Original Research Paper

A Polysaccharide-Rich Fraction Derived from *Oviductus ranae* with Antioxidant and Immunomodulatory Potentials

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Abstract: *Oviductus ranae*, an animal-based traditional Chinese medicinal material listed in Chinese Pharmacopeia is widely consumed as tonic, approximately twenty *Oviductus ranae*-related functional foods have been approved on the market by National Medical Products Administration in China. Proteins are well-defined bioconstituents in *Oviductus ranae*, little information is available on its polysaccharides. In present work, a Polysaccharide-Rich fraction (POR-3) was fractionated from *Oviductus ranae* based on antioxidant activity-guided isolation and its structure was partially characterized as well as its immunomodulatory potential was explored. POR-3 belonged to a heteropolysaccharide-protein fraction mainly consisted of Man, GlcN, GalA, Gal and Fuc at a molar ratio of 1: 2.28: 2.40: 3.00: 2.23 with binding proteins content of 6.42±0.16%. POR-3 possessed polydisperse property with MW of 999.586 kDa and PDI of 4.09. The UV and FT-IR analysis demonstrated that structures of POR-3 correspond with the characteristics of polysaccharide-protein complex. POR-3 elicited higher scavenging capacities against hydroxyl and DPPH radicals and exerted potent *in vitro* immunomodulatory activity via promoting macrophages phagocytosis and via stimulating NO production as well as appeared no proliferation on macrophages. POR-3 possesses high potentials to be consumed as a medicine or functional food with antioxidant and immune modulator properties.

Keywords: *Oviductus ranae*, Polysaccharide-Rich Fraction, Antioxidant Activity-Guided Isolation, Immunomodulatory Activity

Introduction

Reactive Oxygen Species (ROS) play a dual role on immune system. Low ROS production induced by the activation of phagocyte NADPH oxidase 2 complex is considered to regulate immune response and cell proliferation and to control T-cell auto-reactivity (Lam *et al.*, 2010). Nevertheless, excessive accumulation of ROS caused by harmful environmental stresses such as overconsumption of smoking and alcohol, long-term exposure to organic pollutants and heavy metals as well as X-ray irradiation could induce oxidative stress, thereby impairing the activated T cell and destroying the membrane structure of immune cell to cause immune disorder (Amir Aslani and Ghobadi, 2016; Hildeman *et al.*, 2003; Srivastava *et al.*, 2017).

Although endogenous antioxidants, including glutathione, superoxide dismutase, glutathione peroxidase and catalases usually exert certain scavenging capacities against ROS to decrease the damaging effects of oxidative stress (Ighodaro and Akinloye, 2018). However, levels of these endogenous antioxidants are always lower than the minimum concentrations demanded for scavenging ROS (Szuroczki *et al.*, 2016). Therefore, it is of importance to supplement antioxidants exogenously, owing to their beneficial effects on oxidative stress-related disorders, particularly for immune dysfunction.

Natural polysaccharides belong to bio-macromolecules and their antioxidant potentials have been widely recognized (Huang *et al.*, 2017; Wang *et al.*, 2013). Recent studies have revealed that some of antioxidant polysaccharides usually elicit pronounced immunomodulatory properties, such as the polysaccharides from *Gomphidius rutilus* (Gao *et al.*, 2013), *Chenopodium quinoa* (Hu *et al.*, 2017), *Ganoderma lucidum* (Chen *et al.*, 2009) and *Punica granatum* (Joseph *et al.*, 2012). It is therefore feasible and

meaningful to explore the immunomodulatory potentials of the polysaccharides with higher antioxidant capacities.

Rana dybowskii, one of the Chinese brown frogs is a famous economic animal widely farmed in Northeastern China, whose economic value mainly depends on its dried oviduct that is also called *Oviductus ranae* (OR), a traditional Chinese medicinal material listed in Chinese Pharmacopeia. Traditionally, OR was prescribed for the remedies of several ailments, including neurasthenia, insomnia, debilitation, climacteric syndrome and night sweat. OR differs from other traditional Chinese medicinal materials included in Chinese Pharmacopoeia, it is not considered as therapeutic agent but is usually consumed as active constituents for functional foods and nutraceuticals. To this day, approximately twenty OR-related functional foods have been approved on the market by National Medical Products Administration (Zhang *et al*., 2019). OR comprises several bioactive components, including proteins, steroids, fatty acids and polysaccharides. Recent studies have found that OR shows immune enhancement, anti-fatigue, anti-osteoporosis, anti-oxidation, anti-apoptosis, reproductive protection and estrogen-like effects. The proteins and steroids of OR have been well studied (Wang *et al*., 2010; Zhang *et al*., 2018). However, little information is available on the Polysaccharides from OR (POR).

In present work, an antioxidant polysaccharide fraction from OR was isolated from the purified POR based on antioxidant activity-guided fractionation, then the partial characterization as well as immunomodulatory potential were further explored. To the best of our knowledge, it is the first time to investigate POR, which is more likely to enrich the application of natural polysaccharides and to provide evidence and reference for the development and comprehensive utilization of OR as novel drugs and functional foods.

