via* calculating the average optical density in each field.

2.10. Statistical analysis

All parameters were recorded for individuals within all groups. All data were shown as mean ± SD. Statistical comparisons of data were carried out using the ANOVA and t-test of the SPSS 18.0 system. A value of $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Preparation and structural analysis

The crude water-soluble polysaccharides from *P. Multiflori Radix Praeparata* were separated and then purified by DEAE Sephadex A-50 and Sephadex G-100 gel filtration chromatogram. Three fractions were eluted as shown in Fig. 1 and the eluting solution was dried in vacuum condition until a type of white powder was achieved. The major fraction (PMPP) was collected for subsequent analysis. This polysaccharide showed negative Fehling's reagent and iodine–potassium iodide reactions, indicating that it did not contain reducing sugar and starch-type polysaccharide. It also had negative responses to the Bradford test and no absorption at 280 nm in the UV spectrum, indicating the absence of protein. The HPLC profile presented a single eluted peak. The average molecular weight of PMPP was 7.82×10^5 Da. The results of GC quantitative analysis of the acetylated of monosaccharides revealed that PMPP was composed of rhamnose, arabinose, xylose and glucose (Fig. 2) in a molar ratio of 1.64:1.00:1.34:6.06.

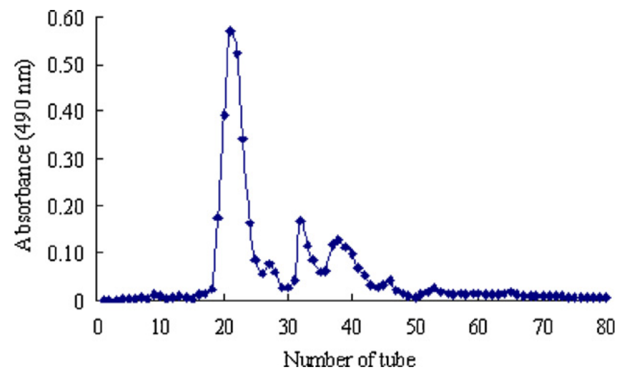

Fig. 1. Gel filtration chromatogram of the crude polysaccharide from *P. Multiflori* Radix Praeparata on Sephadex G-100 column (2.6 cm × 60 cm). The crude polysaccharide was dissolved in deionized water and applied to the column. The eluates were collected and the carbohydrate contents of collected fractions were monitored by phenol–sulfuric acid method.

As shown in Fig. 3, the different absorption bands of the FTIR analysis were assigned as previously described (Nguyen, Do, Nguyen, Pham, & Nguyen, 2011; Zhu et al., 2011). For the three samples, a broad band centered at 3420 cm^{-1} assigned to hydrogen-bonded hydroxyl groups. An intense band centered at

