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Purification, characterization and immunomodulating activity of a polysaccharide from flowers of *Abelmoschus esculentus*

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ABSTRACT

A water-soluble polysaccharide (OFPS11) was obtained from okra (*Abelmoschus esculentus*) flowers using aqueous extraction and purification with DEAE-52 cellulose and Sephacryl™ S-500 column. Its preliminary characterization and immunomodulating activity were investigated. Results showed that OFPS11 is mainly composed of galactose and rhamnose in a molar ratio of 2.23:1 with molecular mass of 1700 kDa. RAW264.7 cells pretreated with OFPS11 significantly inhibited the proliferation of HepG-2 cells. Additionally, OFPS11 enhanced the phagocytic ability and induced the elevation of NO production, TNF-α and IL-1β secretion of RAW264.7 cells. Furthermore, OFPS11 promoted both the expression of iNOS protein and of iNOS and TNF-α mRNA. OFPS11 can strongly increase NF-κB levels in nucleuses, which is an important transcription factor that can modulate expressions of iNOS, NO and TNF-α. These outcomes support that OFPS11 exerts its antitumor activity by probably stimulating macrophage activities through nuclear NF-κB pathway.

1. Introduction

Many plant active ingredients, especially polysaccharides, have gained much popularity on account of their broad spectrum of biological and pharmacological activities, such as antitumor (Cai et al., 2012b; Shen et al., 2012; Wu, Zhu et al., 2012; Zhao et al., 2014), immune-modulatory (Yang, Hsieh, Lu & Lin, 2013; Zhao et al., 2013), antiviral (Jiang et al., 2013; Oliveira et al., 2013), anti-complementary (Di, Zhang & Chen, 2013; Hromádková, Paulsen, Polovka, Košťálová & Ebringerová, 2013), antioxidant (Fan et al., 2011; Mao et al., 2014; Xu, Xiao & Mao, 2013; Zhao et al., 2012), and anti-inflammatory (Liao & Lin, 2013) activities. Among these, interest is growing recently in the antitumor and immune-stimulating activity of natural polysaccharides due to their relatively low toxicities and few side effects (Bai et al., 2012; Cai et al., 2012a). Immune-stimulating is regarded as an important strategy which is used to enhance the body's defense mechanism, especially for elderly people and cancer patients. Studies have demonstrated that the potential immune-stimulating activity and antitumor property are generally related to macrophage activation and modulation of complement system (Han et al., 2006; Kim et al., 2007; Shang et al., 2003). Activation of macrophages can enhance their antitumor activity through secretion of cytokines or by antigen processing and presentation, thereby regulating the immune system (Klimp, De Vries, Scherphof & Daemen, 2002). Thus, it may be one of therapeutical interests to explore new polysaccharides as immune-potentiator to stimulate the macrophage-mediated defense mechanisms.

Okra (*Abelmoschus esculentus* L.), an indigenous crop in Africa (Molfetta, Ceccarini, Macchia, Flamini & Cioni, 2013), is now widely distributed in various parts of the world (Jarret, Wang & Levy, 2011). Published data indicated that okra fruit exhibited promising immune-potentiating effects owing to its thick and slimy polysaccharide (Sheu & Lai, 2012). Due to the high content of similar mucilaginous, okra flowers are therefore expected to be good sources of immune-stimulating polysaccharides. Although numerous activities of polysaccharide from okra fruits have been reported so far, studies have not been reported on the isolation, structural characterization and immune-regulation of polysaccharide from okra flowers. Therefore, the aim of the present study was to isolate

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and preliminarily characterize a water-soluble polysaccharide from okra flowers, evaluate its immune-stimulating effects on murine RAW264.7 macrophage cells and explore whether immunopotentiation is involved in its action. The study will be helpful to develop novel functional foods and drugs.

2. Materials and methods

2.1. Materials and reagents

Okra (*Abelmoschus esculentus* L.) flowers were collected from Zhenjiang, Jiangsu Province in August 2012, and identified by Chen Li (Chief Pharmacist, Food and Drug Administration Bureau of Zhenjiang). The moisture content of the fresh flowers was determined in 2 h after harvest. The flowers were packaged 500 g per polyethylene bag and then stored at −20 °C until use. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO) and lipopolysaccharide (LPS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's minimum essential medium (DMEM) and fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY, USA), respectively. Assay kits for interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), nitric oxide (NO) test kits and BCA were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China). Dextrans of different molecular weights (T-10, T-40, T-70, T-500, and T-2000) were obtained from Pharmacia Co., Ltd. (Uppsala, Sweden). DEAE-52 cellulose and Sephacryl™ S-500 were obtained from Whatman Co., Ltd. (Maidstone, Kent, UK). All other chemicals and solvents used were of analytical reagent grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Cell culture

Human hepatocellular carcinoma HepG-2 and mouse macrophage cell line RAW264.7 cells were obtained from Institute of Cell Biology, Chinese Academy Sciences (Shanghai, China). Cells were cultured at 37 °C in DMEM supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a humidified atmosphere with 5% CO_2 and 95% air.