Materials and Methods

Materials

Freeze-dried OR was purchased from Jilin Huangzhihua Pharmaceutical Co., Ltd (Changchun, Jilin, China). It was authenticated by Prof. Dr. Limei Wang, School of Biology and Food Engineering, Changshu Institute of Technology (Changshu, Jiangsu, China). A specimen with voucher No. KLFB-2019-0016 was deposited at Suzhou Key Laboratory of Food Biotechnology, Changshu Institute of Technology (Changshu, Jiangsu, China).

Major reagents used for the in vitro antioxidant evaluation, including 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH), phenanthroline, ferrous sulfate (FeSO$_4$) and Vitamin C (VC) were obtained from Macklin Biochemical Co., Ltd. (Shanghai, China).

Standard sugars applied for monosaccharide composition analysis were from Sino-pharm Chemical Reagent Co., Ltd. (Shanghai, China), including *L*-Rhamnose (Rha), *L*-Fucose (Fuc), *D*-Glucose (GLu), *D*-fructose (Fru), *D*-Galactose (Gal), *D*-Mannose (Man), *D*-Arabinose (Ara), *D*-Galacturonic acid (GalA), *D*-Glucosamine (GlcN) and *D*-Xylose (Xyl).

T-series dextran standards used for molecular weight determination, including T-500, T-100, T-70, T-40 and T-10 were provided by National Institutes for Food and Drug Control (Beijing, China).

Major reagents applied for immunomodulatory activity were purchased from Sigma Aldrich (St. Louis, MO, USA), including Fetal Bovine Serum (FBS), Lipopolysaccharide (LPS, from *Escherichia coli* O111:B4), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Dimethylsulfoxide (DMSO), Phosphate Buffered Saline (PBS), Roswell Park Memorial Institute (RPMI)-1640 medium, neutral red, penicillin and streptomycin. Assay kit used for the determination of Nitric Oxide (NO) was from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Other materials, such as 1-Phenyl-3-Methyl-5-Pyrazolone (PMP), Trifluoroacetic Acid (TFA), diethylaminoethyl (DEAE)-52 cellulose, papain, ethanol and sodium chloride (NaCl) were obtained from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China).

Cell Culture

Mouse leukaemic monocyte macrophage cell line RAW 264.7 (ATCC TIB-71) was cultured in RPMI 1640 medium containing 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin at 37°C in a humidified incubator with 5% CO$_2$.

Extraction and Purification of Polysaccharides

Freeze-dried OR was grinded into powders and sieved to 60 mesh, which were then soaked in petroleum ether to degrease at room temperature for 12 h. According to the work reported by (Qin *et al*., 2018) with some modifications, ten grams of degreased OR powders were soaked in distilled water at liquid-to-solid ratio of 100: 1 for 12 h, homogenated, adjusted to pH 6.8 and incubated with papain in a ratio of 0.2% (w/w) at 37°C in shaking water bath at 50 cycles/min for 2 h. After extraction, the mixture was placed into boiling water for 10 min to inactivate papain, centrifugated at 10000 rpm for 20 min to obtain the supernatant, which was neutralized with 1 M NaOH, concentrated to a quarter of its volume and mixed with 3-fold 95% (v/v) ethanol at 4°C for 12 h. The precipitant was collected by centrifugation at 4000 rpm for 10 min and freeze-dried to obtain crude polysaccharides, which were then subjected to a deproteinization by the Sevag method (Huang *et al*., 2010) for three times. After removal of Sevag reagent, the

solution was dialyzed for three times, which was freeze-dried to yield purified polysaccharides. Polysaccharide content was determined by the phenol-sulfuric acid method with glucose as standard (Dubois *et al*., 1956). The standard curve was as follows:

$$A = 7.646C + 0.4064 \left(R^2 = 0.9995 \right) \qquad (1)$$

Where:
A = Absorbance
C = Polysaccharide content (mg/mL) linear range was from 0.02 to 0.12 mg/mL

Fractionation of Polysaccharides

Purified polysaccharides were dissolved in distilled water and loaded onto a DEAE-52 cellulose column (2.6×60 cm), followed by a successive elution with distilled water and 0.1 ~ 0.5 mol/L NaCl solution at a flow rate of 1 mL/min, respectively. The obtained eluates (5 mL/tube) were collected and monitored by the phenol-sulfuric acid method. The elution fractions with relatively higher amounts were collected, dialyzed and lyophilized (Meng *et al*., 2018b).

Chemical Analysis of POR-2 and POR-3

The total carbohydrates, uronic acids and proteins of POR-2 and POR-3 were respectively measured by the phenol-sulfuric acid method (Dubois *et al*., 1956), by the *m*-hydroxy biphenyl method (Blumenkrantz and Gustav, 1973) and by the coomassie bright blue method (Bradford, 1976).

Comparison of the In Vitro Antioxidant Activities

The two fractions with relatively higher amounts (POR-2 and POR-3) were screened by comparison of the in vitro antioxidant activities to obtain the fraction with highest antioxidant activity.