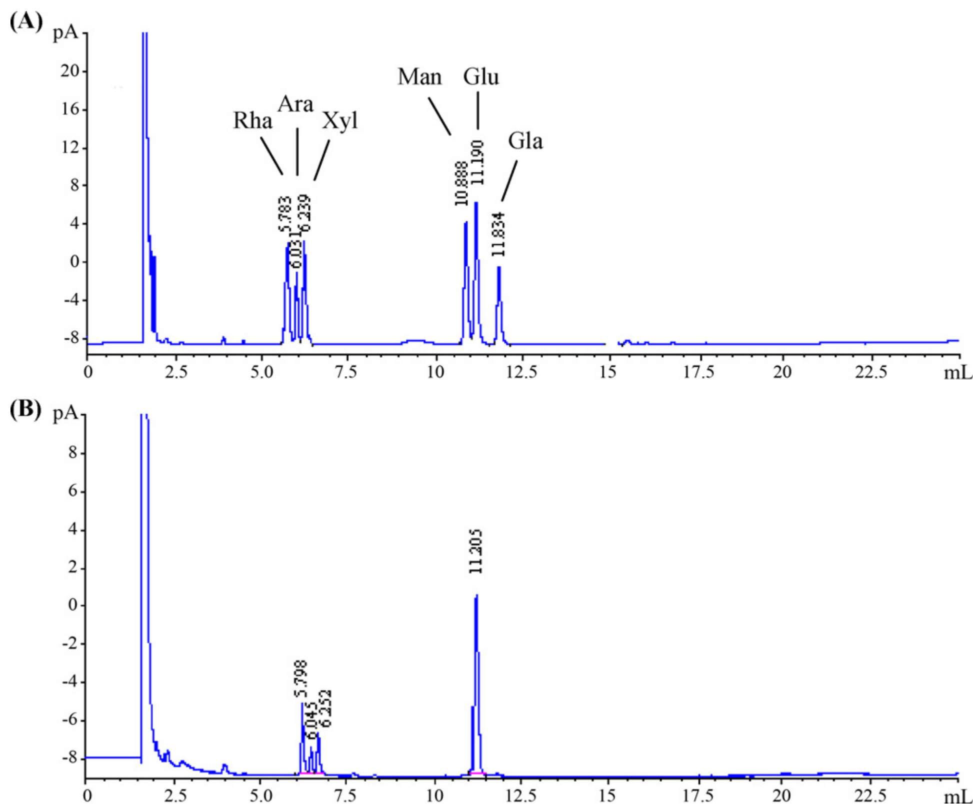

Fig. 2. Gas chromatogram of derivatized standard monosaccharides.

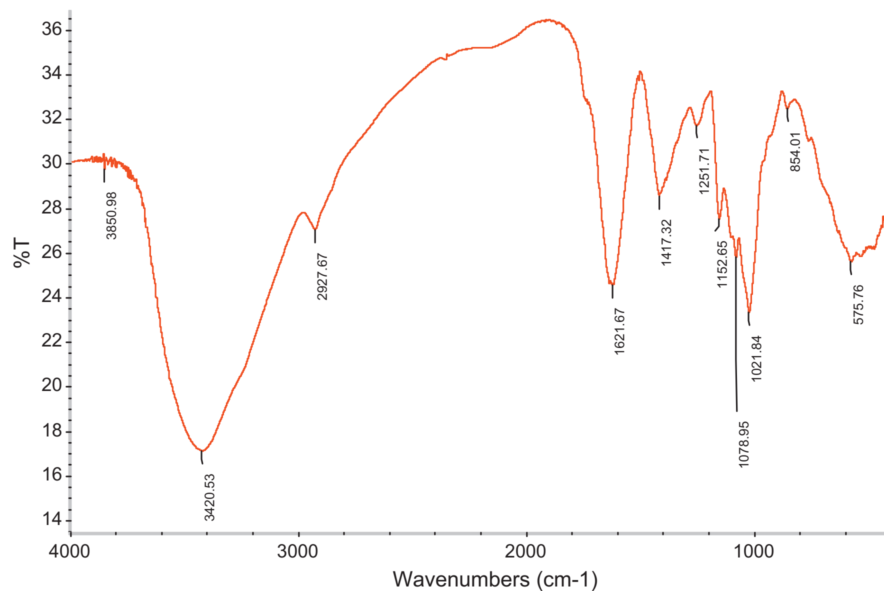

Fig. 3. The infrared spectrum of PMPP.

2928 cm^{-1} was due to the —CH stretching and was a characteristic absorption of polysaccharide. The absorption band centered at 1622 cm^{-1} was caused by the —OH flexural vibrations of the polysaccharide. The absorption band centered at 1417 cm^{-1} assigned to —CH (O—CH_2) flexural vibrations. The group of bands that extended from 1252 cm^{-1} and 1020 cm^{-1} corresponded to C—O stretching vibrations. The absorption band centered at 854 cm^{-1} was due to the α-type glycosidic bond. All the absorption bands listed above were FTIR characteristic peaks of carbohydrate polymer and primarily indicated the nature of PMPP.

3.2. *Effects of PMPP on immunologic profiles of anemic mice*

Spleen is the largest secondary immune organ and responsible for initiating immune reactions in the body. Hematosuppression, one of the most severe side effects of chemotherapy, may lead to immunological inadequacy, severe infections, discontinuation of treatment and even death. Cy is a cytotoxic chemotherapeutic drug for tumor treatment, but long-term administration could cause myelosuppression and immunosuppression, and lead to anemia resulting from erythropoietin deficiencies (Wang, Meng, Qiao, Jiang, & Sun, 2009). The use of erythropoiesis-stimulating agents (ESA) and iron supplements in the treatment of anemia has undergone seismic shift in the past years as a result of adverse outcomes, such as hepatic injury and even higher mortality, associated with targeting higher hemoglobin levels with these agents (Auerbach, Ballard, & Glaspy, 2007). Despite the disappointing experience with ESAs, there is a tremendous interest in other novel agents, such as natural product, to treat anemia in patients with tumor. PMPP is a new polysaccharide isolated from *P. Multiflori Radix Praeparata* and the protective effects of PMPP in ameliorating myelosuppression and immunosuppression induced by Cy treatment were investigated in the present study.