2.3. Extraction, isolation and purification of polysaccharides

500 g frozen sample was cut into small slices and homogenized in double-distilled water (ddH_2O) with a high-frequency homogenizer. The mixture was transferred and extracted twice with ddH_2O for 3 h, and supernatants were collected by centrifugation at 4500 rpm for 30 min. The combined filtrates were concentrated and precipitated with 95% (v/v) alcohol until the concentration of the alcohol reached 80% and kept for 12 h at 4 °C, then centrifuged again as above. The precipitated material was then dissolved and dialyzed through cellulose membrane (Sigma–Aldrich, retaining > Mw 14,000) against flowing water and ddH_2O for 48 h, respectively. The aqueous solution was then collected from the dialysis bag and freeze-dried to obtain the crude polysaccharide (OFPS).

To remove the proteins, OFPS was re-dissolved and subjected to the trichloroacetic acid (TCA) method (Lengsfeld, Titgemeyer, Faller & Hensel, 2004). Then the de-proteined polysaccharides were stepwise eluted with different concentrations of NaCl solutions (0, 0.05, 0.1, 0.15, 0.2, 0.5, 1.0 M) by DEAE-52 (OH^-) cellulose column (1.6 cm × 50 cm). Forty test tubes (5 mL of each) were monitored spectrophotometrically at 490 nm with phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) and collected. One fraction (OFPS1), which was obtained in 0.1 M NaCl, comprised the major components of the elution fraction and was further fractionated with size-exclusion chromatography on a Sephacryl™ S-500 High Resolution (4000–2,000,000) gel column (1.6 cm × 50 cm) using 0.1 M NaCl as eluent at a flow rate of 0.3 mL/min. One single fraction named OFPS11 was collected, dialysis as above, and finally freeze-dried.

2.4. Characterization of OFPS11

2.4.1. Molecular weight determination

The homogeneity and molecular weight distribution of OFPS11 were determined by high-performance gel-permeation chromatography (HPGPC) on a LC-10ATvp instrument (Shimadzu, Tokyo, Japan), equipped with a pre-column of TSKGUARD COLUMN PWH (7.5 mm × 75 mm, Tosoh corporation, Tokyo, Japan) and a column of TSK-GEL G4000PW (7.5 mm × 300 mm, Tosoh corporation, Tokyo, Japan) and eluted with 0.003 M CH_3COONa solution at a flow rate of 1.0 mL/min. The eluents were monitored using refractive index detector (Shimadzu RID-10A, Shimadzu). Standard T-series dextrans were used as standards for calibration (2000, 500, 70, 40 and 10 kDa).

2.4.2. Neutral monosaccharide composition analysis

The monosaccharide compositions of OFPS11 were determined using the method reported by (Yang et al., 2011) with slight modification. Briefly, the OFPS11 was hydrolyzed with 4.0 M H_2SO_4 at 110 °C for 10 h. After neutralization with $BaCO_3$, the supernatants were collected and lyophilized. The hydrolysates were then converted into their corresponding alditol acetates and analyzed with GC using a Shimadzu 2010 instrument, equipped with a HP-5MS column (0.25 mm × 30 m × 0.25 μm) and a flame-ionization detector. The temperature was set as follows: the initial temperature of column was 130 °C, maintained for 5 min and increased to 240 °C at 4 °C/min. The 240 °C was held for 5 min. Standard monosaccharides were then converted to their acetylated derivative and analyzed using the same procedure.

2.4.3. Infrared spectra analysis

To detect functional groups of OFPS11, IR spectrum were obtained with a NEXUS 670 FT-IR (Thermo Nicolet, USA) spectrophotometer. OFPS11 was grounded, mixed with KBr powder (1:100), pressed into pellets and detected in the frequency range of 4000–400 cm^{-1}.

2.5. Macrophage immune-modulating activity

2.5.1. Cytotoxicity assay

The cytotoxic effects of OFPS11 on RAW264.7 cells were measured by MTT assay. In brief, adherent RAW264.7 cells in 96-well plates (2×10^4 cells/well) were cultured with different concentrations of OFPS11 (0–1000 μg/mL) prepared in DMEM (10% FBS). The LPS (10 μg/mL) was used as a positive control (Wang et al., 2013). The treated cells were incubated for 24 and 48 h to estimate the viability. 1 mg/mL of MTT was prepared and 100 μL MTT was added to each well and incubated for 4 h. After incubation, the observed purple color of mazone crystals was dissolved with 100 μL DMSO for 1 h. The absorbance was detected at 570 nm by microplate ELISA reader (Spectra MAX 190, Molecular Devices Corporation, USA).

