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Immune-enhancement of oligosaccharides from *Codonopsis pilosula* on cyclophosphamide induced immunosuppression in mice

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Abstract

As an important edible traditional Chinese medicine, *Codonopsis pilosula* has good immunomodulation effects. It has been said that the sweeter the *C. pilosula* tastes, the better. This study focused on *C. pilosula* oligosaccharides (CPO), which is the sweetness component of *C. pilosula.* CPO was obtained through systematic separation and purification (the yield is 14.3%), and the effect of CPO on the immunological activities of immunocompromised mice induced by cyclophosphamide (CTX) was evaluated. The results showed that CPO could increase immune organs indices, phagocytic index and contents of immunoglobulins, stimulate proliferation of splenic lymphocytes (coordinating with ConA and LPS), enhance earlap swelling of DTH reaction, promote productions of NO and cytokines (IL-2 and IFN-γ) and the expressions of corresponding mRNA. In addition, CPO upregulated the protein expression of phosphorylated p38, phosphorylated ERK1/2 and phosphorylated JNK, which indicated CPO might exert immunomodulatory activities through the MAPKs signaling pathway. These findings indicated that CPO was an important immunomodulatory component in *C.pilosula* and could be developed as immunomodulator in medicine or functional food areas.

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1. Introduction

Immunosuppression is a condition of long-lasting or temporary immunity disfunction. Because the immune system is damaged, it makes the organism more sensitive to pathogens [1]. Nowadays, many clinical immunomodulators have some side effects such as levamisole and Corynebacterium parvum bacterium[2]. Therefore, developing safer immunomodulators is important to prevent and cure immunosuppressive diseases. Meanwhile, Chinese medicine has obtained a wealth of theoretical and practical knowledge in its application as immunomodulators, such as *ginseng* [3], *Schisandra chinensis* [4], *Glycyrrhiza uralensis* [5], and *Astragalus membranaceus* [6]. Modern medical research have confirmed that TCM could exert immunomodulatory activities through innate and acquired immunity [7, 8, 9].

Codonopsis pilosula, as one kind of important TCMs, is widely used to strengthen the middle warmer, invigorate spleens and nourish lungs[10]. As medicine and food, it is very popular in China, Korea and Japan[11]. Carbohydrates are important components of *C. pilosula*. It is reported that polysaccharides of *C.pilosula* could exert immunomodulatory activities by provoking the proliferation of lymphocytes [12], regulating the proportion of T lymphocyte subsets[13, 14], promoting the secretion of cytokines [13, 15], enhancing macrophage activity[11] and exhibiting complement fixing activity[16]. At present, the reported polysaccharides from *C.pilosula* all have

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been obtained by water-extration and alcohol-precipitation (final concentration 70~80%) and then further purified. The molecular weight range is 4.84 KDa[17]~133.20 KDa[18]. The supernatant are often discarded after total polysaccharides are precipitated, but oligosaccharides are usually in this part. Until now, there were no reports about the immunomodulatory activities of the oligosaccharides from *C.pilosula.* In addition, it has been said that the sweeter the *C. pilosula* tastes, the better, which has been recorded in many ancient medical writings, such as "Bai Cao Jing" and "Yao Long Xiao Pin". Oligosaccharides not only taste sweet but also have been proved to have various physiological activities, such as stachyose[19], raffinose[20]. Therefore, we made a hypothesis that the oligosaccharides were important active components in *C.pilosula*.

Thus, we studied the immunomodulatory effects of CPO on cyclophosphamide (CTX) -treated mice through innate and adaptive immunity. It proved for the first time that the oligosaccharides from *C.pilosula* had good immunomodulatory activities and had potentiality to develop as immunomodulator in functional foods and pharmaceuticals.

2.Materials and methods

2.1 Materials

Dried *Codonopsis pilosula* (Franch.) Nannf. was collected from Weiyuan County (Gansu Province, China), which was identified by Prof. Yin-suo Zhou, School of Pharmacy, Lanzhou University. Thymosin and

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CTX were purchased from the First Hospital of Lanzhou University (Lanzhou, Gansu Province, China). ELISA kits of interferon-γ (IFN-γ), interleukin-2 (IL-2), immunoglobulin G and M (IgG and IgM) were purchased from Meimian Biotechnology Co. Ltd (Nanjing, Jiangsu Province, China); Indian ink , heparin sodium and ECL Western Blotting Substrate were purchased from Beijing Solarbio Science & Technology Co.,Ltd. (Beijing, China); Dinitrofluorobenzene was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China); Assay kit for nitric oxide (NO) was purchased from Beyotime Institute of Biotechnology (Shanghai, China); TRIzol® Reagent, Hifair™ II First Stand cDNA Synthesis SuperMix (11123ES60) and Hieff™ qPCR SYBR® Green Master Mix (11201ES60) were purchased from Yeasen Biotech Co., Ltd. (Shanghai, China); Antibody SAPK/JNK, Phospho-SAPK/JNK (Thr183/Tyr185), p38 MAPK, Phospho-p38 MAPK (Thr180/Tyr 182), p44/p42 MAPK (Erk 1/2), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.2Extraction and purification of CPO

The *C. pilosula* (500 g) was crushed into powder and then defatted twice with 95% EtOH for 2 h. The residue was extracted thrice with hot water for 45 min each time. After filtration by defatted cotton and concentration under reduced pressure, the polysaccharides were

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precipitated with 80% ethanol (final concentration) overnight. The supernatant was collected after centrifugation at 10600 g for 15 min and concentrated under reduced pressure to remove ethanol. The crude product of oligosaccharides was obtained after freeze-drying, named RCPO.