As shown in Table 1, thymus index and spleen index of MC mice were both much lower than those of NC mice (both $P < 0.01$), respectively. Compared with MC, there were observably increases in the thymus and spleen indexes of PMPP-treated mice (all $P < 0.01$). Moreover, Cy showed a trend of decrease in the mouse body weight, while treatment with PMPP at the high dose (80 mg/kg) ameliorated this action ($P < 0.01$). To further elucidate immunological effect of PMPP, IL-2 levels in the serum of mice were determined by ELISA as presented in Fig. 5. It is well known that IL-2 as an important immune factor is secreted by helper T lymphocytes, which can promote immune cell proliferation and differentiation. In this study, PMPP could significantly increase levels of serum IL-2 by more than 2-fold compared with Cy-treated immunosuppressed mice. It suggested that PMPP has an adjuvant role as well as immunomodulatory functions on improving cellular immune of Cy-inhibited mice.

3.3. *Effects on blood cell counts and hemoglobin concentration*

Cy, which causes alkylation of cell functional groups in protein, acts by restraining medulla hemopoietic function and causing leucopenia by decreasing numbers of WBC in blood. Particularly, a high dose of Cy could also lay significant restraints on building blood in

Table 1
Effects of MPMM on body weight, spleen index, thymus index and antioxidant profiles in Cy-induced anemic mice.

Group	Doses (mg/kg)	Body weight (g)	Spleen index (mg/g)	Thymus index (mg/g)	SOD (U/mL)	MDA (nmol/mL)	T-AOC (U/mL)
NC	–	24.1 ± 1.1[b]	5.40 ± 0.35[c]	2.98 ± 0.54[c]	70.0 ± 6.1[c]	1.09 ± 0.17[c]	5.42 ± 1.33[c]
MC	–	22.8 ± 1.9	4.11 ± 0.60	2.02 ± 0.37	56.1 ± 10.9	4.27 ± 0.78	3.11 ± 0.84
ID	5[a]	23.4 ± 1.7	4.96 ± 0.32[c]	2.90 ± 0.31[c]	61.0 ± 4.9	4.43 ± 1.14	6.45 ± 1.14[c]
PMPP	20	22.7 ± 1.7	4.96 ± 0.55[c]	2.67 ± 0.64[c]	61.6 ± 6.2	2.65 ± 0.58[c]	7.97 ± 1.04[c]
	40	22.8 ± 1.4	5.02 ± 0.21[c]	2.83 ± 0.79[c]	65.0 ± 8.8[b]	2.08 ± 0.37[c]	8.74 ± 1.78[c]
	80	24.7 ± 1.0[c]	5.39 ± 0.81[c]	3.17 ± 0.32[c]	67.1 ± 10.9[c]	1.74 ± 0.43[c]	9.97 ± 1.42[c]

Data were expressed as mean ± SD ($n = 10$).
[a] The content calculated by using ferric ion as the standard.
[b] Compared with the MC group, $P < 0.05$.
[c] Compared with the MC group, $P < 0.01$.