2.5.2. Macrophage-mediated cytotoxicity

Briefly, adherent RAW264.7 cells in 96-well plates (2×10^4 cells/well) were cultured with different concentrations of OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL). After 48 h incubation, the supernatants were collected by centrifugation at 1500 rpm for 15 min. Then the collected supernatants and treated macrophages were analyzed, respectively. On the one hand, the collected supernatants were added to another 96-well plates with adherent HepG-2 cells (4×10^3/well). After culturing for 48 h, the

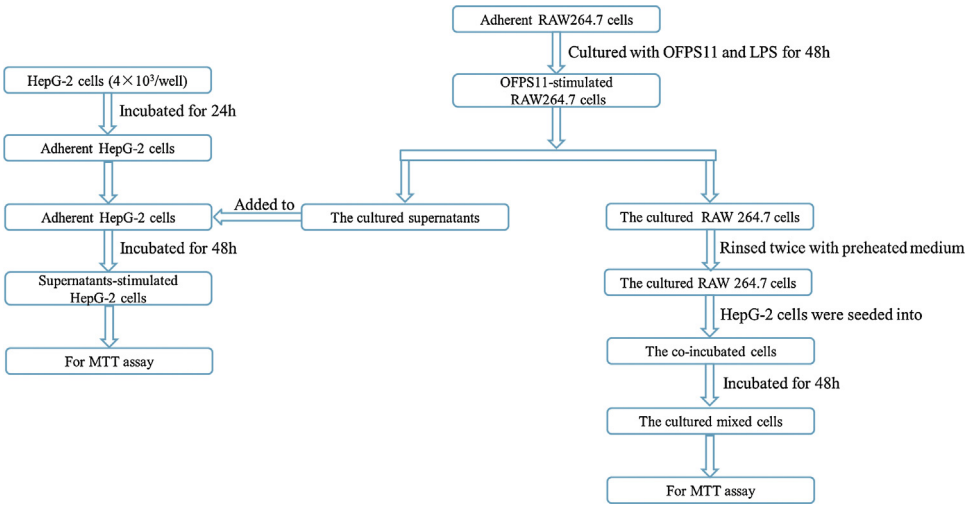

Fig. 1. Flowchart scheme of macrophage-mediated cytotoxicity on HepG-2 cells.

supernatant-stimulated HepG-2 cells were then used for MTT assay as described above. On the other hand, the OFPS11-treated cells were rinsed twice with preheated DMEM (10% FBS) to remove stimulants (as shown in Fig. 1). The stimulated RAW264.7 (effector cells) were then co-incubated with 100 μL HpeG-2 cells suspension (target cells, 2×10^4 cells/mL, 1:10 of the density of effector cells) for another 48 h incubation. After incubation, an MTT assay was used for measuring the inhibition of tumor cells.

2.5.3. Neutral red uptake by RAW264.7

Phagocytic activity was determined with neutral red uptake assay. In brief, adherent RAW264.7 cells in 96-well plates (2×10^4 cells/well) were treated with different concentrations of OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) for 48 h, and then the culture medium was abandoned. One hundred microliter of 0.075% (g/mL) neutral red solution was added to each well and cultured for a further 4 h. After cultivation, the pretreated macrophage was washed three times with phosphate buffered saline (PBS) and 100 μL of cell lysis buffer (acetic acid/ethanol; 1:1) was added and incubated at 4 °C for 2 h. The absorbance was determined at 540 nm using microplate ELISA reader.

2.5.4. Measurement of NO and cytokine

RAW264.7 cells were treated with OFPS11 in different concentrations and LPS (10 μg/mL) as above. After 48 h, the cultured supernatants were collected for the nitrite assay with Nitric oxide test kits (Kim et al., 2012; Zhang & Dai, 2011) and cytokine secretion assay using an enzyme-linked immunosorbent assay (ELISA), respectively. The concentration of nitrite was calculated using a standard curve and cytokine concentration was measured following the manufacturer's instructions.

2.5.5. Western blot analysis

The effect of OFPS11 on iNOS protein expression in RAW264.7 cells was performed using whole-cell extracts. Cells treated with OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) were rinsed twice with ice-cold PBS, lysed using ice-cold extraction buffer (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1% CHAPS, and 1% Triton X-100) containing 1% of protease inhibitor cocktail for 30 min at 4 °C. Protein concentrations were determined using the BCA protein assay kit. The protein extract was then boiled with isotonic loading buffer for 10 min, and an equal amount of protein (20 ng) for each sample was separated by a 12.5% SDS-PAGE and followed by electrically transferring to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk in TBS-0.1% Tween 20 (TBST), the membranes were treated with rabbit anti-iNOS monoclonal antibody (Santa Cruz Biotechnology, Inc., USA) and rabbit anti-β-actin (Santa Cruz Biotechnology, Inc., USA) overnight at 4 °C, respectively, then the membranes followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz) for 3 h at room temperature. The blot was visualized using the ECL-detection with chemiluminescence system. Analysis of NF-κB using nuclear extracts with β-actin as internal reference was also performed as above. The lysis buffer contains 20 mM Tris (pH 7.5), 2 mM EDTA, 135 mM NaCl, 2 mM DTT, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM PMSF. The primary antibody was rabbit anti-NF-κB p65 monoclonal antibody (Santa Cruz Biotechnology, Inc., USA), and the secondary antibody was horseradish peroxidase-conjugated anti-rabbit antibody.