RCPO (200 mg) was dissolved in distilled water and applied to a column (2.6 × 60 cm,i.d.) packed with Sephadex G-25. The column was pre-equilibrated with water and eluted with distilled water. Fractions (10 mL/tube) were collected by a fraction collector and the content of carbohydrates were analyzed through the phenol-sulfuric acid assay. ESI-MS method was used to determine the molecular weight distribution of CPO. The purified product of RCPO was named CPO and the production steps of CPO from *C.pilosula* were shown in Fig.S1. The yield of CPO was calculated as:

$$Y(\%) = (W_{oligosaccharides} / W_{powder}) \times 100\%$$

Y is the yield of oligosaccharides (%, w/w), $W_{oligosaccharides}$ is the weight of oligosaccharides (g), W_{powder} is the weight of *C. pilosula* (g).

2.3 Animals and experimental design

72 male kunming mice (18~22 g) were provided by the Experimental Animal Center in Lanzhou University, China (the license number SCXK（Gan）2018-0002). Throughout whole experiment, the mice were reared at room temperature (25 ± 2 ℃), humidity (55-60%) and a 12 h light-dark cycle with arbitrary water and food. All procedures were accomplished

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according to the code of ethics of the World Medical Association and approved by the Ethics Committee of School of Pharmacy, Lanzhou University (approved in 20 Nov, 2018 with approve number of LZU-YX-2018-11-20-1).

After a week of adaptive breeding, the mice were randomly divided into six groups with 12 mice in each group. From the 1st day to the 20th day, the daily gavage of mice was as below: normal group (NC): saline; model group (MC): saline; thymosin group (XXT): 10mg/kg body weight thymosin; three CPO groups (CPO50, CPO100, CPO150): 50,100 or 150 mg/kg body weight CPO. From the 15th day to the 18th day, all mice (except NC group) were immunosuppressed by intraperitoneal (i.p.) injection of 40 mg/kg body weight CTX.

2.4 Calculation of immune organs indices

At the end of the experiments, mice were weighed and sacrificed by cervical dislocation. The thymus and spleen were excised and weighed. Immune organ index was calculated according to the formula:

Thymus or spleen index = (weight of thymus or spleen × 1000) / (body weight × 100).

2.5 Determination of phagocytic index

Carbon clearance assay was used to access the function of macrophage according to the literature of Niu et al[21]. The mice were injected with 100 μL of 4-fold dilution of Indian ink via tail vein. At the

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point of 2 min (t_A) and 10 min (t_B), blood samples were taken from the posterior orbital vein and 20 μL sample was mixed with 2 mL 0.1% Na_2CO_3. The absorbance of each sample at 600 nm was measured (OD_A for t_A and OD_B for t_B). The phagocytic index (α) was calculated as below:

$$K = (\lg OD_A - \lg OD_B) / (t_B - t_A)$$

$$\text{Phagocytic index } \alpha = K^{(1/3)} \times W_B / (W_L + W_S)$$

Where W_B, W_S and W_L are body weight, liver weight and spleen weight, respectively.

2.6 Determination of splenic lymphocytes proliferation

As described by Zhang et al[13], the splenic lymphocytes were seeded in a 96-well plate (5×10^6 cell/mL) with ConA (20 μg/mL) or LPS (40 μg/mL). After 72 h cultured at 37 °C in 5% CO_2, 20 μL of 5 mg/mL MTT was added to each well and incubated for another 4 h. After discarding the supernatant, DMSO (150 μL) was added into each well. The absorbance at 570 nm was measured.

2.7 Delayed-type hypersensitivity (DTH) reaction

The DTH reaction is a method to evaluate the cell-immunity function[22]. At the first day of intraperitoneal (i.p.) injection of CTX, mice were covered with 20 μL 5% dinitrofluorobenzene dissolved in acetone–olive oil (acetone: olive oil=1:1) on the shaved belly skin. The second sensitization was made after 5 days and 10 μL 5% dinitrofluorobenzene was evenly applied to both sides of the right ear of the mouse. After 24 h,

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the mice were sacrificed and the 8 mm ear patches were removed and weighed. The extent of the DTH reaction was evaluated by the change between the weight of the right and left ear.

2.8 Measurements of serum immunoglobulin (Ig)

The serum was obtained from eyeball blood after centrifugation. The concentrations of IgG and IgM in serum were determined by the ELISA kit according to the instructions of manufacturer.

2.9 Determination of IL-2, IFN-γ and NO

The splenic lymphocytes were incubated (1×10^6 cells/mL) with 20 μg/mL ConA for 48 h in 24-well plates in a 37 °C in 5% CO_2 atmosphere. The supernatants were gathered to detect the level of IL-2, IFN-γ and NO according to commercial kits.

2.10 Real time-PCR (RT-RCR) assay

The total RNA was extracted from spleen using the Trizol reagent. The resulting RNA was reverse transcribed using a Hifair™ Ⅱ 1st Strand cDNA Synthesis SuperMix Kit (Yeasen, China). The RT reaction was performed at 42 °C for 15 min and 85 °C for 2 min to prepare cDNA, which was then stored at −80 °C for use. Real-time PCR was carried out using an LightCycle 480 real-time PCR system and a real-time PCR master mix (SYBR Green) reagent kit (Yeasen, China). Custom-made primers are present in Table 1. The conditions for real-time PCR were 95 °C for 5min, then 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 95 °C for 15 s, 60 °C

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for 1 min, 95 °C for 15 s. Relative mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method.

