

# Immunomodulatory and antioxidant effects of polysaccharides from the parasitic fungus *Cordyceps kyushuensis*

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# Abstract

Ascomycete *Cordyceps* genus has been used as valued traditional Chinese medicines. *Cordyceps kyushuensis* is a unique species of *Cordyceps*, which parasitizes on the larvae of *Clanis bilineata* Walker, and its major component cordycepin and aqueous extract are known to have many pharmacological effects. However, the physiological function of water soluble polysaccharides has not been explored in detail. In this study, to resolve these doubts, we extracted and separated *Cordyceps*-derived polysaccharides, then evaluated the immunomodulatory and antioxidant activities. Four polysaccharide fractions were purified from *Cordyceps* cultured stroma by DEAE-cellulose 23 and Sephadex G-150 column chromatography. Basic structural information was elucidated on the basis of physicochemical property and spectroscopic evidences. The antioxidant activities were evaluated by DPPH radical method and protective effect of DNA damage. The qualified immunologic activities were also determined *in vivo* and *in vitro*. The polysaccharides could stimulate the proliferation of mouse splenocytes whether ConA and LPS existed or not, strengthen peritoneal macrophages to devour neutral red, and increase the content of IL-2 and TNF- $\alpha$  in serum. The research provides the corresponding evidence for *Cordyceps* polysaccharides as a potential candidate for functional foods and therapeutic agents.

## Introduction

It is reported that about a total of 110,000 fungal species had been recognized (Wasser 2010). Macrofungi, also known as mushrooms, have been extensively used as foods, nutraceuticals, and medicines since time immemorial (Friedman 2016). The edible and medicinal mushrooms are recognized as one of the most important food supplements and have been recently studied for bioactive metabolites because of their vital roles in human health, nutrition, and various illnesses. Fungal-derived polysaccharides, a kind of natural biological macromolecules, recognized as “biological response modifiers”, mainly derived from the mycelium, fruiting bodies, and fermentation broth of the Basidiomycetes family, and some from the Ascomycetes. Depending on their chemical composition, molecular weight, conformation, glycosidic bond, degree of branching, etc (Methacanon et al. 2005), fungal polysaccharides presented good nutritional values and pharmaceutical properties, such as immunomodulatory (Fan et al. 2018; Cheung et al. 2009; Li et al. 2019), antitumor (Rashid et al. 2011; Fisher and Yang 2002; Wu et al. 2012), antioxidant (Sun et al. 2009), hypocholesterolemic (Jin et al. 2019; Liu et al. 2019), hepatoprotective (Zhang et al. 2012), and anti-inflammatory activities (Gondim et al. 2012).

The genus *Cordyceps* belongs to the entomopathogenic fungi, Clavicipitaceae, Ascomycotina (Tang and Eisenbrand 1992). *Cordyceps sinensis* and *C. militaris*, have been used as traditional Chinese medicines for the effectiveness in improving lung and kidney functions, restoring health after prolonged sickness, and enhanced physical performance (Xia et al. 2017; Bi et al. 2020). Continuous attentions had been paid to *Cordyceps*-derived polysaccharides (Chen et al. 2015; Wang et al. 2014; Wu et al. 2012; Hu et al. 2019). Polysaccharides from the stroma and mycelia culture of *Cordyceps* fungus have extensive health effects and pharmacological activities, such as stimulating the innate and adaptive immune responses,

activated macrophages production (Chiu et al. 2014; Meng et al. 2014). *C. kyushuensis* is a unique species of Cordyceps, and it is worth of further investigation. As far as we know, the only host of *C. kyushuensis* is the larvae of *Clanis bilineata* walker (Shimizu 1994). Two polysaccharides purified from the cultured stroma of *C. kyushuensis* were reported previously by our group, showed remarkably antioxidant effects by assays of various antioxidant *in vitro* systems (Zhang et al. 2015).

Up to now, there are only a few research papers on purification of polysaccharides from *C. kyushuensis* and their immunostimulatory activity. Therefore, in the present study, four other water-soluble polysaccharides of *C. kyushuensis* were extracted, purified and preliminarily characterized. Moreover, immune regulation and antioxidant properties of the fractions were also evaluated *in vitro* and *in vivo*. The research would serve as a good foundation for further investigation, development and industrial application of Cordyceps-derived polysaccharides in functional food and therapeutic agents.

## Materials And Methods

### Materials and reagents

The anamorph strain JY1A of *C. kyushuensis*, originally isolated from fresh natural specimen, was confirmed by means of both morphological and molecular methods, and conserved by our lab. Cultured *C. kyushuensis* grew on solid rice medium and was obtained after about 90 days. The dried stroma was shattered to powder (80 mesh) for further experiment. DEAE-cellulose 23 and Sephadex G-150 were purchased from Amersham Biosciences (Uppsala, Sweden). The standard monosaccharide (D-mannose, D-galactose, D-arabinose, D-fructose, L-rhamnose, D-glucuronic acid, D-glucosamine, D-galactosamine), TFA, PMP, LPS and ConA were obtained from Sigma-Aldrich (St. Louis, MO, USA). The RPMI-1640 medium and fetal bovine serum was provided by Gibco (Vienna, NY, USA). All other chemicals and solvents used were of analytical grade and obtained from Sinopharm (Shanghai, China).

### Purification of polysaccharides

The extraction of 300 g stroma powder was carried out three times, each time with 3 L distilled water at 90 °C for 2 h. The mixtures were concentrated to one-third volume and precipitated by adding four volume of 95% ethanol (v/v) and kept at 4 °C for overnight. The precipitate was collected and washed with 95% ethanol, acetone, and ethyl ether, respectively. The resulting fraction was dialyzed in cellulose membrane tubing (exclusion limit 3500 Da) against de-ionized water and lyophilized. Protein was removed by Sevag method (Matthaei et al. 1962). Crude polysaccharide was preliminarily separated by sub-fractionated with gradient final concentrations of ethanol (15%, 30%, 50% and 95%). The fraction at final concentration of 50% ethanol, named as CKPS, was obtained by lyophilization, and selected for further study. CKPS powder (100 mg) was dissolved in 2 mL water, the supernatant was applied to a DEAE-23 column (50×2.0 cm i.d.), which was eluted with water and followed by a 4-step gradient of 0-0.32 M NaCl (0.06, 0.1, 0.16 and 0.32 M). Guided by the phenol-sulfuric acid method, the NaCl eluting fraction with high content of sugar was collected, dialyzed, lyophilized, and purified by Sephadex G-150 column (80×2.0 cm i.d.).

## Analysis of physicochemical properties

Total sugar content was determined by Phenol-sulfuric acid method (Chaplin and Kennedy 1994) with glucose as the standard. Protein concentration was measured with a [Bradford protein assay kit](#) (Beyotime, Shanghai, China). Sulfate content was evaluated using the barium chloride-gelatin method (Kawai et al. 1969) and the content of uronic acid was assessed by the method of m-hydroxydiphenyl using galacturonic acid as standard (Bitter and Muri 1962).