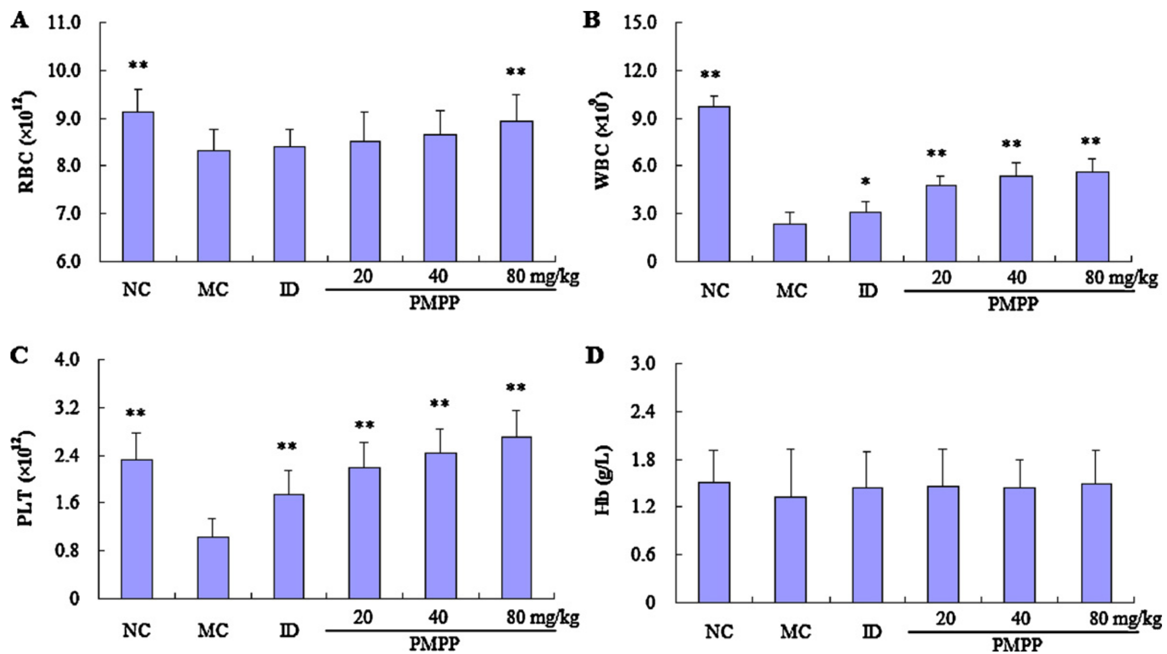

Fig. 4. Effects of PMPP on hematological parameters in Cy-induced anemic mice.

the medulla and extramedulla. Compared with NC, the hematologic parameters (including RBC, WBC and PLT counts) in MC decreased significantly (all $P < 0.01$). As shown in Fig. 4, over a period of 7 days, compared with MC, the levels of RBC, WBC and PLT counts were increased significantly (all $P < 0.01$) with PMPP treatment at a dose of 80 mg/kg. ID is rich in iron, which is necessary for cells building blood to breed and grow and synthesize hemoglobin. Oral administration of ID could also increase hematologic parameters (except RBC counts), but it is weaker to ameliorate immunosuppression induced by Cy compared with those in PMPP-treated group.

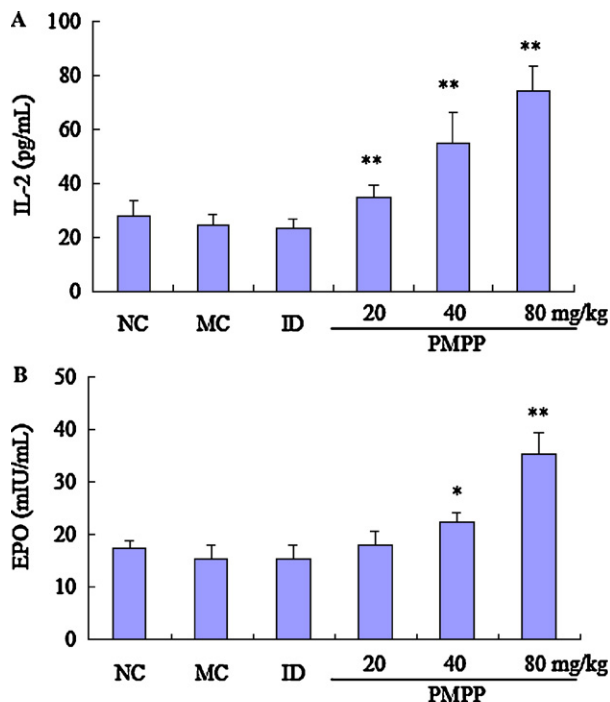

Fig. 5. Effects of PMPP on serum levels of EPO and IL-2 in Cy-induced anemic mice. Values were expressed as mean ± SD ($n = 10$). Compared with MC, * $P < 0.05$, ** $P < 0.01$.

Meanwhile, there was a decreasing trend in the levels of Hb in MC but no significant difference was obtained between MC and NC ($P > 0.05$). Although PMPP had an increasing trend in Hb, which were higher than that in MC, these differences were also no significant ($P > 0.05$).

Erythropoiesis is a complex multistep process encompassing the differentiation of hematopoietic stem cells (HSC) into mature erythrocytes, which have the potential to proliferate rapidly in response to anemia through EPO. The erythropoietin receptor (EPOR), which is expressed abundantly in bone marrow and spleen, plays a crucial role in promoting the erythropoietic response. EPO-EPOR triggers signal cascades leading to the proliferation and differentiation of erythroid progenitors, at least in part by up-regulating the erythroid-specific transcription factor GATA-1 (Aispuru et al., 2008; Juaristi, Aguirre, Todaro, Alvarez, & Brandan, 2007). As shown in Fig. 5, due to the immunosuppression induced by Cy, serum levels of EPO in MC were lower but no significant difference compared with that in NC ($P > 0.05$). After PMPP treatment, serum levels of EPO were significantly increased in a dose-dependent manner. Especially, serum level of EPO of anemia mice treated with PMPP at a dose of 80 mg/kg was 2-fold more than that of MC mice ($P < 0.01$). Simultaneously, EPOR and GATA-1 protein was detected in spleen tissue, whose expressions decreased significantly in MC (both $P < 0.01$). In contrast, both of them were found to be expressed significantly in spleen tissue with a much higher optical density than those of MC mice (Fig. 6).