2.5.6. RT-PCR

To evaluate the mRNA expression levels of inducible nitric oxide synthase (iNOS) and TNF-α, total RNA from LPS and OFPS11-treated RAW264.7 cells were prepared by adding RNAiso Plus (Takara, China), according to the manufacturer's instructions. RT-PCR was carried out by using two-step RT-PCR System kit (Takara, China) with following primer sequences: ′5-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (forward) and ′5-GGCTGTCAGAGCCTCGTGGCTTTGG-3′ (reverse) for mouse iNOS, ′5-ATGAGCACAGAAAGCATGATC-3′ (forward), ′5-TACAGGCTTGTCACTCGAATT-3′ (reverse) for mouse TNF-α, ′5-TGGAATCCTGTGGCATCCATGAAAC-3′ (forward) and ′5-TAAAACGCAGCTCAGTAACAGTCCG-3′ (reverse) for mouse β-actin. The β-actin primer was used as an internal control. PCR was performed with 1 cycle of 180 s at 94 °C, 25 cycles of 30 s at 94 °C, 40 s at 56 °C, 60 s at 72 °C and 1 cycle of 180 s at 72 °C in a 50 μL reaction mixture, and the products were separated on a 1.5% agarose gels and visualized by ethidium bromide staining. The gel slabs were scanned and analyzed with image analysis software (Kodak Digital Science, Kennesaw, GA, USA).

2.6. Statistical analysis

All experiments were repeated at least three times. The statistical values were presented as mean ± standard deviation and datas were analyzed by the Tukey method multiple comparison tests to evaluate significant differences. A value of $p < 0.05$ denoted the

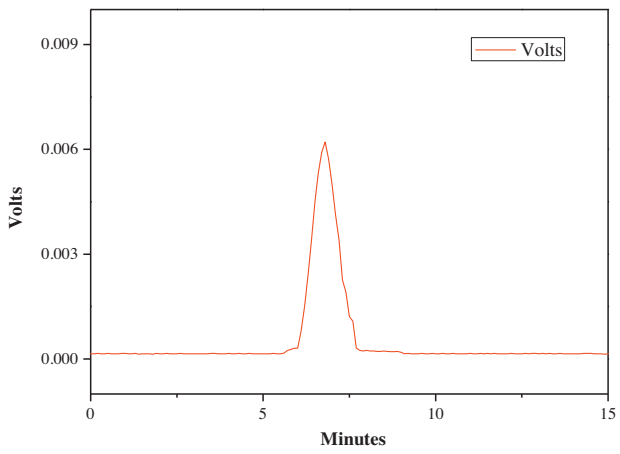

Fig. 2. HPSEC chromatogram of OFPS11.

presence of a statistically significant difference. Statistical analyses were carried out by SPSS version 16.0 (SPSS Inc, Chicago, USA).

3. Results and discussion

3.1. Isolation, purification and molecular weight of OFPS11

The moisture of the fresh okra flowers was (90.42 ± 0.11) %, and the yield of OFPS was calculated to be 15.4% by dry basis. After TCA treatment, no absorption was detected by the UV spectrum at 280 and 260 nm, which indicates the absence of protein and nucleic acid. OFPS was then fractionated through DEAE-52 cellulose and Sephacryl™ S-500 to obtain a white fluffy polysaccharide named OFPS11. The molecular weight of OFPS11 was determined by HPGPC, which has been shown to be an effective method (López-Barajas, López-Tamames & Buxaderas, 1998). As shown in Fig. 2, a single and symmetrical peak indicates a high purity of the OPFS11 and the average molecular weight of OFPS11 was calculated as 1700 kDa according to the calibration curve.

3.2. Monosaccharide composition and infrared spectroscopy

To further investigate the composition of OFPS11, GC was used. As shown in Fig. 3A, the neutral sugars of OFPS11 was mainly composed of galactose and rhamnose in a ratio of 2.23:1, and it also contains some other sugars such as arabinose, mannose, and glucose with little content. The infrared spectra (Fig. 3B) showed strong and wide stretching peak around 3387.76 cm^{-1} for O—H stretching vibrations, a weak absorption peak at 2937.80 cm^{-1} was induced by C—H stretching vibration. The intense peak which appeared in 1611.67 cm^{-1} was the C=O asymmetric stretching of —COO, and the band at 1421 cm^{-1} was attributed to bending vibration of C—H or O—H (Cao, Yuan, Sun & Sun, 2011). Particular polysaccharide has specific bands in the 1000–1200 cm^{-1} region, and this region is dominated by ring vibrations overlapped with stretching vibrations of (C—OH) side groups and the (C—O—C) glycosidic band vibration. Absorptions at 1146.99, 1095.39, 1073.56, and 1044.07 cm^{-1} indicated a pyranose form of sugar (Wu, Mao et al., 2012; Zhang, Liu, Park, Xia & Kim, 2012).