Hydroxyl Radical-Scavenging Activity

POR-2 and POR-3 were respectively dissolved in distilled water to prepare solutions at different concentrations (0.2 ~ 1.0 mg/mL). Two milliliters of sample solution and 1 mL of PBS solution (pH 7.4) containing 0.75 mmol/L phenanthroline were mixed, then 1 mL of $FeSO_4$ solution (0.75 mmol/L) and and 1 mL of H_2O_2 solution (0.12%, v/v) were added. After incubation at 37°C for 60 min, the absorbance at 536 nm (A_s) was determined. The mixtures without H_2O_2 and tested sample were served as normal control (Ac) and blank control (A_0), respectively. VC was used as positive control. The hydroxyl radical-scavenging rate was calculated according to the following equation (Zhang *et al*., 2018):

$$\begin{aligned} &Hydroxyl\ radical-scavenging\ rate\ (\%) \\ &= \left(A_s - A_0\right) \times 100 / \left(A_c - A_0\right) \end{aligned} \qquad (2)$$

DPPH Radical-Scavenging Activity

POR-2 and POR-3 were respectively dissolved in distilled water to prepare solutions at different concentrations (0.2 ~ 1.0 mg/mL). Two milliliters of sample solution and 2 mL of ethanol solution containing 0.1 mmol/L DPPH were mixed, followed by a reaction in the dark for 30 min at room temperature, the absorbance at 517 nm (A_s) was then measured. The mixtures in absence of DPPH and tested sample were used as normal control (A_c) and blank control (A_0), respectively. VC was served as positive control. The DPPH radical-scavenging rate was obtained based on the following equation (Zhang *et al*., 2018):

$$\begin{aligned} &DPPH\ radical-scavenging\ rate\ (\%) \\ &= \left(A_s - A_c\right) \times 100 / A_0 \end{aligned} \qquad (3)$$

Partial Characterization of POR-3

Molecular Weight

Twenty microliters of 2 mg/mL POR-3 solution was filtered by using a 0.45 μm-pore Millipore filter, then the filtrate was injected into a Shodex SUGAR KS-804 column (8.0×300 mm) and analyzed by a High-Performance Liquid Chromatography (HPLC) (Elite P230IIHPLC, Elite Analytical Instruments Co. Ltd., Dalian, China) equipped with a Refractive Index Detector (RID) (RI2000, A, Schambeck SFD GmbH, Germany). Data was recorded and processed by using a N2000 GPC chromatographic work station. A series of dextran standards with different molecular weights were used to establish calibration curve. The analytical conditions were: Ultrapure water was served as mobile phase at a flow rate of 1.0 mL/min; the temperatures of column and RID were set as 50 and 35°C, respectively and the run rate was set as 30 min (Hu *et al*., 2019).

Monosaccharide Compositions

Two milligrams of POR-3 was hydrolyzed by 2 mL of 2 M TFA at 110°C for 5 h to obtain monosaccharides. After removal of the residual TFA, the hydrolysate was reacted with 200 μL of 0.5 M PMP (dissolved in methanol) and 200 μL of 0.3 M NaOH at 70°C for 1 h. The reaction mixture was neutralized by 0.3 M HCl and extracted with chloroform for three times to obtain the solution containing PMP-labeled monosaccharides, which was then passed through a 0.45 μm-pore Millipore filter and injected into the Supersil ODS2 column (5 μm, 4.6×250 mm) of an Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA)

equipped with a diode array detector (DAD). The analytical conditions were: the mobile phase consisted of PBS (pH 6.8) and acetonitrile (82:18, v/v) at a flow rate of 0.8 mL/min; column temperature-30°C; detector wavelength-245 nm; run rate-85 min. Different commercial monosaccharides were served as standards (Hu *et al*., 2019).

Ultra Violet (UV) and Fourier Transform-Infrared (FT-IR) Analysis

UV spectrum of POR-3 was determined by using a 2700 UV-vis spectrophotometer (Shimadzu, Kyoto, Japan) at 200 ~ 400 nm. POR-3 was ground with dried KBr and pressed into pellets, then FT-IR spectrum of POR-3 was recorded on a FTIR-650 Fourier transform-infrared spectrophotometer (Gangdong Sci. and Tech. Development Co., Ltd, Tianjin, China) at 4000 ~ 400 cm^{-1} (Wei *et al*., 2019).

The In Vitro Immunomodulatory Activity of POR-3

Macrophages Proliferation Assay

Effects of POR-3 on the proliferation of RAW264.7 cells were estimated by MTT method with some modifications (Hu *et al*., 2019). Accordingly, RAW264.7 cells were seeded in 96-well plates containing RPMI-1640 medium and 10% FBS. One hundred microliters of POR-3 solution ranging from 25 to 800 μg/mL were added and incubated at 37°C with 5% CO_2 for 24 h. The complete medium without tested sample and the LPS at concentration of 25 μg/mL were served as negative and positive controls, respectively. After incubation, 10 μL of MTT solution at concentration of 5 mg/mL were added to each well, followed by another incubation at 37°C with 5% CO_2 for 4 h. Then supernatant was discarded and 100 μL of DMSO were added to each well to dissolve formazan crystals. The absorbance at 490 nm was recorded by a microplate reader (Epoch2, BioTek, America).