Identification and quantification of monosaccharide was carried out by HPLC method (Sun et al. 2009) with some modification. The polysaccharide (5 mg) was hydrolyzed with 2 M TFA at 120 °C for 4 h in a sealed-tube fulfilled with N<sub>2</sub>. Then excessive acid was removed by methanol. The dried sample was dissolved with solution containing 0.3M aqueous NaOH (0.5 mL) and 0.5 M methanol solution of PMP (0.5 mL), and incubated at 70 °C for 100 min, then neutralized with 0.5 ml of HCl (0.3 M). The resulting solution was extracted by chloroform for three times. The aqueous layer was filtered through a 0.22 µm nylon membrane (MSI, Westborough, MA, USA), and injected in to a Kromasil 100-C<sub>18</sub> column (250×4.6 mm i.d., 5 µm) at 260 nm and at a column temperature of 25 °C. The mobile phase, a solution of 0.02 M phosphate buffer (pH 6.7): acetonitrile= 80:20 (v/v), was eluted at a flow rate of 0.9 mL/min. Identification of the target compounds was based on comparison with reference sugars. Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide.

Homogeneity and absolute measure of molecular weight of the purified fractions were obtained by HPSEC (Agilent Technologies, 1200 series LC system, St. Clara, CA, USA) coupled with MALLS (Wyatt Technology DAWN HELEOS II, St. Barbara, CA, USA) at 690 nm. The sample was dissolved with mobile phase (5 mg/mL) and filtered through a 0.22 µm MSI nylon syringe filter before injection. A serial column which combined TSK-Gel G-6000 PWXL (300×7.8 mm i.d., Tokyo, Japan) with TSK-Gel G-5000 PWXL columns was then employed to separate the samples at 30 °C. The pre-degassed 0.2 M NaCl aqueous solution was applied as the elution buffer at a flow-rate of 0.6 mL/min. Three injection operations of the polysaccharide were performed and molecular mass values were determined by averaging these results.

FTIR spectra were recorded in the region 4000-400 cm<sup>-1</sup> on Thermo Nicolet 20sx spectrometer at 4 cm<sup>-1</sup> resolution. The samples were blended with KBr powder, grounded and pressed into a 1 mm pellets. The <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Bruker AM-400 MHz NMR spectrometer (Rheinstetten, Germany) at 25 °C. The polysaccharide sample (25 mg) was exchanged 3 times with DMSO-d<sub>6</sub> upon freeze-drying, re-dissolved in 0.5 mL DMSO-d<sub>6</sub>, and centrifuged prior to analysis.

## Vertebrate Animal Study Methods

### Animals care, feeding, housing, and grouping

A total 60 male healthy Kunming mice (8 weeks old, 20.0 ± 2.0 g), SPF grade, were purchased from Laboratory animal center of Shandong University. The mice were housed on a 12-h-dark/12-h-light cycle

at  $22 \pm 1$  °C and 50-60% relative humidity, free to access to standard laboratory rodent diet and water during the experiments. All procedures involving animal study were approved by the Ethics Committee of School of Life Science of Shandong University. After being adapt to the environment for one week, the mice were randomly divided into test and control groups (10 for each).

#### Assay of immunity activity *in vivo*

The polysaccharide samples were dissolved in physiological saline, and given intraperitoneally to mice at doses of 10 mg/kg/d for 7 consecutive days. Control group was treated with 0.2 mL physiological saline instead of the polysaccharide solution. The mice were sacrificed via cervical vertebra dislocation 24 h after the last administration. The spleen was removed aseptically and then was placed in aseptic PBS buffer. Spleen cells were harvested by gently mincing and grinding the spleen fragment through sterilized meshes (200 meshes), and centrifuged at 3000 rpm/min at 4 °C for 5 min. After red blood cells were removed by erythrocyte lysis buffer, the remaining cells were washed twice and suspended to  $1 \times 10^6$  cells/mL by RPMI 1640 complete medium containing 10% fetal bovine serum. The Spleen cells (100  $\mu$ L/well) were placed in a 96-well plate with a total volume of 200  $\mu$ L per well, in the presence of mitogen (5.0  $\mu$ g/mL ConA, or 10.0  $\mu$ g/mL LPS, final concentration), or RPMI 1640 medium, and incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 48 h. Cells proliferation was determined by CCK-8 assay (Dojindo, Kumamoto, Japan).

The blood samples were obtained from eye-orbital sinus under light ether anaesthesia prior to being sacrificed. After centrifugation at 2000 $\times$ g for 10 min, the serum samples were collected. The IL-2 and TNF- $\alpha$  concentration were measured with Mouse IL-2 or TNF- $\alpha$  Sunny ELISA kit (MultiSciences, Hangzhou, China) according to the indication of the manufacturer.

#### Assay of immunity activity *in vitro*

8-12 weeks old SPF mice were sacrificed by cervical dislocation, spleens were collected under aseptic conditions in RPMI-1640, and spleen cells were prepared and adjusted to  $1 \times 10^6$  cells/mL. 100  $\mu$ L/well of splenocyte suspension were seeded into a 96-well culture plate, and mixed with 100  $\mu$ L polysaccharide solutions (62.5, 125, 250, 500  $\mu$ g/mL, final concentration, respectively) in triplicate. The RPMI 1640 medium was added as blank control, ConA (5.0  $\mu$ g/mL, final concentration), and LPS (10.0  $\mu$ g/mL, final concentration) was used as positive control, respectively. The plate was incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 48 h. The viable cells were determined at 450 nm. Cell proliferation rate (%) was calculated as the absorbance of sample-treated cells divided by the absorbance of control cells. Cell viability of the control group was 100%.

The resident macrophages of mice were harvested by peritoneal lavage, and the cells were subsequently cultured in RPMI 1640 complete medium and diluted to a density of  $2 \times 10^6$  cells/mL. The purity of macrophages was tested by adherence. Macrophage suspension (100  $\mu$ L/well) was pipetted into a 96-well culture plate and incubated for 3 h (37 °C, 5% CO<sub>2</sub>). The adherent macrophages were

washed twice by complete medium and then incubated with 100  $\mu$ L various concentrations (125, 250, 500, 1000  $\mu$ g/mL) of polysaccharides for 24 h. The stimulated cells were washed twice by PBS, and 100  $\mu$ L neutral red (0.1%, w/v) was used to assess the phagocytosis. The plate was incubated for 3 h. After the removal of un-phagocytized neutral red by PBS, 200  $\mu$ L cell lysate (the volume ratio of acetic acid to ethanol was 1:1) was added in and kept for 3 h. The OD value of each well was read at 540 nm using the Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). DMEM medium and LPS (10  $\mu$ g/mL) were used as the blank and positive control, respectively.

#### Dislocation euthanasia method

At the end of the experiments, the cervical vertebrae of all surviving animals were dislocated by external force, and the spinal cord was severed to make them die painlessly.