3.3. Cytotoxicity assay

Many kinds of polysaccharides (Jin, 2012; Zhao et al., 2012), as natural biological macromolecules, have been proved to exhibit significant antitumor activities with little toxicity. The cytotoxicity of OFPS11 on HepG-2 cells was examined with MTT using 5-fluorouracil (5-FU) as the positive control (Shi, Nie, Chen, Liu & Tao, 2007). However, OFPS11 was not able to inhibit the proliferation of HepG-2 cells at the tested concentrations. Thus, it might indicate that the potential antitumor activity of OFPS11 showed in an indirect way, for example, by boosting the immune system. The cytotoxicity effect of OFPS11 on RAW264.7 cells was also determined. As seen in Fig. 4A, after treatment with OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) for 24 and 48 h, there were disorderly changes in the OD values. The data demonstrated that OFPS11 showed no significant effects on RAW264.7 cells up to 1000 μg/mL. Therefore, due to the hardly any cytotoxicity of OFPS11 on RAW264.7 cells, these macrophages were selected as test cells in the following study.

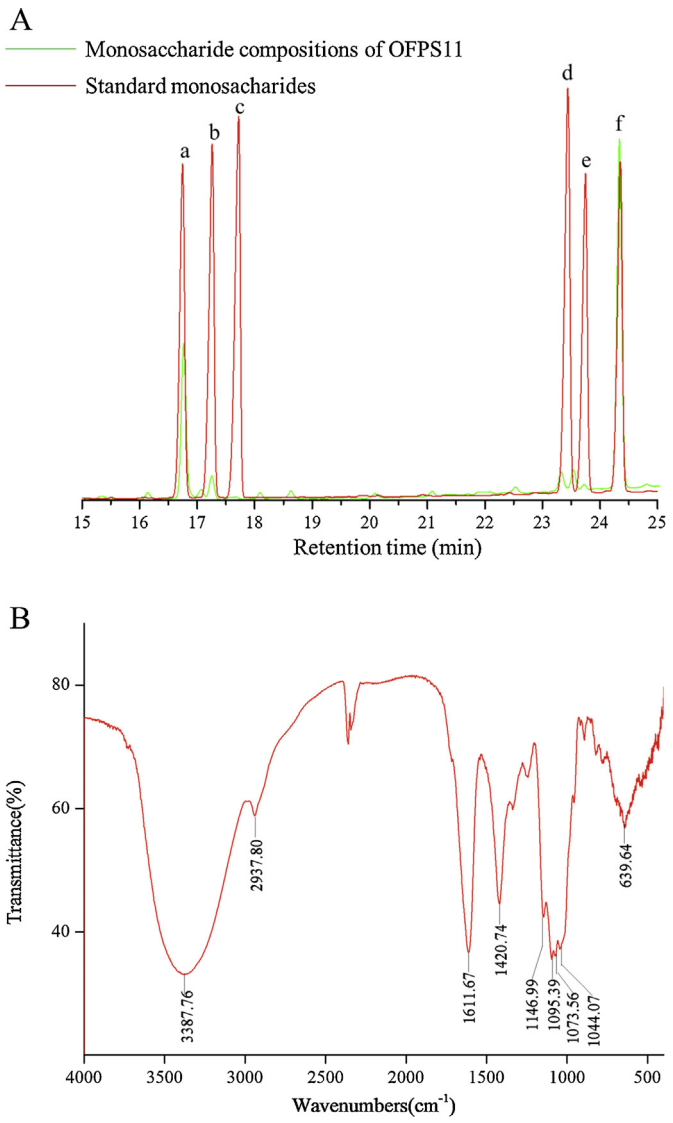

Fig. 3. (A) Gas chromatograms of acetate-derivatized standard monosacharides and monosaccharide compositions of OFPS11. (a) L-rhamnose (Rha), (b) L-arabinose (Ara), (c) D-xylose (Xyl), (d) D-mannose (Man), (e) D-glucose (Glu) and (f) D-galactose (Gal). (B) The FTIR spectra of OFPS11.

3.4. Proliferation inhibition with OFPS11-M-CM and macrophage-mediated cytotoxicity activity with OFPS11

Our study above indicated that OFPS11 exhibited an indirect cytotoxic activity against HepG-2 tumor cells. To investigate whether macrophage-conditioned (M-conditioned) media induced by different concentrations of OFPS11 could affect the proliferation