Macrophages Phagocytosis Assay

Effects of POR-3 on the macrophages phagocytosis were evaluated by the neutral red uptake method with minor modifications (Hu *et al*., 2019). In brief, RAW264.7 cells were seeded in 96-well plates and exposed to different concentrations of POR-3 solution or complete medium alone (negative control) or LPS solution (positive control) and incubated for 24 h. After that, the supernatant was transferred into another 96-well plate for the determination of NO content (see section of NO assay) and non-adherent cells were removed by washing twice with PBS, followed by adding 100 μL of neutral red solution to each well and by being incubated for 1 h. After removal of the supernatant and excessive neutral red solution, cells were washed twice with PBS. Then, 100 μL of cell lysate (1.0 mol/L acetic acid: Ethanol = 1: 1) were added and kept overnight at room temperature and the absorbance at 490 nm was determined.

NO assay

Effects of POR-3 on NO release were appraised according to the method described in NO assay kit (Hu *et al*., 2019). In short, the supernatant was mixed with equivalent amounts of Griess reagent I (1% sulfanilamide) and Griess reagent II (0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid) at room temperature for 10 min. The absorbance at 540 nm was recorded and nitrite concentration was calculated by reference to sodium nitrite standards.

Statistical Analysis

Data was expressed as means ± SD (standard deviation). Statistical analysis was conducted with t-test or one-way Analysis Of Variance (ANOVA) by using SPSS 19.0 software (SPSS Inc., Chicago, USA). $P <$ 0.05 was deemed as statistically significant.

Results and Discussion

Extraction and Purification of Polysaccharides

Extraction yield of crude POR was 5.09±0.21% and polysaccharide content was found to be 23.08±1.56%. After deproteinization and dialysis, polysaccharide content in purified POR was upgraded to 55.42±1.21%.

Fractionation and Chemical Analysis of Polysaccharide Fractions

Purified POR was fractionated by a DEAE-52 cellulose column chromatography and the elution curve was presented in Fig. 1. Three fractions, namely POR-1, POR-2 and POR-3 were fractionated with 0.1 to 0.3 M NaCl solution. The two major fractions, accordingly POR-2 and POR-3 were collected, respectively. After dialysis and lyophilization, their chemical constituents were roughly determined, which were summarized in Table 1. It can be seen that contents of total carbohydrates ($P < 0.05$) and uronic acids ($P < 0.01$) in POR-3 were significantly higher than those in POR-2, while content of proteins in POR-3 was remarkably lower ($P < 0.05$) than that in POR-2. It has been reported that uronic acids enable polysaccharides negatively charged (Sun *et al*., 2018) and POR-3 contained more uronic acids than that of POR-2 (Table 1), which may be the reason that fractionation of POR-3 required relatively higher concentration of NaCl solution as eluent.

Values were expressed as means ± SD for three experiments. Different superscript letters in the same column represented statistically significant differences ($P < 0.05$, or $P < 0.01$).

Table 1: General chemical constituents in the major polysaccharide fractions of purified POR

Fraction	Total carbohydrates/%	Uronic acids/%	Proteins/%
POR-2	75.05±1.24[a]	4.91±0.28[a]	8.39±0.29[a]
POR-3	80.87±1.17[b]	10.19±0.43[b]	6.42±0.16[b]
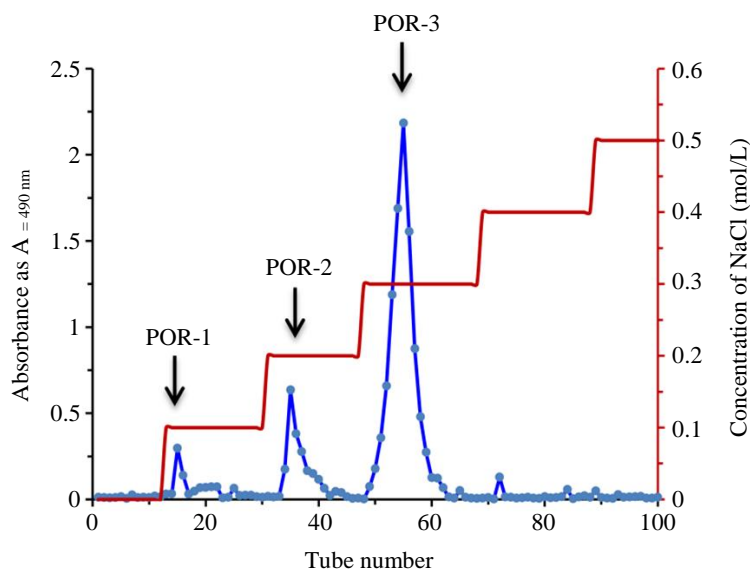
Fig. 1: The elution curve of purified POR on DEAE column chromatography
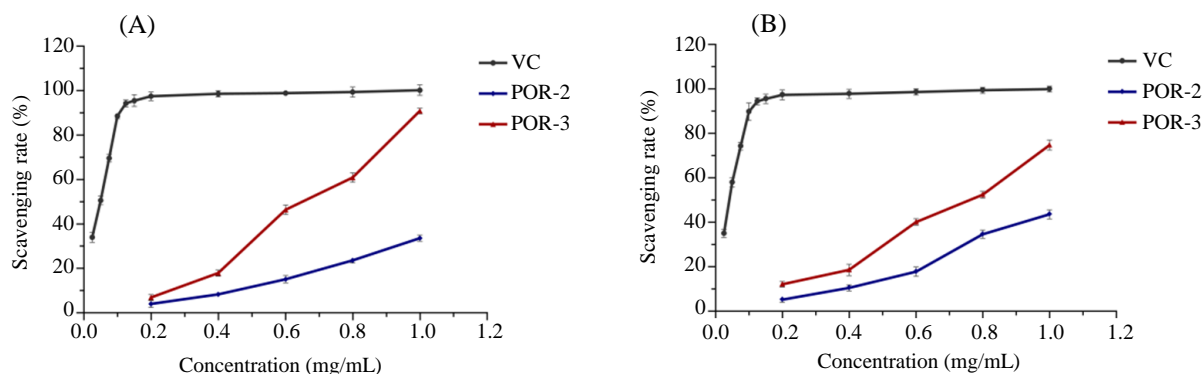
Fig. 2: The comparison of in vitro antioxidant capacities of POR-2 and POR-3 using VC as positive control. (A) Hydroxyl radical-scavenging capacity; (B) DPPH radical-scavenging capacity