#### DPPH radicals scavenging assay

The radical scavenging effects of the polysaccharides were estimated by using DPPH free radicals method (Blois 1958). DPPH solution (50  $\mu$ L, 0.1 mM) in 50% ethanol was added in 96-well plate with equivalent aliquot sample solution at different final concentrations (0.5, 1, 1.5, 2, 2.5 mg/mL). The reaction solution was shaken vigorously and incubated at room temperature for 30 min, and the absorbance at 517 nm was measured. VC was used as a positive control. The DPPH scavenging rate ( $R$ ) was calculated as the following formula,  $R(\%) = [1 - (A_s - A_i) / A_0] \times 100$ . As indicated as absorbance of sample or VC. Sample reference solution, which contained equivalent 50 % ethanol instead of the DPPH solution, was recorded as  $A_i$ , while distilled water instead of sample was used for the blank  $A_0$ . All tests were performed in triplicate and the mean of Abs was used in the equation above.

#### Determination of DNA damage protective effect

DNA damage protection activities of polysaccharides were determined with pUC19 plasmid DNA, isolated from *Escherichia coli* DH5 $\alpha$  by SanPrep column plasmid mini-preps kit (Sangon Biotech, Shanghai, China). pUC19 plasmid was damaged by H<sub>2</sub>O<sub>2</sub> and UV treatment using the method of Yang (Yang et al. 2014). Rutin was used as a positive control. Different structural or conformational forms of plasmid DNA were separated by electrophoresis. The reaction mixture (10 mL) contained 3 mL of plasmid DNA, 5 mL of 5 mg/mL polysaccharide or 0.4 mg/mL rutin, 1 mL of 10 mmol/mL H<sub>2</sub>O<sub>2</sub> and 1 mL of water. The mixtures were located in super clean bench with ultraviolet lamp (20 W). After UV irradiation lasted for 5 min at room temperature, reaction samples along with 10 $\times$ gel loading dye were analyzed on a 1% agarose gel in TBE buffer at pH 8.0 for 30 min (100 V).

#### Statistical analysis

All bioassay results were expressed as means  $\pm$  standard deviation (SD). The experimental data were subjected to an analysis of variance (ANOVA) for a completely random design. A probability of  $P < 0.05$  and  $P < 0.01$  was considered as significant.

Add your materials and methods here.

## Results

### Isolation and purification

The crude polysaccharide was obtained by water extraction, ethanol precipitation, deproteinization and lyophilization. As shown in Fig. 1A showed that 50% ethanol portion (CKPS) with a yield of 4.86% was further fractionated on a DE-23 column eluting with de-ionized water, and different concentrations of stepwise NaCl solution (0.06, 0.1, 0.16 and 0.32 M). Guided by the phenol-sulfuric acid method, The NaCl elutes F1, F2, F3 and F4 were further purified by Sephadex G-150 column, respectively. Four resulting fractions named as CKPS-1, CKPS-2, CKPS-3 and CKPS-4 (Fig. 1B) were collected and treated for follow-up research.

### Physicochemical characterization

Positive response to the Bradford method, and the adsorption detected by UV spectrum at 280nm, indicated the presence of protein. The results proved that CKPS-1, CKPS-2, CKPS-3 and CKPS-4 all contained minor amounts of protein (0.45, 1.07, 1.53 and 4.34%, respectively) and uronic acid (0.51, 0.77, 0.86 and 1.22%, respectively), and did not have any sulfate ester. Uronic acid was found in all four fractions, which suggested that these fractions were starch-like polysaccharides. The total carbohydrate contents of the samples were 84.35, 77.33, 84.29, and 78.22%, respectively. Neutral monosaccharide constitutions of the polysaccharides were analyzed by reversed-phase HPLC. CKPS-1 was mainly composed of Fru, Man, Glu and Gal with molar ratios of 1:0.92:1.09:0.72. CKPS-2, CKPS-3 and CKPS-4 was consisted of Fru, Man and Gal in a molar percentage of 1:0.63:0.61, 1:1.65:1.4 and 1:2.06:1.97, respectively. Results showed that fructose, mannose and galactose were the main monosaccharide components in four samples with different molar ratios, and glucose was only found in CKPS-1, strongly indicating that the polysaccharides were heterogeneous. Large amounts of fructose components were found in both 50% ethanol and 90% ethanol precipitates (Zhang et al. 2015) of *C. kyushuensis*, which is quite different from the reports on polysaccharide of other Cordyceps species. HPSEC equipped with MALLS was considered to be a powerful, effective and reliable technique for determining molecular characteristics of macromolecules without any calibration standard. Single and symmetrical peaks indicated that the four fractions were homogeneous polysaccharides. The Mw of the purified polysaccharides was estimated to be 7153, 5945, 5643 and 5642 kDa, respectively. The IR spectra of four fractions exhibited the characteristic absorption of polysaccharides. All the fractions had similar infrared absorption bands indicating similarities in their structural features. The strong and broad peak between  $3600\text{ cm}^{-1}$  and  $3200\text{ cm}^{-1}$  was due to the stretching vibration of O-H. The bands at  $2924$  and  $2854\text{ cm}^{-1}$ , which corresponded to C-H stretching vibration in  $-\text{CH}_2$  and  $-\text{CH}_3$  groups (usually present in hexoses, like glucose or galactose, or deoxyhexoses like rhamnose or fucose), is further proved that what we are

dealing with is polysaccharide containing glucuronic acid (Leandro et al. 2003). The band at  $1645\text{ cm}^{-1}$  corresponds to the stretching vibration of the carbonyl bond that is a part of amide group and the band at  $1545\text{ cm}^{-1}$  is related to the N-H bending vibration of the same group. Occurrence of these two vibrations due to amide group indicates the presence of protein. The signal at  $1408\text{ cm}^{-1}$  could be assigned to stretch vibration of C-O within COOH (Kacurakova et al. 2000). The signal at  $1225\text{ cm}^{-1}$  accounted for asymmetric stretching vibration of sulfate group (Zhang et al. 2005). The absorptions in the range of  $1000\text{-}1200\text{ cm}^{-1}$ , attributed to the stretching vibrations of C-O-C and C-O-H, were observed. It indicated the strong absorptions at around  $1048\text{ cm}^{-1}$  due to stretching vibration of the pyranose ring. In addition, the absorption band at  $811\text{ cm}^{-1}$  and  $880\text{ cm}^{-1}$  indicated the presence of  $\alpha$ -mannopyranose and galactose units (Shingel 2002).