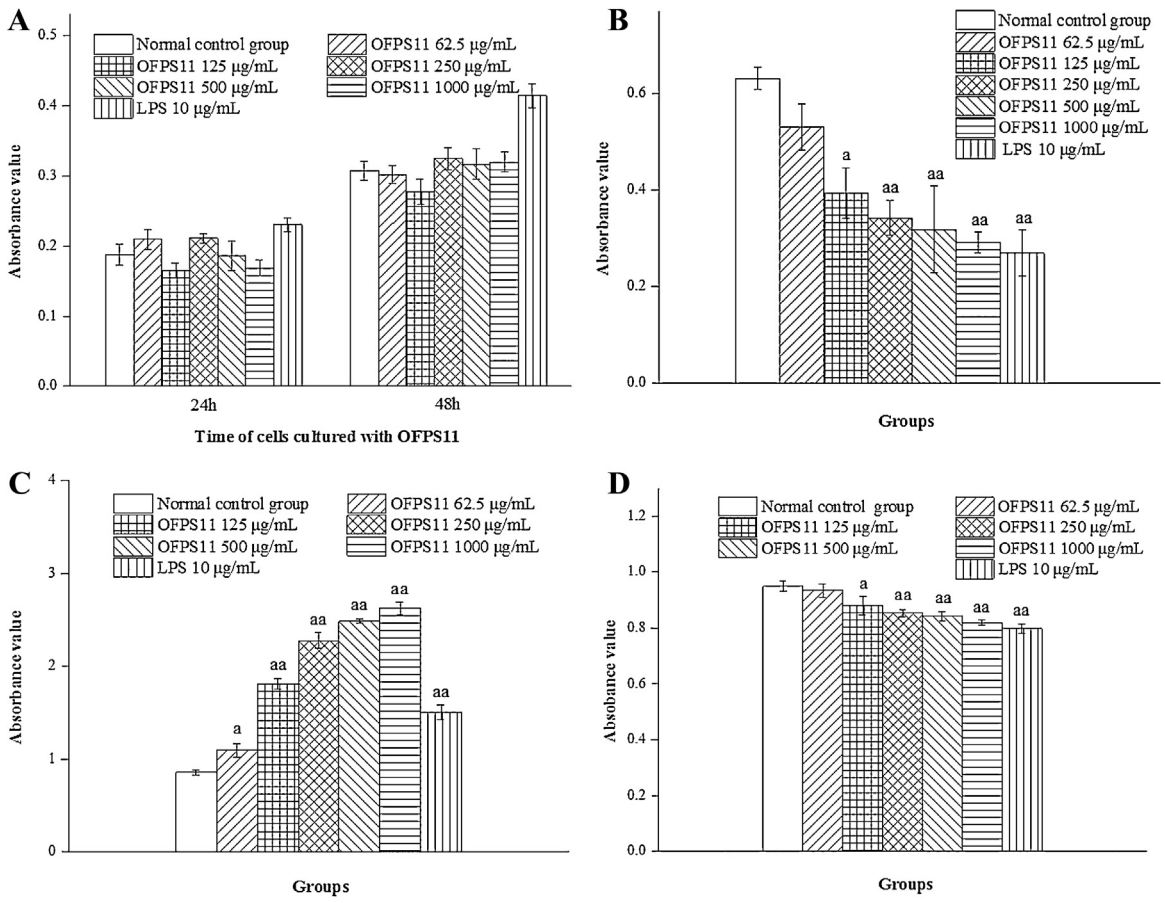

Fig. 4. (A) Effects of OFPS11 on the proliferation of RAW264.7 macrophages. RAW264.7 cells (2×10^4 cells/well) were incubated with different concentrations of OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) for 24 h or 48 h. Effect of OFPS11-induced M-conditioned media (B) and OFPS11-activated macrophage (D) on the proliferation of HepG-2 cells. RAW 264.7 Cells (2×10^4 cells/well) were incubated with OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) for 48 h. Antitumor activity was determined as described in 2.5.2 at an initial effector/target ratio of 10:1. Each value is presented as mean ± SD of 5 separate experiments. *P* values are shown as $^{a}p < 0.05$, $^{aa}p < 0.01$ compared with normal control group. A value of $p < 0.05$ demoted the presence of a statistically significant difference according to Tukey method. (C) Effect of OFPS11 on phagocytosis of RAW264.7 macrophages by neutral red uptake assay. RAW264.7 cells (2×10^4 cells/well) were incubated with OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) for 48 h. The absorbance at 540 nm was determined.

of HepG-2 cells, an MTT assay was performed. As seen in Fig. 4B, M-conditioned media induced by OFPS11 (62.5–1000 μg/mL) inhibited the proliferation of HepG-2 cells in a distinct dose-dependent manner. Moreover, the inhibition of all concentrations was significant compared with normal control group.

To assess the cytotoxic activity of OFPS11-activated macrophages, the HepG-2 cells were co-incubated with OFPS11 and LPS-treated RAW264.7 respectively. From Fig. 4D, the anti-tumor activity was observed in the OFPS11-induced macrophage groups, and the higher concentrations (250–1000 μg/mL) of OFPS11 significantly enhanced cytotoxic activity of RAW264.7 cells against HepG-2 cells compared to normal control group. These results indicated that OFPS11 exhibited a great immunological competence by stimulating macrophage responses, and the immune-modulating activity was one of the important mechanisms in the antitumor activity of OFPS11.

3.5. Pinocytic activity assay

Since phagocytic activity is one of the most important functions of macrophages in innate immune response (Zhao, Dong, Chen & Hu, 2010), the effect of OFPS11 on phagocytic activities of RAW264.7 cells was assessed by neutral red assay in this study. As shown in Fig. 4C, OFPS11 appeared to enhance the phagocytic function of RAW264.7 in a prominent dose-related increment. Except for the 62.5 μg/mL OFPS11 and 10 μg/mL LPS, the enhancement at other concentrations was significant compared with the normal control group. From the data, it is suggested that OFPS11 can play action on phagocytic function of macrophages in some degree.