3.4. *Effects on serum antioxidant profiles in anemic mice*

Recent studies have indicated that Cy would induce free radical production, followed by cytotoxicity and oxidative stress (Tripathi & Jena, 2009). As shown in Table 1, there were significant decreases ($P < 0.01$) in the activities of SOD, but there was a significant increase in the levels of MDA in the serum of mice in MC ($P < 0.01$), comparison with those of mice in NC. However, compared with the mice in MC, intraperitoneal injection of PMPP at the doses of 40–80 mg/kg could cause a significant increase in the activities of SOD ($P < 0.05$ and $P < 0.01$, respectively) and a significant decrease in the

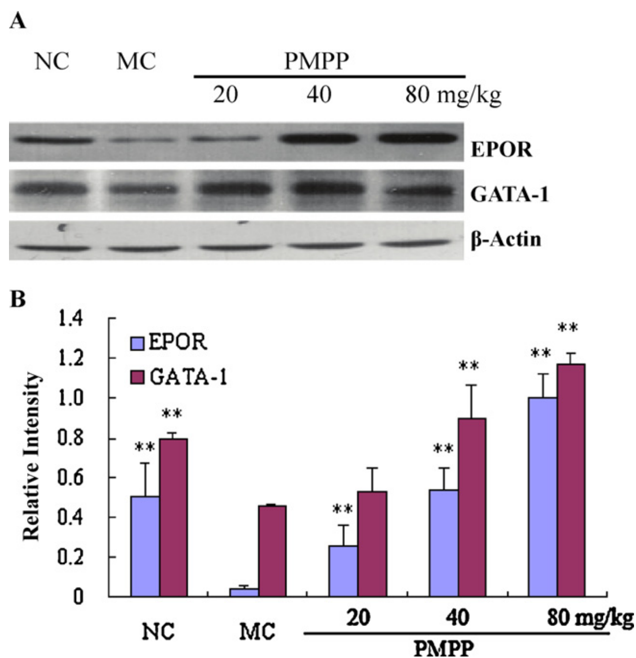

Fig. 6. Protein expression of EPO and GATA1 in spleen tissues by Western blot analysis. (A) Representative immunoblot results. (B) Densitometric analysis of blots for EPO and GATA1 protein. Values were expressed as mean ± SD ($n = 10$). Compared with MC, * $P < 0.05$, ** $P < 0.01$.

accumulation of serum MDA (all $P < 0.01$) in the mice of the PMPP-treated group in a dose-dependent manner.

T-AOC reflects the capacity of the non-enzymatic antioxidant defense system. As shown in Table 1, serum levels of T-AOC decreased remarkably with Cy-treatments, but PMPP administration greatly elevated serum levels of T-AOC. Serum levels of T-AOC in PMPP-mice at the doses of 20–80 mg/kg were increased by 2.6–3.2-fold compared with that in MC mice. The obtained results suggested that PMPP may ameliorate oxidative response through increasing levels of TAOC and enhancing antioxidant activities in the challenged tissue.

4. Conclusion

In the present study, a polysaccharide from *Polygoni Multiflori Radix Praeparata* (PMPP) was obtained by hot water extraction and then purified by DEAE Sephadex A-50 and Sephadex G-100 column. Preliminary structural characterizations were conducted by using physicochemical property and FTIR analyses. The average molecular weight of PMPP was 7.82×10^5 Da. FTIR spectrum indicated that PMPP had α-type glycosidic bond and results of GC analysis showed that it was composed of rhamnose, arabinose, xylose and glucose in a molar ratio of 1.64:1.00:1.34:6.06. Furthermore, intraperitoneal administration of PMPP could increase Th1 type cytokine productions (IL-2) and hematological parameters (RBC, WBC and PLT counts), enhance antioxidant profiles and promote hematopoiesis of splenocytes though up-regulating levels of EPOR and GATA-1 proteins in Cy-induced anemic mice. Efficacy of PMPP provided evidence that this functional plant extract could be developed as a potential immunomodulatory agent for treatment of anemia and immunosuppression.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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