The anomeric protons from each monosaccharide can give recognizable signals depending on  $\alpha$ - or  $\beta$ -configurations. Most of  $\alpha$ -anomeric protons usually appear in the 5-6 ppm region in  $^1\text{H}$  NMR while most of the  $\beta$ -anomeric protons in the 4-5 ppm range (Cui 2006). The signals at 5.53 and 5.46 ppm of Fig. 2A were attributed to  $\alpha$ - configuration pyranose units of CKPS-1. The resonance at 4.91 ppm may be attributed to glucosyl residues (Seymour 1979), and  $^1\text{H}$  signals at 4.53 ppm conformed to the  $\beta$ - form of D-galactopyranosyl residues. The chemical shifts from 3.4 to 4.2 ppm were assigned to protons of C-2–C-6 of hexose glycosidic ring (Chauveau et al. 1996). Thus there were possibly both  $\alpha$ - or  $\beta$ -type glycosidic linkages in CKPS-1. In a  $^{13}\text{C}$  spectrum, the signals derived from  $\alpha$ -anomeric carbons usually appear in the 95-101 ppm region while most of the  $\beta$ - anomeric carbons will appear in the range 101-105 ppm (Cui 2006). The major resonance in the anomeric region occurs at 97-101 ppm rather than at 90 ppm of Fig. 2B, indicating that C-1 of  $\alpha$ -monosaccharide residue is linked (Uzochukwu et al. 2002). The signal at 172.79 ppm was due to carboxyl resonance signal of **uronic acid**, which was consistent with the IR results. As judged by the absence of signals within  $\delta$  82-88, all sugar residues were in the pyranose form. The NMR data of other three fractions were similar with those of CKPS-1 (result not shown). The detail structural features of the four polysaccharides should be further investigated by 2D NMR, periodate oxidation, and methylation analysis.

### **Assay of immunity activity *in vivo***

In this study, no deaths and other clinical strange were observed throughout the experimental period. Lymphocytes are the key effector cells of mammalian immune system. Proliferation of splenocytes is an indicator of immune activation, being related to immunity improvement of T-lymphocyte or B-lymphocyte (Zhao et al. 2014). Effects of CKPS-1, CKPS-2, CKPS-3 and CKPS-4 on splenocyte proliferation with or without mitogen (ConA or LPS) were shown in Fig. 3. Spleen lymphocyte proliferation induced by ConA *in vivo* has been used to evaluate T lymphocyte activity, while that induced by LPS has been used to examine B lymphocyte activity. The data of Fig.3 (A) and (B) proved that, with the administration of the four polysaccharides at the doses of 10 mg/kg, the splenocyte proliferation induced by ConA or LPS was

significantly enhanced ( $P < 0.01$ ), respectively. Fig. 3C demonstrated that four polysaccharides still stimulated lymphocyte proliferation even without mitogenic stimuli (ConA or LPS), the experiment results were markedly higher than the control medium group ( $P < 0.01$ ). The present data also indicated that the effect of CKPS-2 on the proliferation of mixed lymphocytes (Fig. 3C) and B lymphocytes induced by LPS (Fig. 3B) was greater than that of the other three components, while CKPS-4 had a stronger effect on the proliferation of T lymphocytes induced by ConA (Fig. 3A).

The IL-2 and TNF- $\alpha$  expression levels were measured to determine the stimulation properties of immune response of the purified polysaccharide fractions. The mice blood samples were taken from orbit at the 24 th hour after the last administration, the serum samples were collected and ready to determine IL-2 and TNF- $\alpha$  level by extrapolation from cytokine standard curve, according to the manufacturer's protocol. As shown in Fig. 4A, compared with the control group, the stimulating effects on secretion of TNF- $\alpha$  was strongly enhanced by all the four fractions ( $P < 0.01$ ). The IL-2 expression levels of Fig. 4B were found to be elevated by the polysaccharides CKPS-1, CKPS-2 and CKPS-3 ( $P < 0.05$ ). Additionally, CKPS-4 significantly promoted the secretion of IL-2 in serum ( $P < 0.01$ ). IL-2 is essential for the growth, proliferation, and differentiation of T cells, and is produced by T cells normally during an immune response (Malek 2008). TNF- $\alpha$  is a cytokine with tumor necrosis activity that is secreted mainly by macrophages and has been recognized as an important host regulatory molecule (Vilcek and Lee 1991). The experiment data demonstrated that the four polysaccharides could enhance the immune function by promoting cytokine expression levels for both T-lymphocytes and peritoneal macrophages *in vivo*.

### **Assay of immunity activity *in vitro***

By performing the CCK-8 assay, the effects of CKPS on normal (without mitogen) and mitogen-induced splenic lymphocyte proliferation were investigated in the final dose range of 62.5-500  $\mu\text{g}/\text{mL}$ . As shown in Fig. 5, both ConA and LPS could greatly stimulate lymphocyte proliferation compared with the blank. Compared with the mitogen control, CKPS-4 had excellent activities on normal proliferation ( $P < 0.01$ ). CKPS-1, CKPS-3 and CKPS-4 exhibited significant stimulation on normal proliferation at the final concentration of 62.5-500  $\mu\text{g}/\text{mL}$  ( $P < 0.01$ ), but the promotion on the proliferation of lymphocytes had not shown a dose-dependent suppressive effect. At the lowest concentration of 62.5  $\mu\text{g}/\text{mL}$ , the proliferation rate of CKPS-2 was significantly higher than that of ConA or LPS, however, in the range of 250-500  $\mu\text{g}/\text{mL}$ , the high concentration of CKPS-2 did not show stimulation effect on normal proliferation ( $P > 0.05$ ).

One of the most distinguished features of activated macrophages is an increase in phagocytosis. The CKPS fractions were evaluated with regard to the effect on the phagocytic activity of macrophages using a neutral red uptake assay. As seen in Fig. 6, each fraction had various enhancing effects on macrophage phagocytosis in the dose range of 62.5-500  $\mu\text{g}/\text{mL}$ . The phagocytic indexes of macrophages under the sample treatments all exceeded 1.0. Compared with the blank control, the fractions could considerably stimulate the phagocytosis of macrophages ( $P < 0.05$  or  $P < 0.01$ ) after administration, as well as LPS

action (10 µg/mL,  $P < 0.01$ ). Macrophages played an important role in immune system and could phagocytose aging cells, necrotic tissues, malignant cells and pathogens invading the body, and production of cytokines. The phagocytosis of macrophages was thought as one of the most important indicators of the body's non-specific immunity (Thambiraja et al. 2015; Schepetkin and Quinn 2006; Laskin 2009). Our present results proved that the benefit effect of the polysaccharides on immune and inflammatory diseases might be partly attributed to the improvement of defective or deficient phagocytosis of macrophage.

## Antioxidant properties

Natural antioxidants are known to play an important role against various diseases and aging processes. Polysaccharides were generally considered to have potential antioxidant activity. Thus it is essential to determine the antioxidant capacities of four fractions from the stroma of *C. kyushuensis*. DPPH radical methods were often conducted to evaluate the free radical scavenging ability of natural compounds (Amarowicz et al. 2004). As a stable free radical, DPPH showed the maximum absorption at 517 nm with violet color due to its odd electron. When DPPH encountered antioxidant scavengers, the resulting decolorization was stoichiometric with respect to the ability to bleach the DPPH radical. The scavenging effect was measured and shown in Fig.7A. All the polysaccharide fractions showed good scavenging effect against DPPH radical in a dose-dependent manner at each concentration level. The scavenging ratio at the highest concentration of CKPS-3 and CKPS-4 were 63.5 % and 59.7 %, respectively. And compared with other samples, especially CKPS-4 had much stronger antioxidant activity even at the low concentration of 1 mg/mL.