2.11 Western blot assay

To prepare total protein sample, the spleen homogenates of each group were lysed for 5 min in ice bath and centrifuged (12000g, 30 min) at 4℃. The concentration of protein was determined by BCA method. After boiling for 5min in SDS sample buffer, the proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with primary antibody against p-JNK (1:2000), JNK (1:1000), p-ERK (1:2000), ERK (1:1000), p-p38 MAPK (1:1000) and p38 MAPK (1:1000, CST,USA) according to the manufacturer's instructions. 5% BSA (prepared in TBST containing 0.1% Tween 20) was used to block the membranes at 4 °C with gentle shaking, overnight. The membranes were incubated with the appropriate second antibodies at room temperature for 1 h. The antigen-antibody complexes were visualized using an ECL kit (Solarbio, Beijing). To quantify protein levels, the protein bands were scanned, and their intensities and areas were analyzed using ImageJ (NIH, USA).

2.12 Statistical analysis

Results were expressed as mean ± SD. All data were analyzed by one-way ANOVA with Tukey post-test. Significance was defined as $P < 0.05$.

3. Results

3.1 Characterization of CPO

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CPO was brownish yellow and hygroscopic powder. The yield of CPO was 14.3 g/100 g and the sugar content was 92.7%. CPO had no UV absorption at 280 nm, indicating that there was no protein contained in the prepared CPO. As shown in Fig.1, The result of ESI-MS indicated that CPO was mainly composed of saccharides with DP from 1 to 7. Many natural carbohydrates with low degree of polymerization are sweetness, such as maltose, lactose, trehalose, maltulose, isomaltulose (palatinose), lactulose, cellobiulose, melibiulose and fructo-oligosaccharides[23]. Therefore, we thought CPO was sweet. In addition, we produced a small amount of CPO using food-grade reagents and found it tasted sweet.

3.2 Effect of CPO on innate immunity in CTX-treated mice

3.2.1 Spleen and thymus indices

The immune organs indices reflect the immune organ function to a certain extent[24]. As displayed in Fig.2a，compared with the normal group, spleen and thymus of the model group significantly decreased ($P < 0.01$). In comparison with the model group, the CPO administration (50, 100 and 150 mg/kg) remarkably increased the spleen and thymus indices of the laboratory mice (50 mg/kg，$P < 0.05$；100, 150 mg/kg，$P < 0.01$). These findings suggested that CPO could alleviate the immune organ atrophy caused by CTX to a certain extent.

3.2.2 Phagocytic index

The phagocytosis of monocytes can be assessed by the carbon

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clearance assay. The faster removal of carbon particles is linked to stronger phagocytosis[25]. As shown in Fig. 2b, compared with the normal control, the phagocytic index (α) of the model group significantly decreased ($P < 0.01$). Treatment with CPO (100 and 150 mg/kg body weight) could significantly enhance the phagocytic index (α) in comparison with model group. The phagocytic activities of CPO100 and 150 even exceeded the normal level, from 4.26 to 5.82 and 5.66. These results indicated that CPO could improve the carbon clearance ability of CTX-treated mice.

3.3 Effect of CPO on adaptive immunity in CTX-treated mice

3.3.1 The proliferation of splenic lymphocytes

The cell mitogens ConA and LPS can motivate lymphocytes proliferation reactions[26]. As shown in Fig. 2c, compared with the control group, splenic lymphocytes proliferation was substantially decreased attributing to ConA or LPS stimulation to the CTX-treated mice ($P < 0.01$). In addition, the CPO-treated group significantly upregulated the splenic lymphocytes proliferation induced by ConA or LPS (50 mg/kg，$P < 0.05$；100, 150 mg/kg，$P < 0.01$), especially at the dose of 100 mg/kg body weight.

3.3.2 DTH reaction mediated by T cells

The earlap swelling indicates the strength of DTH reaction, which is mediated by T cells involved in cellular immunity[22]. As shown in Fig. 2d, compared with the normal group, it showed a significant decrease of earlap

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swelling in the model group ($P < 0.01$), which indicated that the DTH model induced by DNFB was successfully established. Moreover, CPO treatment could effectively increase earlap swelling in comparison with the model group ($P < 0.01$), which indicated that CPO could reinforce DTH reaction in CTX-treated mice. The results demonstrated that the immunomodulatory activity of CPO might be associated with cellular immunity.

3.3.3 The contents of serum Ig

The serum immunoglobulins are critical markers of humoral immunity[27]. The serum Ig G and Ig M levels were measured by ELISA to assess the effect of CPO on the humoral immunity of CTX-treated mice. As the Fig. 3 showed, the concentration of Ig M and Ig G of model group decreased remarkably ($P < 0.01$), while CPO treatment group (100 and 150 mg/kg) had a significantly increasment of the levels of Ig M and Ig G ($P < 0.05$). Thus, the immunomodulatory effects of CPO might be related to humoral immunity in CTX-treated mice.