3.6. NO and cytokine levels

As a significant part of host defense system, macrophages can produce various mediators and cytokines, which plays potential roles in defense against cancer cells (Bontà & Ben-Efraim, 1993). NO, an intracellular messenger and regulates cellular functions, has been identified as the major effector molecule involved in the destruction of tumor cells by activated macrophages (Gordon & Martinez, 2010; Lorsbach, Murphy, Lowenstein, Snyder & Russell, 1993). NO contributes to the killing of virally infected cells, tumor cells and some pathogens remains unknown, partly because it inactivates their mitochondrial respiratory chain enzymes. In addition, a variety of cytokines that can regulate the cellular and humoral immune responses were produced by activated macrophages. TNF-α plays a crucial role in immune-regulation and can also induce tumor cell apoptosis. Similarly, IL-1β is an important cytokines. The cytokine from activated macrophages, as a part of immune response, plays an important role in the inhibition of cancer cells. Thus, the immunological activities of OFPS11 was evaluated by monitoring its effects on the production of NO and cytokines of RAW264.7 cells. As shown in Table 1, NO, TNF-α and IL-1β induced by RAW264.7 cells indicated

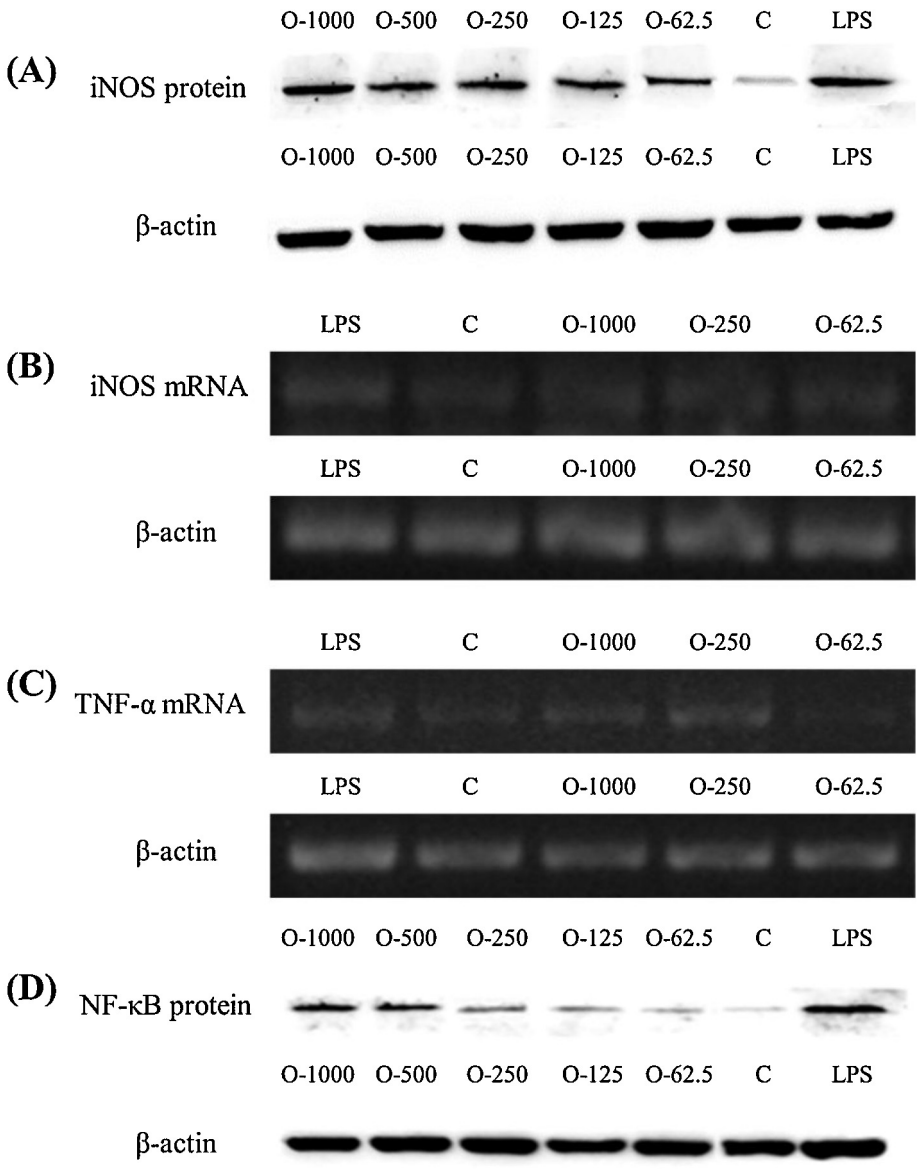

Fig. 5. (A) iNOS protein expression in RAW264.7 cells treated with OFPS11. Adherent RAW264.7 cells were incubated with different concentrations of OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) at 37 °C for 48 h. After incubation, the whole-cell lysates were prepared from the treated cells, and analyzed by western blot analysis as described in the text. iNOS mRNA (B) and TNF-α mRNA (C) in RAW264.7 cells treated with OFPS11.Adherent RAW264.7 cells were incubated with OFPS11 (0, 62.5, 250, 1000 μg/mL) and LPS (10 μg/mL) at 37 °C for 24 h. After incubation, iNOS and TNF-α mRNA levels in treated RAW264.7 cells were analyzed by RT-PCR method as described in the text. (D) NF-κB protein expression levels in RAW264.7 cells treated with OFPS11. Adherent RAW264.7 cells were incubated with different concentrations of OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) at 37 °C. After 48 h, the nuclear lysates were prepared from the treated cells, and analyzed by western blot analysis as described in the text. OFPS11 (0–1000 μg/mL): C, O-62.5, O-125, O-250, O-500, O-1000; LPS: lipopolysaccharide.