The protective effects of the polysaccharides on the damage induced by co-action of  $H_2O_2$  and UV were studied on pUC 19 plasmid. Figure 7 (B) demonstrated that the electrophoretic pattern of DNA after UV photolysis of  $H_2O_2$  (2.5 mmol/L) in the absence or presence of CKPS-1 to CKPS-4, and rutin. DNA derived from pUC19 plasmid showed the band corresponding to the native form of supercoiled circular DNA (Sc DNA) on agarose gel (Lane 7). After the UV irradiation of DNA with  $H_2O_2$ , the graph of Lane 6 proved the result of the cleavage of Sc DNA to open circular form (Oc DNA) (Kumar et al. 2004). With the addition of rutin and CKPS-1 to CKPS-4, Lanes 1-5 revealed the protection effect of the polysaccharides to the damage of native Sc DNA. Lane 2 and Lane 3 of the gel showed clearly Sc DNA band, which indicated CKPS-1 and CKPS-2 had a relatively stronger capacity to suppress the formation of Oc DNA than other polysaccharides (Lane 4 and Lane 5). The positive control of rutin (Lane 1) had almost the same protective effect. Cordyceps-derived polysaccharides of CKPS-1 and CKPS-2 could be proved to reduce the damage of  $\cdot OH$  generated by UV-photolysis of  $H_2O_2$  produced DNA strand scission.

## Discussion

It has been reported that the immune response to fungal polysaccharide mixture may differ from that observed with purified ones (Snarr et al. 2017), and as we know, the presence of other compounds, such as proteins, polyphenols and lipids, can affect the biological activity of the fungal components. Purification and structural characterization of fungal polysaccharides is thus very important for their further application as selective and effective immune modulators (Baeva et al. 2019). The present study was undertaken to elucidate the antioxidant and immune stimulatory activities of the polysaccharides from the stoma of *C. kyushuensis*. Four water soluble homogeneous polysaccharide fractions were isolated at final ethanol concentration of 50% and purified by column chromatography. Preliminary structural characterizations were conducted, and DPPH scavenging activity and protection to DNA damage *in vitro* were carried out to evaluate the antioxidant potential of these fractions. The four polysaccharides could significantly enhance the splenocyte proliferation with or without mitogen (ConA or LPS) *in vivo* and *in vitro*. The effects on the production of cytokines IL-2 and TNF- $\alpha$  were investigated. The results showed that the levels of serum IL-2 and TNF- $\alpha$  was increased significantly by the fractions administration compared with those of control group, suggesting the physiological effect of the polysaccharides were implemented by increasing the immune response. Moreover, the tests of macrophage phagocytosis offered demonstrative evidence that these polysaccharides could effectively activate macrophages response. The results indicated that the polysaccharides of *C. kyushuensis* could be applied to the potential health and functional food source. This may provide new strategies for the discovery of effective and safe approaches for cancer treatment from natural resources.

## Abbreviations

**CKPS**, *Cordyceps kyushuensis* polysaccharide; **ConA**, concanavalin A; **DPPH**, 1,1-diphenyl-2-picrylhydrazyl; **HPSEC**, high performance size exclusion chromatography; **IL-2**, interleukin-2; **LPS**, lipopolysaccharide; **MALLS**, multi-angle laser light scattering; **PMP**, 1-phenyl-3-methyl-5-pyrazolone; **SPF**, specific pathogen free; **TFA**, trifluoroacetic acid; **TNF- $\alpha$** , tumor necrosis factor-alpha; **VC**, vitamin C; **Mw**, weight-average molecular weight;

## Declarations

### Acknowledgements

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### Author's contributions

JL supervised, conceived and designed the experiments. JS and GZ conducted experiments.

All authors analyzed data. JL and GZ wrote the manuscript. All authors read and approved the manuscript.

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## **Availability of data and materials**

The authors declare that all data supporting the findings of this study are available from the corresponding authors upon request.

## **Ethics approval and consent to participate**

All experimental procedures involving animal study were approved by the Ethics Committee of School of Life Science of Shandong University.

## **Consent for publication**

All authors read the manuscript and expressed their consent for publication.

## **Consent to participate**

Not applicable.

## **Competing interests**

The authors declare that have no competing interests.