Comparison of the In Vitro Antioxidant Activities

The *in vitro* scavenging capacities of POR-2 and POR-3 against hydroxyl and DPPH radicals were appraised and compared to define the polysaccharide fraction with relatively higher antioxidant activity. As shown in Fig. 2A, the hydroxyl radical-scavenging activities of POR-2 and POR-3 were elevated with the increase of sample concentration ranging from 0.2 ~ 1.0 mg/mL, the highest scavenging rates of POR-2 and POR-3 against hydroxyl radical were 33.65±1.39% and 90.94±1.25%, respectively. The IC_{50} (half-inhibitory concentration) value of POR-3 against hydroxyl radical

was 0.65±0.008 mg/mL, significantly lower ($P < 0.01$) than that of POR-2 (1.49±0.05 mg/mL), but higher ($P < 0.01$) than that of VC (0.045±0.0008 mg/mL).

As shown in Fig. 2B, in the range of 0.2 ~ 1.0 mg/mL, POR-2 and POR-3 also elicited scavenging activities against DPPH radical, which were raised with the increase of sample concentration, the highest scavenging rates of POR-2 and POR-3 against DPPH radical reached 43.63±2.04% and 74.78±2.22%, respectively. The IC_{50} value of POR-3 against DPPH radical was 0.73±0.01 mg/mL, still markedly lower ($P < 0.01$) than that of POR-2 (1.15±0.03 mg/mL), but higher ($P < 0.01$) than that of VC (0.037±0.004 mg/mL).

These results indicated that POR-3 outperforms POR-2 on scavenging activities against hydroxyl and DPPH radicals, which might be contributed partially by the relatively higher content of uronic acids in POR-3 (Table 1), due to the fact that uronic acids are well acknowledged as an important role in antioxidant activity (He *et al.*, 2016).

Partial characterization of POR-3

Considering that POR-3 exhibits a stronger *in vitro* antioxidant activity deserving of deep study, POR-3 was therefore selected for further investigations.

POR-3 was a heteropolysaccharide fraction that mainly consisted of Man, GlcN, GalA, Gal and Fuc at a molar ratio of 1: 2.28: 2.40: 3.00: 2.23. The weight-average molecular weight (MW) was 999.586 kDa and number-average Molecular weight (MN) was 244.366 kDa. Polydispersity Index (PDI) calculated by MW/MN was found to be 4.09, indicating that POR-3 belongs to polydisperse polysaccharide fraction (Castro *et al.*, 2016).

UV spectrum of POR-3 was presented in Fig. 3, from it, a noticeable absorption peak can be observed at around 280 nm, suggesting the existence of residual free proteins or conjugated proteins in POR-3 (Meng *et al.*, 2018a), which was consistent with the findings in Table 1, where both POR-2 and POR-3 were observed to contain certain amounts of proteins, further corroborating the suspicion of conjugated proteins, because free proteins can be basically removed by the Sevag reagent (Gu *et al.*, 2020).

FT-IR spectrum of POR-3 was showed in Fig. 4, to be specific, broad peak at 3417 cm^{-1} was assigned to the stretching vibration of -OH, the asymmetric and symmetric stretching vibrations of -CH$_2$- were registered at 2925 and 2854 cm^{-1}, respectively. The strong absorption peaks at 1646 and 1548 cm^{-1}, representing the stretching vibrations of carbonyl group, amides I and II, were from uronic acids and bound proteins in POR-3 (Liu *et al.*, 2013; 2018), which were in accordance with the results reported by (Gu *et al.*, 2020). Three absorption peaks in the range of 1200 ~ 1000 cm^{-1}, namely 1121, 1078 and 1036 cm^{-1} were assigned to the stretching modes of pyran ring, implying the presence of C-O-C glycosidic band (Liu *et al.*, 2018). The weak absorption peak at about 765 cm^{-1} was recorded as the ring stretching and/or deformation of α-D-(1-4) and α-D-(1-6) bonds (Gu *et al.*, 2020). These results showed that POR-3 possesses the characteristic groups of polysaccharides and could be classified as a kind of pyranose.