3.4 Effect of CPO on cytokines and NO secretion in CTX-treated mice

In order to further demonstrate the immunomodulatory activity of CPO, the secretion of cytokines (IL-2, IFN-γ) and NO produced by splenic lymphocytes were measured. As shown in Fig. 4, IL-2, IFN-γ and NO levels in the supernatants of splenic lymphocytes cultures were significantly decrease in CTX-treated mice compared with the normal

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group ($P < 0.01$). The concentrations of IL-2, IFN-γ and NO in CPO treatment groups increased markedly in comparison with the model group. At the doses of 50, 100 and 150 mg/kg, CPO treatment significantly increased the IL-2 concentration by 3.5-, 4.5- and 4.2-fold of the model group, respectively. Correspondingly, when processing with CPO (100 and 150 mg/kg), the amount of NO secretion significantly increased, which was 5.4- and 4.2-fold of the model group, respectively. IFN-γ production could be enhanced after CPO administration (100 mg/kg). Therefore, CPO could synergize with Con A to stimulate splenic lymphocytes, resulting in increased levels of IL-2, IFN-γ and NO.

3.5 Effect of CPO on gene expression in CTX-treated mice

As shown in Fig. 5, RT-PCR analysis displayed that CTX-induced mice had lower expression of related genes than that in the normal group ($P < 0.01$). CPO treatment remarkably increased the related gene expression in comparison with the model group, which was consonant with the levels of IL-2, IFN-γ and NO. When the dosage of CPO was 100 mg / kg, the mRNA expression levels of iNOS, IL-2 and IFN-γ were 3.4, 5.1, and 4.2 times that of the model group, respectively. In general, CPO could promote the sceretion of cytokines (IL-2 and IFN-γ) and NO by enhancing the gene expression of IL-2, IFN-γ, and iNOS in CTX-treated mice.

3.6 Effect of CPO on protein expression in CTX-treated mice

MAPK is a family of serine / threonine-specific protein kinase which

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is involved in regulating many cell life activities [25]. Furthermore, the activation of MAPKs affects the cellular response to cytokines and expression of related genes[28]. Western blot analysis was used to analyze MAPKs protein expression, including phosphorylated JNK, phosphorylated ERK, and phosphorylated p38. As shown in Fig. 6, phosphorylation of JNK, ERK and p38 decreased significantly in CTX-treated mice in comparison with the normal group ($P < 0.01$). Nevertheless, compared to the model group, the protein expression of phosphorylated p38, phosphorylated ERK1/2 and phosphorylated JNK in CPO treatment were significantly increased. After dosing with 50 mg/kg of CPO, the ratio of the protein contents of phosphorylated p38/p38 increased from 0.16 (model group) to 0.58. After treated with 100 mg/kg of CPO, the ratio of the protein contents of phosphorylated ERK/ERK increased from 0.40 (model group) to 2.32. The administration of 150 mg/kg CPO could increase the ratio of the protein contents of phosphorylated JNK/JNK from 0.50 (model group) to 6.26. To sum up, CPO might improve CTX-induced immune suppression by activating MAPKs signaling pathway.

4. Disscussion

CPO was obtained from *C.pilosula* by water extraction and alcohol precipitation combined with column chromatography. The yield of CPO was as high as 14.3% and the sugar content was 92.7%. It has been said the

sweeter the *C.pilosula* tastes, the better. Therefore, we focused on CPO and studied its immunomodulatory effect on CTX-induced immunosuppressed mice.

CTX is an alkylating agent and an important chemotherapeutic drug for the treatment of malignant tumors. However, CTX can damage the DNA of normal cells and cause immunosuppression[29]. In our research, CTX was used to build an immunodeficiency model in mice. Not surprisingly, the results showed that CTX could reduce the immune organs indices, arrest proliferation of splenic lymphocytes, decrease secretion of cytokines and NO and inhibit DNFB-induced DTH reaction, which indicated that the immunosuppression model induced by CTX was established successfully.

The spleen and thymus are important immune organs and the immune organs indices indicated the function of innate immunity to a certain extent[30]. The treatment of CPO could remarkably increase the thymus and spleen indices in CTX-treated mice. Meanwhile, macrophages can perform phagocytosis through the innate immune response[31]. The increase in phagocytic index (α) of CTX-treated mice treated with CPO (100 and 150 mg/kg) indicated that CPO could enhance phagocytic ability of macrophages.

Adaptive immune system, including cell and humoral immune responses, can protect the body from the invasion of specific pathogens.

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Lymphocyte proliferation plays a critical role in activating process of adaptive immune [32]. Lymphocytes proliferation induced by mitogen ConA or LPS has been widely used as a method to evaluate T or B lymphocyte activity because of its high sensitivity. The present results indicated that CPO (50, 100 and 150 mg/ kg) could enhance ConA- and LPS-induced splenic lymphocytes proliferation. DNFB-induced DTH reaction is a type of cellular immunity mediated by T lymphocytes, the character of which is the large aggregation of non-specific inflammatory cells[33]. CPO treatment could enhance DTH reaction by increasing earlap swelling in CTX-treated mice. This result indicated that CPO might play a role in assisting cellular immune response. Immunoglobulins play an important role in humoral immunity and can recognize and neutralize foreign substances [27]. In this study, it is observed that CPO (100 and 150 mg/kg) showed significant increments in Ig G and Ig M contents in comparison with the model group. Thus, CPO might exert immunomodulatory effects through humoral immunity in CTX-treated mice.