## **References**

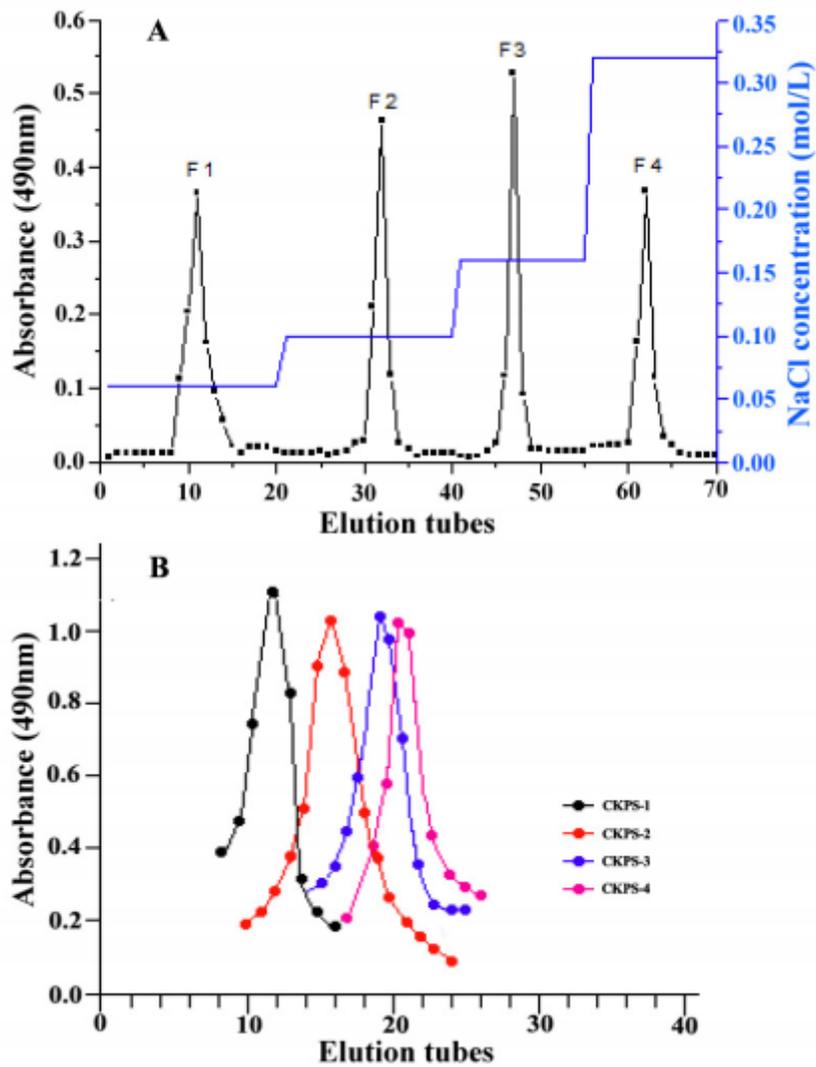
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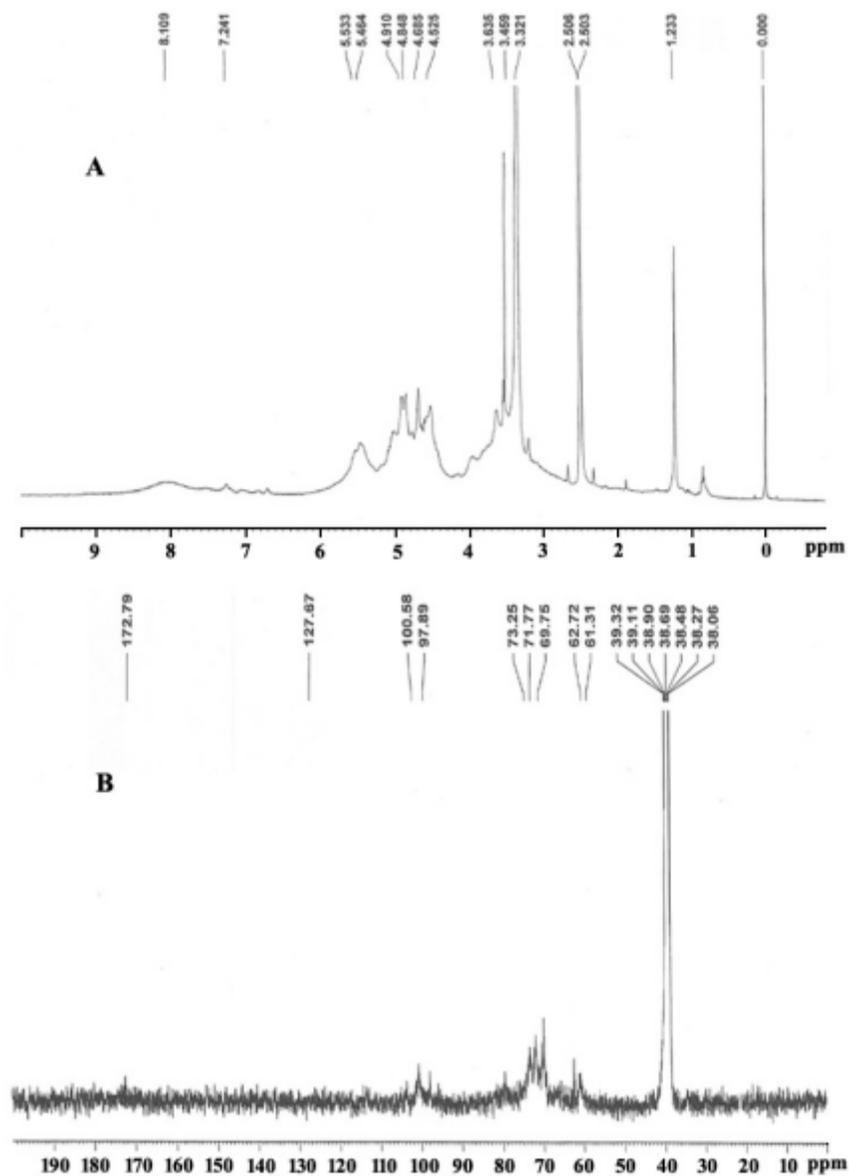
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## Figures



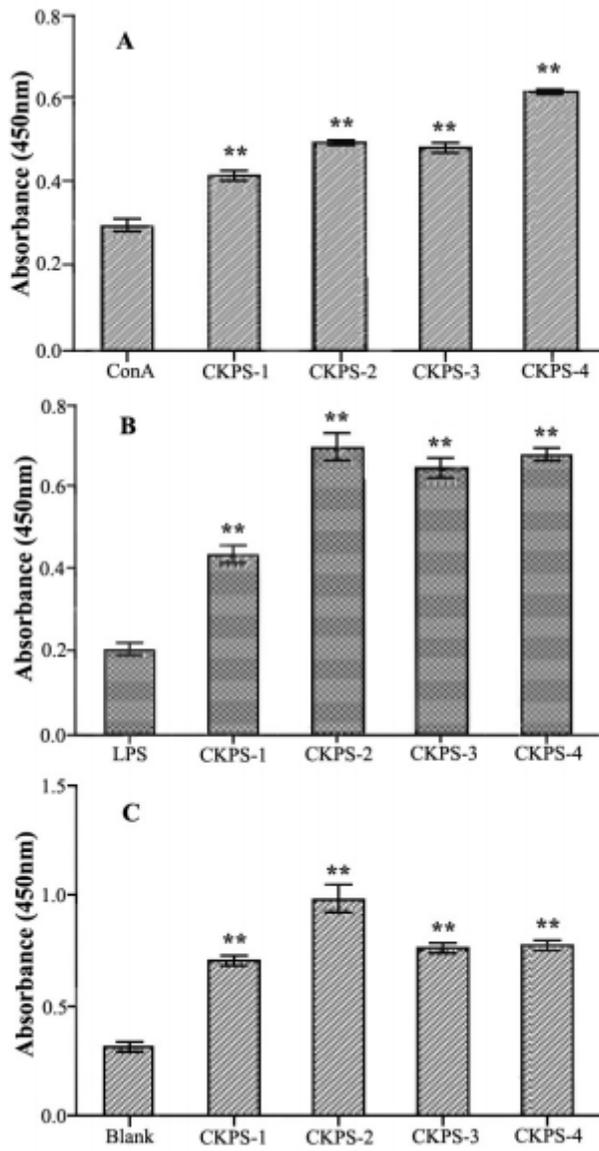
**Figure 1**

Separation and purification of polysaccharides of *Cordyceps kyushuensis*. (A) DE-23 chromatographic profile for CKPS eluted with different NaCl solution. (B) Sephadex G-150 chromatographic profile for CKPS eluted with water.