The In Vitro Immunomodulatory Activity of POR-3

Effects of POR-3 on Macrophages Proliferation

Macrophages play a crucial role in immune system, where they can activate innate immune and fight against infection and inflammation (Sun *et al.*, 2015). As shown in Fig. 5, there were no significant differences ($P > 0.05$) in macrophages proliferation between NC and POR-3 treated groups even up to the concentration of 800 μg/mL. The results showed that POR-3 has no proliferative or suppressive effects on macrophages, implying that treatment of POR-3 in the concentration range of 25 ~ 800 μg/mL could be no toxicity to macrophages.

Fig. 3: UV spectrum of POR-3
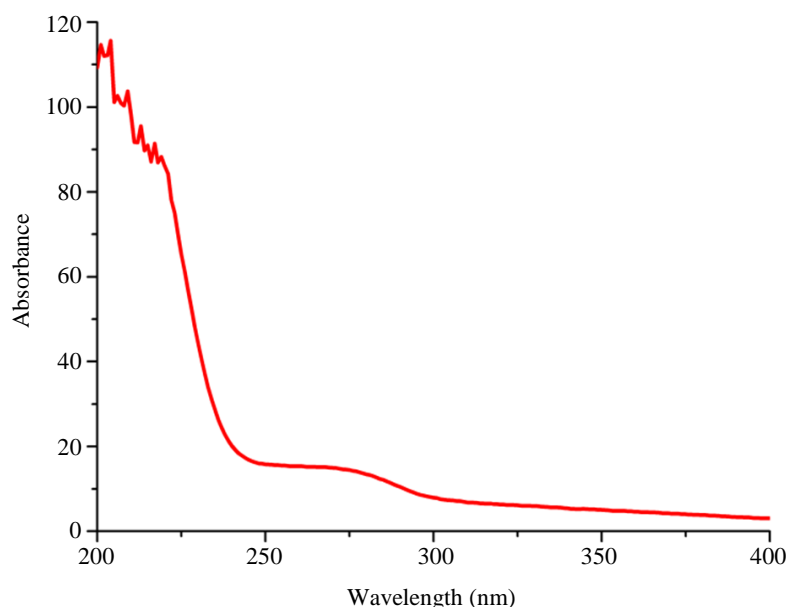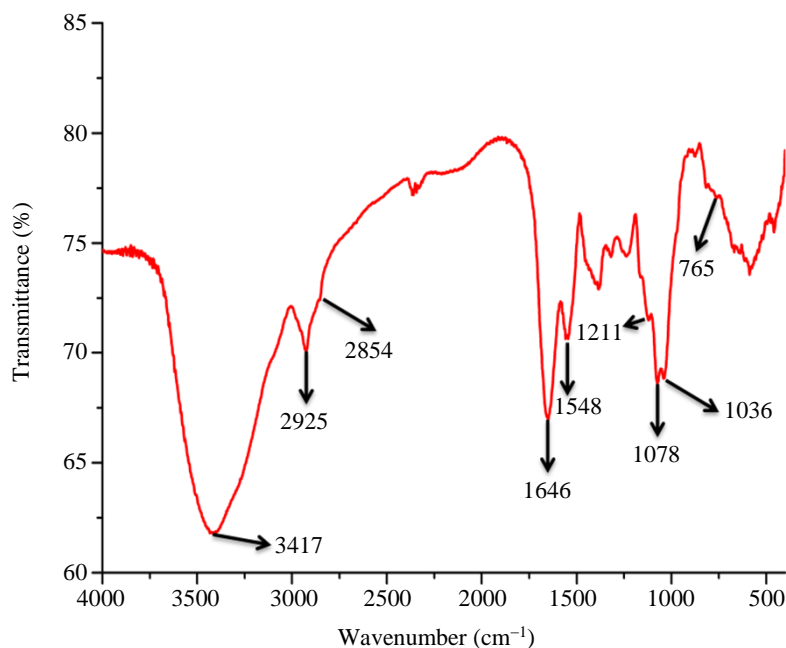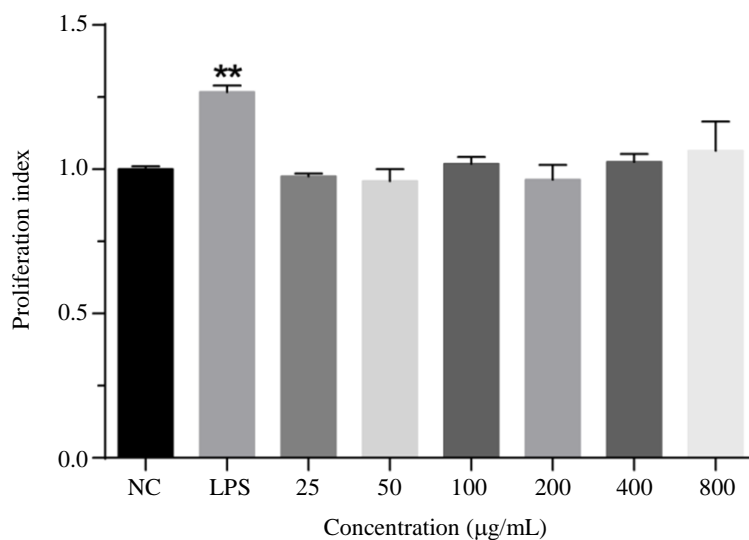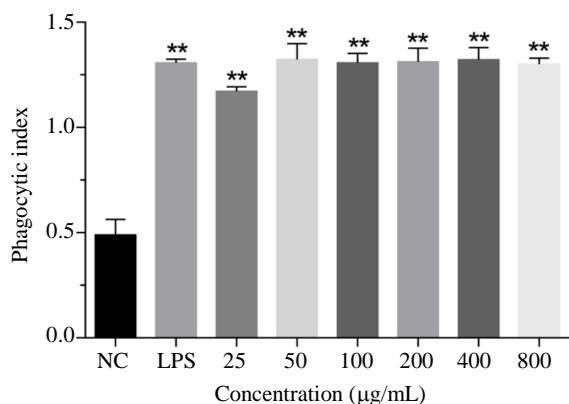
Fig. 4: FT-IR spectrum of POR-3