Cytokines play vital roles in the immune system, which are involved in the preservation or restoration of homeostasis via coordination of lymphoid cells, inflammatory cells, and hematopoietic cells[34]. IFN-γ and IL-2 are primary immunity-related cytokines. IFN-γ could induce the generation of T cells, activate macrophages, regulate crossly Th1 and Th2 cells[35]. IL-2 is a pleiotropic cytokine that promotes T cell growth, enhances

NK cell lytic activity, and induces the differentiation of Treg cells[36]. NO is an important signaling medium in organisms involved in many biological functions, such as host defense and neural transmission[31]. Our results showed that CPO treatment could increase the levels of IFN-γ, IL-2 and NO in the supernatants of splenic lymphocytes cultures in CTX-treated mice, which is in accordance with the mRNA expression of IFN-γ, IL-2 and iNOS.

MAPK is a family of serine / threonine protein kinases, including JNK, ERK1/2 and p38[37]. MAPKs signaling pathways regulate immune response by regulating the compound and releasing of cytokines and the related mRNA expression. For example, glycoprotein from *Dioscorea opposita* Thunb can stimulate macrophages to secret IL-6, TNF-α and NO via MAPKs and NF-κB pathways[21]. In this study, CPO could enhance the protein expression of p-p38,p-JNK and P-ERK1/2, which indicated that CPO might exhibit immunomodulatory activities through up-regulating various transcription factors in MAPKs signaling pathway. These findings are meaningful to understand the potential immunomodulatory molecular mechanisms of CPO . The possible mechanism was shown in Fig 7.

The results showed that CPO had good immunomodulatory activity, which had been firstly proved the scientific of the saying " the sweeter the *C.pilosula* tastes, the better it is ". We demonstrated that not only polysaccharides but also oligosaccharides were important active ingredient

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in *C.pilosula*. Compared with polysaccharide fraction, the separation and purification of oligosaccharides were simpler, the water solubility was better, bioavailability was higher and quality control was easier.

5. Conclusion

To sum up, our results firstly showed that oligosaccharides from *Codonopsis pilosula* were important active components and had protective effects against immunosuppression induced by CTX. These findings provided the basis for the development of CPO as a functional food or drug with immunomodulatory activity. Meanwhile, it was the first time to clarify that the quality of *C.pilosula* might be closely tied to its sweet oligosaccharides. However, although the sugar content of CPO is as high as 92.7%, it is still a mixture of glycans and it needs further investigation about which glycans play the most important role.

Conflicts of interest

The authors declare no conflicts of interest.

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Figure captions

Fig. 1: ESI-MS spectrum of CPO.

Fig. 2: Effects of CPO on immune organ index (a), phagocytic index (b), ConA- and LPS-induced splenocyte proliferation (c), ear swelling in DTH reaction (d) in CTX-treated mice. NC, normal control group; MC, CTX-induced mice group; XXT, positive control group (thymosin, 10mg/kg bw); CPO, oligosaccharides from *Codonopsis pilosula*(50,100,150mg/kg bw). Values are given as means ± S.D. *P < 0.05, **P < 0.01 vs. Normal group; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 vs. Model control.

Fig. 3: Effect of CPO on IgG (a) and IgM (b) contents in serum of CTX-treated mice. NC, normal control group; MC, CTX- induced mice group; XXT, positive control group (thymosin, 10mg/kg bw); CPO, oligosaccharides from *Codonopsis pilosula*(50,100,150mg/kg bw). Values are given as means ± S.D. *P < 0.05, **P < 0.01 vs. Normal group; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 vs. Model control.

Fig. 4: Effect of CPO on IL-2 (a), IFN-γ (b) and NO (c) levels in CTX-treated mice. NC, normal control group; MC, CTX- induced mice group; XXT, positive control group (thymosin, 10mg/kg bw); CPO, oligosaccharides from *Codonopsis pilosula*(50,100,150mg/kg bw). Values are given as means ± S.D. *P < 0.05, **P < 0.01 vs. Normal group; $^{\#}$P <

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0.05, ##P < 0.01 vs. Model control.

Fig. 5: Effect of CPO on mRNA expression levels of IL-2 (a), IFN-γ (b) and iNOS (c) in spleens of CTX-treated mice. NC, normal control group; MC, CTX- induced mice group; XXT, positive control group (thymosin, 10mg/kg bw); CPO, oligosaccharides from *Codonopsis pilosula* (50,100,150mg/kg bw). Values are given as means ± S.D. *P < 0.05, **P < 0.01 vs. Normal group; #P < 0.05, ##P < 0.01 vs. Model control.

Fig. 6: Effect of CPO on the phosphorylation of mitogen activated protein kinase (MAPK) in spleens of CTX-treated mice. (a) The proteins isolated from splenocytes were analyzed by western blot with anti-p-ERK, anti-p-JNK, anti-p-p38 antibodies. ERK, p38 and JNK were used as loading controls. (b) Levels of p-p38/p38, (c) Levels of p-ERK/ERK and (d) Levels of p-JNK/JNK. Histogram represents quantification of protein expression levels using ImageJ software. NC, normal control group; MC, CTX-induced mice group; XXT, positive control group (thymosin, 10mg/kg bw); CPO, oligosaccharides from Codonopsis pilosula (50,100,150mg/kg bw). Values are given as means ± S.D. *P < 0.05, **P < 0.01 vs. Normal group; #P < 0.05, ##P < 0.01 vs. Model control.