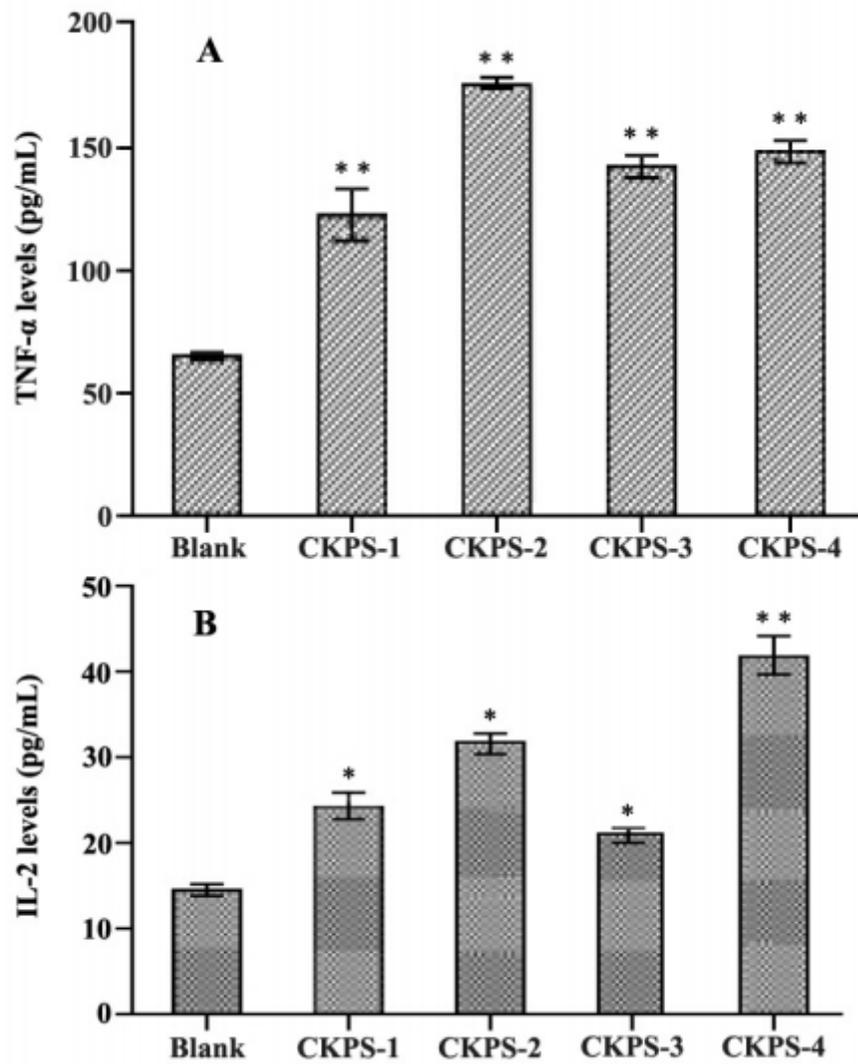
**Figure 2**

$^1\text{H}$  NMR spectra (A) and  $^{13}\text{C}$  NMR spectra (B) of the purified CKPS-1 of *Cordyceps kyushuensis*.



**Figure 3**

Splenocyte proliferation of polysaccharide fractions with mitogens (A) ConA, (B) LPS, or without (C) in vivo. Values are means $\pm$ S.D. (n=3). \*\*p<0.01 vs. control.



**Figure 4**

Effects of polysaccharide fractions on levels of serum TNF- $\alpha$  (A) and IL-2 (B) in mice. Values are mean  $\pm$  S. D. (n=3). \*p<0.05, \*\*p<0.01 vs. control.

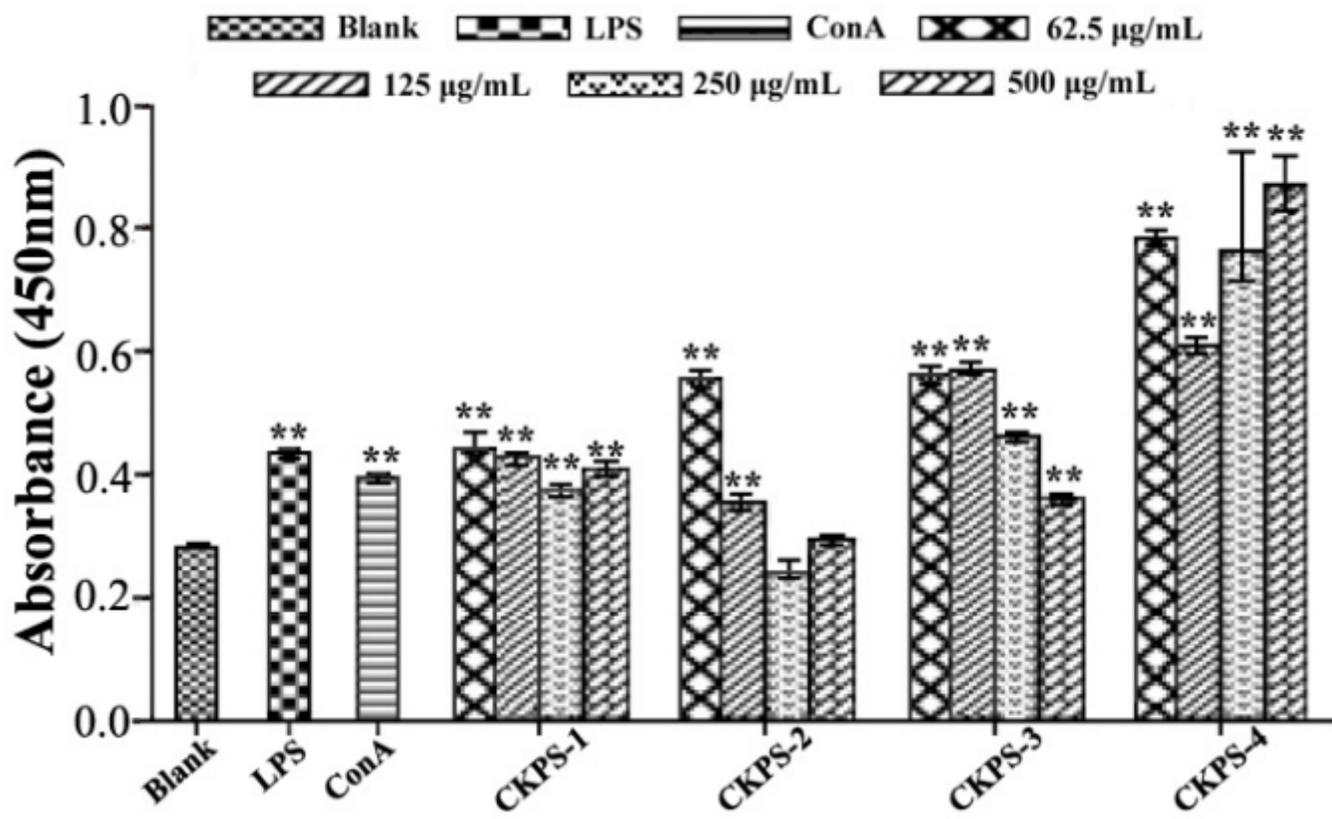


Figure 5

Splenocyte proliferation of polysaccharide fractions in vitro. Values are means±S.D. (n=3). \*\*p<0.01 vs. control.