Fig. 5: Effects of POR-3 on macrophages proliferation. [**]$P < 0.01$ compared with the NC (negative control)

## *Effects of POR-3 on Macrophages Phagocytosis*

Phagocytosis, a fundamental cellular process that has been defined as the leading function of macrophages is recognized as a major mechanism of immune responses to pathogens (Meng *et al.*, 2018a). As shown in Fig. 6, when compared with NC, significant differences ($P < 0.01$) in phagocytic index were found in all POR-3 treated groups, which was raised with the increase of concentration ranging from 25 to 50 µg/mL. When the concentration of POR-3 was 50 µg/mL, phagocytic index

reached highest level of 1.32±0.074, which was almost equivalent to that of LPS (1.31±0.017), after that, it was not elevated with the increase of sample concentration, no remarkable differences ($P > 0.05$) were observed in the concentration range of 50 ~ 800 µg/mL. These results indicated that POR-3 exerts potent promoting effects on macrophages phagocytosis, however, likely due to the inability of proliferating macrophages, this beneficial activity would be not enhanced in a dose-dependent manner. Exact reasons and mechanisms should be explored in the near future**.**

Fig. 6: Effects of POR-3 on macrophages phagocytosis. $^{**}P <$ 0.01 compared with the NC (negative control)
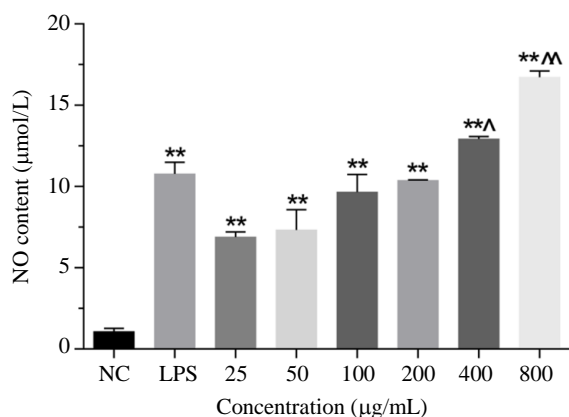
Fig. 7: Effects of POR-3 on NO release. $^{**}P < 0.01$ compared with the NC (negative control). $^{\wedge}P < 0.05$ compared with LPS. $^{\wedge\wedge}P < 0.01$ compared with LPS

Effects of POR-3 on NO release

NO is one of the vital signaling molecules that widely participate in diverse physiological processes, particularly in defending against the invasion of pathogens. The release of NO usually depends on exogenous stimulating agents (Li *et al*., 2017). As shown in Fig. 7, POR-3 simulated NO production in a dose-dependent manner ($P < 0.01$) in the concentration range of 25 ~ 800 µg/mL. The NO content in RAW 264.7 cells being treated with POR-3 in the concentration of 800 µg/mL reached the highest level of 16.72±0.38 µmol/L, which was evidently higher ($P < 0.01$) than that of LPS (10.77±0.72 µmol/L). Significant difference ($P < 0.05$) in NO production was even observed between POR-3 in the concentration of 400 µg/mL and LPS (12.93±0.14 µmol/L for POR-3 *Vs*. 10.77±0.72 µmol/L for LPS). The results demonstrated that POR-3 equally elicits potent stimulating effects on NO production, suggesting a potential of being used for the remedies of various

disorders in addition to immune dysfunction, owing to the reason that NO needs to be involved in the regulation of a widely ranges of pathophysiological processes (Achike and Kwan, 2003).