Fig. 7: Possible mechanisms by which CPO activate splenic lymphocytes.

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Table

Table 1. Primers used for qPCR analysis of genes

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	GTGCTATGTTGCTCTAGACTTCG	ATGCCACAGGATTCCATACC
iNOS	CAAGCTGAACTTGAGCGAGGA	TTTACTCAGTGCCAGAAGCTGGA
IL-2	CCCAGGATGCTCACCTTCA	CCGCAGAGGTCCAAGTTCA
IFN-γ	CGGCACAGTCATTGAAAGCCTA	GTTGCTGATGGCCTGATTGTC

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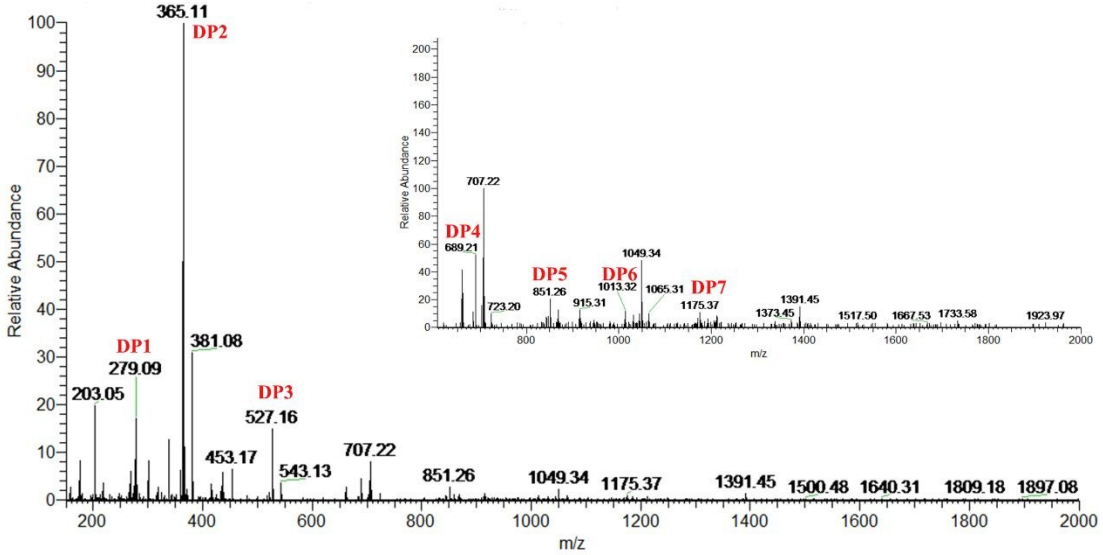
Fig. 1



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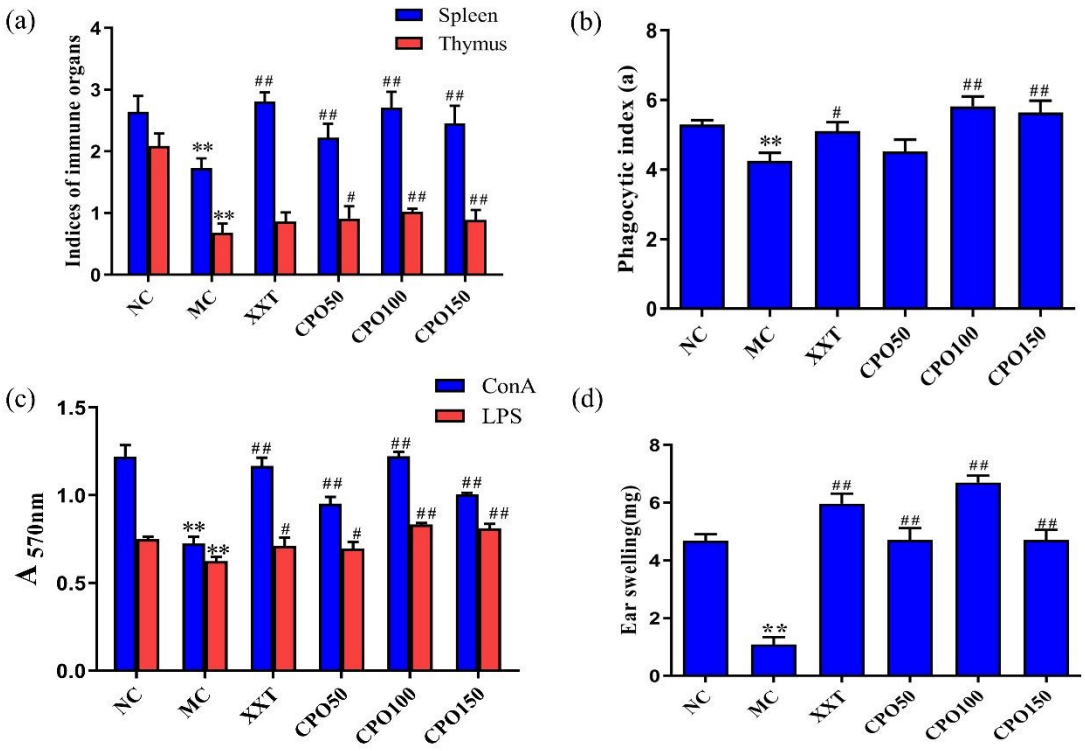
672 Fig. 2