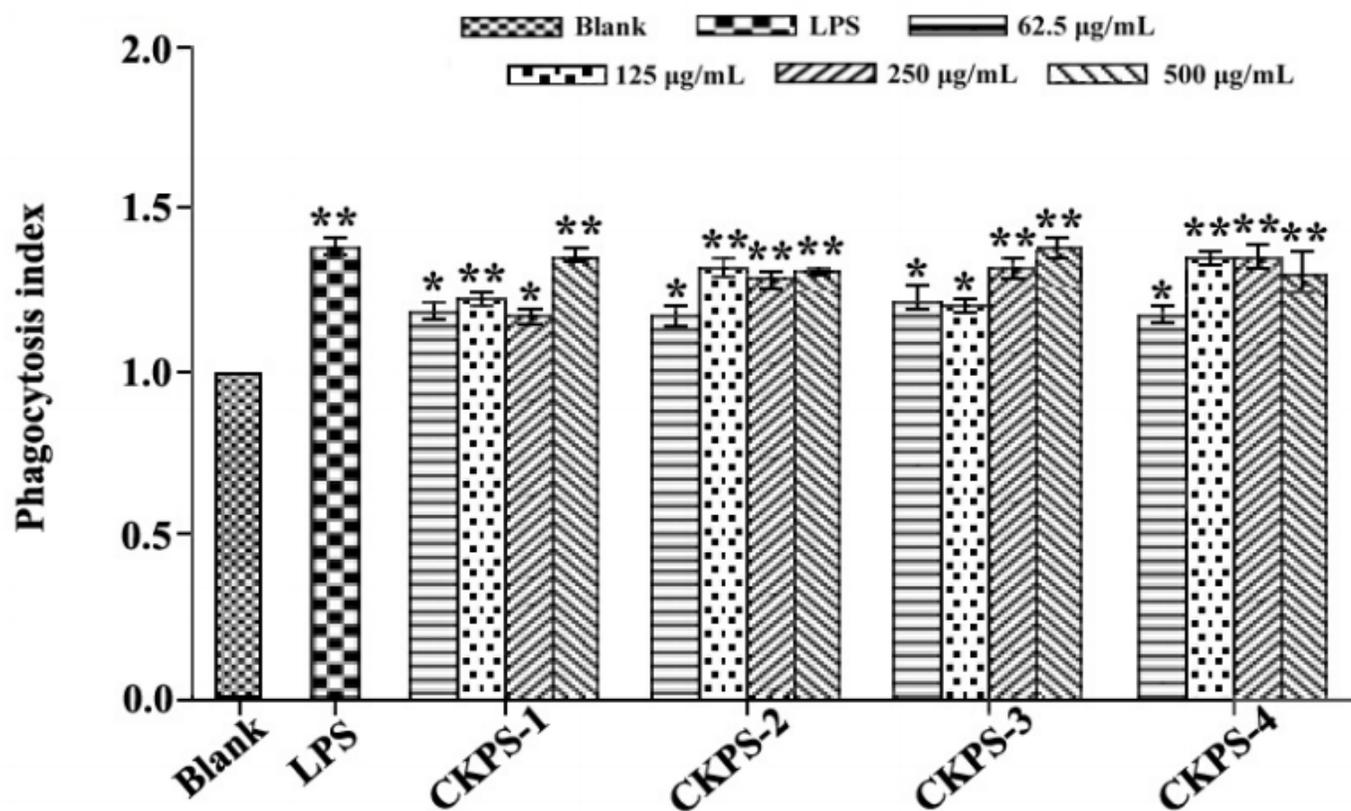
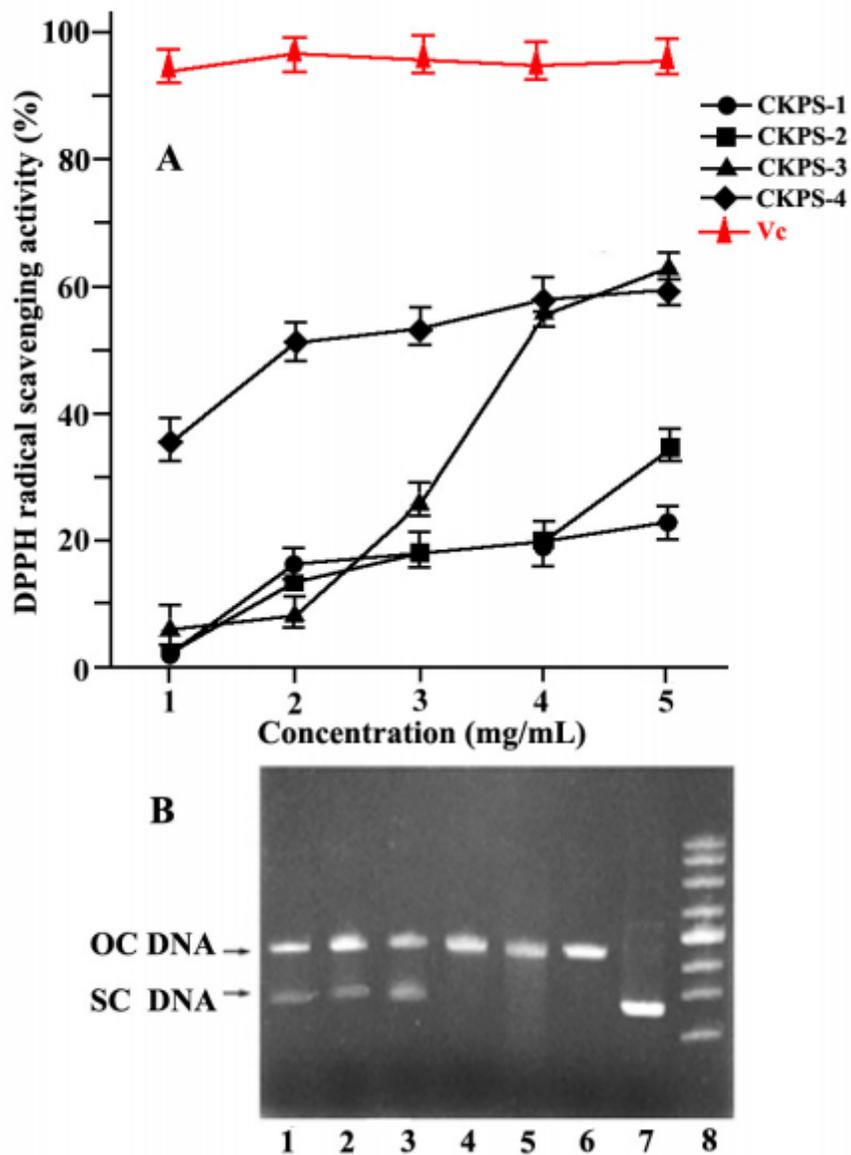


Figure 6

Macrophage phagocytosis of polysaccharide fractions and LPS by neutral red uptake assay. Values are means  $\pm$  S. D. (n=3). \*p<0.05, \*\*p<0.01 vs. control.



**Figure 7**

Evaluation of antioxidant activity of polysaccharides extracted from *Cordyceps kyushuensis*. (A) Scavenging activity on DPPH radical of polysaccharide fractions from *Cordyceps kyushuensis*. Values are means  $\pm$  S.D. (n=3). (B) Effects of polysaccharide fractions on protection of supercoiled DNA (plasmid pUC19). Lanes 1-5, H<sub>2</sub>O<sub>2</sub>+UV treated with rutin, CKPS 1-4, respectively; Lane 6, H<sub>2</sub>O<sub>2</sub>+UV treated without polysaccharide; Lane 7, untreated DNA (control); Lane 8, Supercoiled DNA marker.