In present investigation, POR-3 exhibited potent in vitro immunomodulatory activities via promoting macrophages phagocytosis and via stimulating NO production, as well as appeared no toxicity to macrophages.

Immunomodulatory activity is one of the most beneficial functions of polysaccharides (Cao *et al*., 2018). Althrough a broader range of structure-function relationships of immunostimulatory polysaccharides has not been well established owing to the insufficiency of precise separation and full characterization, actually several physicochemical properties such as monosaccharides composition, uronic acids content and molecular weights distribution have been recognized to be highly associated with the immunomodulatory activity of polysaccharides (Hu *et al*., 2019).

It has reported that polysaccharides with relatively abundant monosaccharides including Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glu) and Fucose (Fuc) commonly exhibit higher macrophage stimulatory activities, because on the membrane of macrophages, specific receptors that can recognize these monosaccharides such as Complement Receptor 3 (CR3), Mannose Receptor (MR), Toll-Like Receptors (TLRs), Scavenger Receptors (SRs) and Mannose/Fucose Receptor (MFR) are highly expressed (Ferreira *et al*., 2015; Lo *et al*., 2007; Meng *et al*., 2018a; Shepherd *et al*., 1982). In present investigation, POR-3 mainly comprised Man, GlcN, GalA, Gal and Fuc at a molar ratio of 1: 2.28: 2.40: 3.00: 2.23 and total moles of the reported immunostimulatory monosaccharides were 6.23, accounting for approximately 57%.

As mentioned above, uronic acids can enable polysaccharides negatively charged (Gu *et al*., 2020), which could enhance the interactions with immune-related receptors and immunoglobulins (Jimenez-Dalmaroni *et al*., 2009; Manjula *et al*., 1982). As shown in Table 1, the content of uronic acids in POR-3 was up to 10.19±0.43%, which was in accordance with the monosaccharides composition analysis, where GalA was observed to be present in POR-3.

With regard to the effects of molecular weight distribution on the immunomodulatory activity of polysaccharides, different voices can be heard. Im *et al*. (2005) found that polysaccharides with molecular weights ranging from 5 to 400 kDa exhibit the most potent macrophage-activating activity using modified Aloe polysaccharides as model members. However, to summarize and analyze the molecular weight distributions of immunomodulatory polysaccharides reported by literatures, it can be inferred that the immunomodulatory activity of polysaccharides seems to be less connected with their molecular weights (Ferreira *et al*., 2015). This

controversial issue might be explained by the different immune system activation modes of different immunomodulatory polysaccharides. As stated above, several receptors such as CR3, MR, TLRs, SRs and MFR are highly expressed on the membrane of macrophages and they can recognize the specific monosaccharides of immunomodulatory polysaccharides to trigger immune system (Ferreira *et al.*, 2015; Shepherd *et al.*, 1982). While, for certain non-specific immunomodulatory polysaccharides, entrance into macrophages would be the first step of action, which requires that polysaccharides should possess lower molecular weights, thereby facilitating the entrance via transporters such as glucose transporters and oligosaccharide transporters (Fu *et al.*, 2004; Sun *et al.*, 2013). In this study, the MW of POR-3 was found to be 999.586 kDa, larger than 400 kDa (Im *et al.*, 2005), combining its monosaccharides composition, it can be conjectured that POR-3 would elicit immunomodulatory activity via binding to certain specific receptors expressed on the surface of macrophages, particularly MR and MFR. Nevertheless, details remain to be unravelled and deepened.

In addition, due to the well-known immune-enhancing function of proteins (Mason *et al.*, 2014), the binding proteins in POR-3 could also potentiate its immunomodulatory activity, which has been confirmed by Zhang *et al.* (2014) who observed that polysaccharide-protein complex from *Lycium barbarum* L. showed higher immunological enhancement than that of polysaccharide. Furthermore, the branched structure, spatial conformation and the branching degree equally affect the immunomodulatory activity of polysaccharides (Ferreira *et al.*, 2015). It is therefore important to explore the structure-immunoregulatory function relationship of POR-3 in future investigations.

Conclusion

In presnt investigation, three fractions POR-1 ~ 3 were isolated from the purified polysaccharides of OR, the two major fractions POR-2 and POR-3 were compared on the aspect of *in vitro* antioxidant activities to obtain the fraction deserving of further studies. POR-3 outperformed POR-2 on scavenging activities against hydroxyl and DPPH radicals, thus POR-3 was analyzed and partially characterized. Results indicated that POR-3 belongs to a polydisperse heteropolysaccharide-protein fraction. In RAW 264.7 macrophages, POR-3 elicited potent promoting effects on macrophages phagocytosis and NO release, but no proliferating or inhibiting effects on macrophages growth, exhibiting a desirable safety property for macrophages. Finally, the probable structure-immunomodulatory function relationships and underlying mechanisms of POR-3 were discussed and speculated. It is hoped that present work might provide

evidence that animal-derived polysaccharides could be an emerging source of bioactive components for nutraceutical and medicinal purposes.

Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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