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685 Fig. 3

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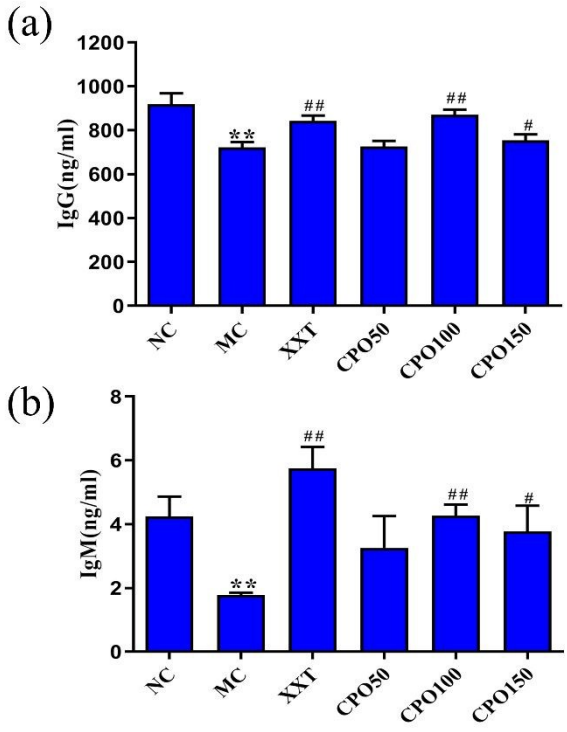

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698 Fig. 4

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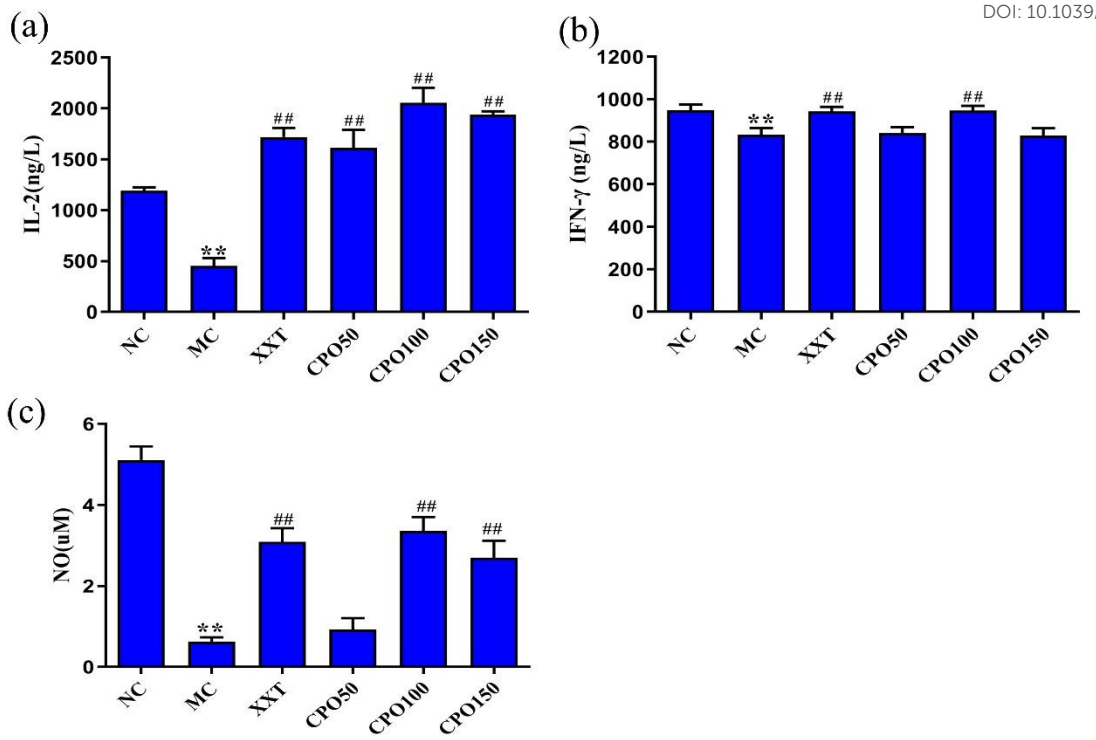

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711 Fig. 5

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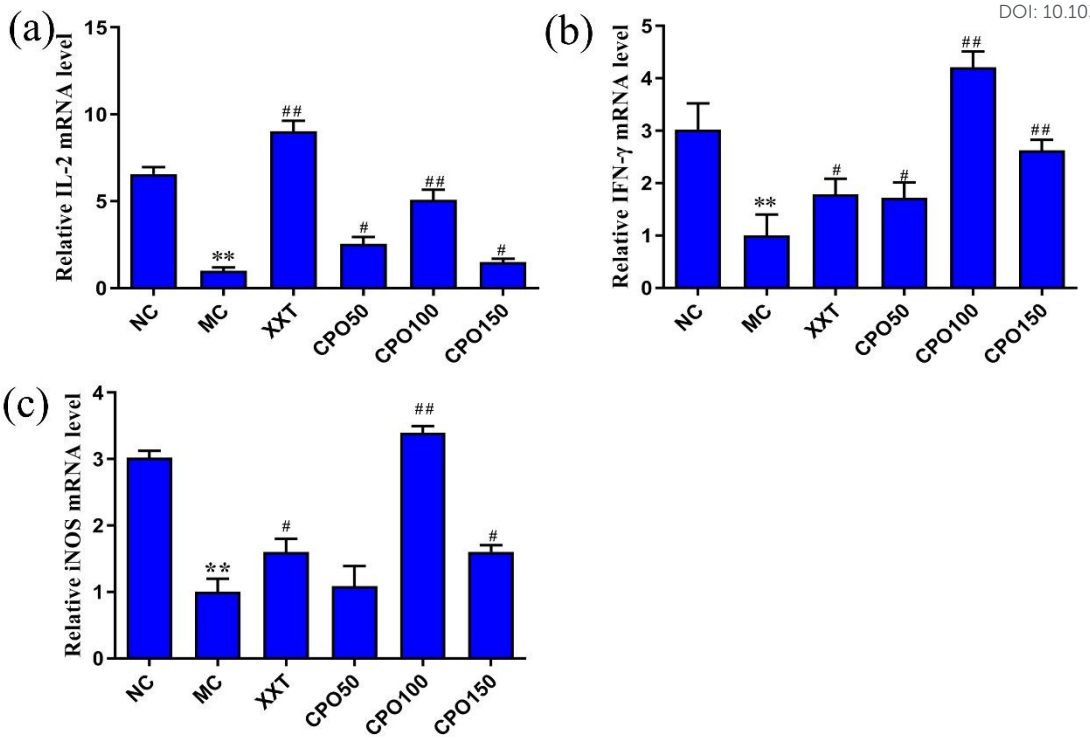

Fig. 6

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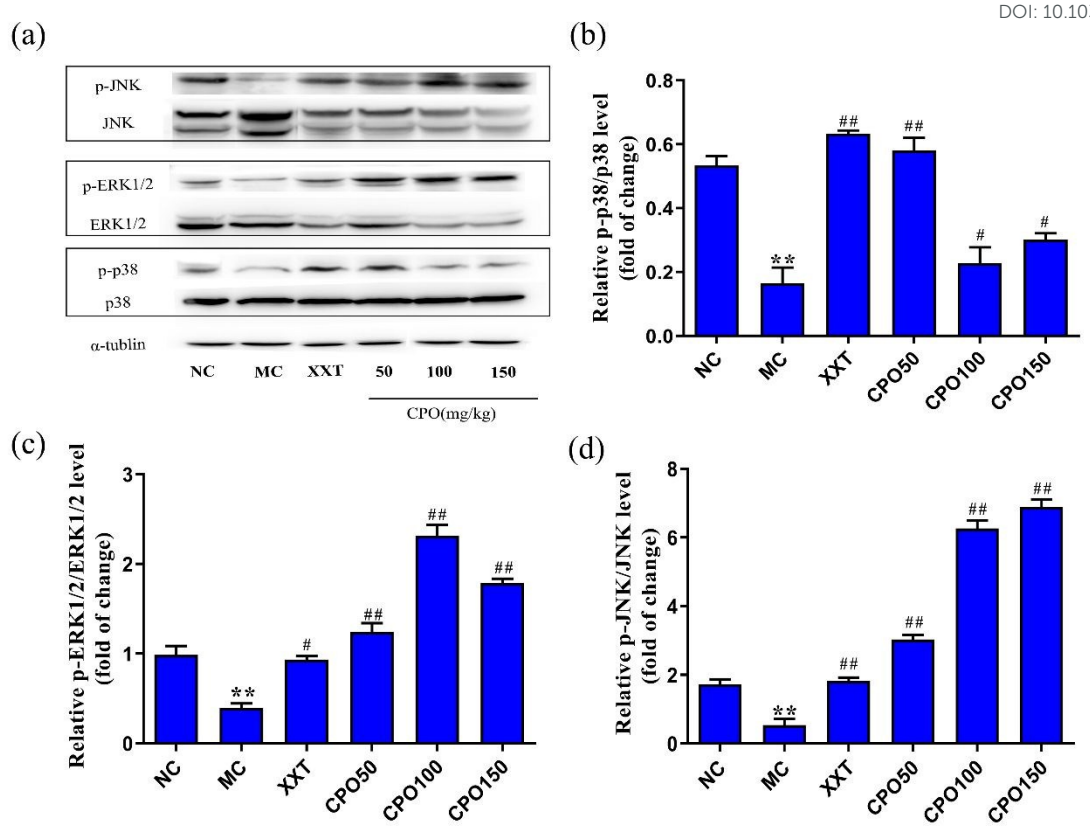

Fig. 7

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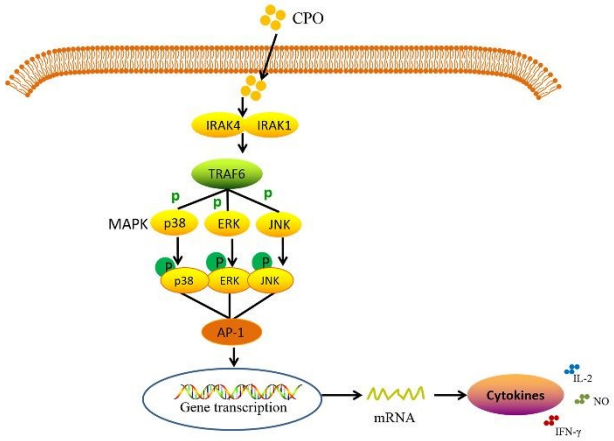

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The oligosaccharides are the main components of *C.pilosula* and exert excellent immunomodulatory activities.