Table 1
Effect of OFPS11 on inducing NO, TNF-α and IL-1β production of RAW264.7 cells.

Groups	NO levels (μmol/mL)	TNF-α levels (pg/mL)	IL-1β levels (pg/mL)
Normal control group	2.79 ± 0.18	200.03 ± 7.18	65.96 ± 2.07
OFPS11 (62.5 μg/mL)	3.07 ± 0.15	207.39 ± 5.83	80.27 ± 3.18^{a}
OFPS11 (125 μg/mL)	3.22 ± 0.08	225.51 ± 7.65^{a}	123.58 ± 2.36^{aa}
OFPS11 (250 μg/mL)	3.33 ± 0.65	268.09 ± 6.59^{aa}	130.92 ± 3.98^{aa}
OFPS11 (500 μg/mL)	4.87 ± 0.25^{a}	344.96 ± 4.70^{aa}	147.60 ± 4.16^{aa}
OFPS11 (1000 μg/mL)	10.37 ± 0.98^{aa}	436.93 ± 4.86^{aa}	150.77 ± 2.03^{aa}
LPS (10 μg/mL)	11.83 ± 0.79^{aa}	348.04 ± 2.25^{aa}	122.78 ± 1.68^{aa}

RAW 264.7 Cells (2×10^4 cells/well) were stimulated with OFPS11 (0–1000 μg/mL) and LPS (10 μg/mL) for 24 h. The supernatant NO, TNF-α and IL-1β levels were determined using commercial test kit. Each value is presented as mean ± SD of 5 separate experiments. *P* values are shown as $^{a}p < 0.05$, $^{aa}p < 0.01$ compared with normal control group. A value of $p < 0.05$ denotes the presence of a statistically significant difference according to Tukey method.

significant dose-response increases in the secretion amount. The difference in NO production between OFPS11 (500–1000 μg/mL) and LPS (10 μg/mL) treated groups and normal control group were statistically significant ($^{a}p < 0.05$, $^{aa}p < 0.01$). This suggests that OFPS11 may stimulate the NO production of RAW264.7 cells. In addition, OFPS11 and LPS treated-RAW264.7 cells secreted more TNF-α and IL-1β than that in the normal control group ($^{a}p < 0.05$, $^{aa}p < 0.01$). These results suggest that OFPS11 shows beneficial effects on enhancing the production of NO and the secretion of cytokines.

3.7. Effects of OFPS11 on protein expression of iNOS and mRNA expressions of iNOS and TNF-α in RAW264.7 cells

iNOS is a significant isoform of enzyme nitric oxide synthase (NOS) which induces the synthesis of NO (Davis, Martin, Turko

& Murad, 2001). In addition, the published data showed that polysaccharide induce NO production from RAW264.7 cells by up-regulating the expression of iNOS protein and iNOS Mrna (Nakamura, Suzuki, Wada, Kodama & Doi, 2006). Thus, the possible mechanism involved in the up-regulating of NO production was examined in the protein and mRNA expressions of OFPS11-treated RAW264.7.

As shown in Fig. 5A, western blot analysis revealed that levels of protein were significantly expressed in OFPS11-treated and LPS-stimulated RAW264.7 cells. However, in normal control group, macrophage cells showed very low levels of iNOS protein, which indicates that OFPS11 could significantly stimulate the expression of iNOS protein of macrophages. Consistent with the NO production and iNOS protein expression, RT-PCR analysis exhibited higher iNOS mRNA expression in OFPS11-induced group than that in normal control group, though the expressions were not in a dose-related way (Fig. 5B). Furthermore, the LPS (10 μg/mL) could up-regulate the expression of iNOS mRNA significantly. The results suggested that the increasing of NO levels in activated macrophages might be due to the enhancement of protein and mRNA expression of iNOS. The enhanced expression of mRNA level of TNF-α was also observed from Fig. 5C, after treatment with various doses of OFPS11 (62.5, 250 and 1000 μg/mL) and LPS (10 μg/mL) for 24 h, the higher concentrations of OFPS11 (250 and 1000 μg/mL) enhanced expression of mRNA markedly comparing with normal control group, though all the expressions were almost trace level. Consistent with the increase of TNF-α secretion, this observation suggests that OFPS11 could enhance TNF-α production by up-regulated TNF-α gene expression. Since gene expressions are regulated by mediators, the results may indicate that OFPS11 increased the NO production and TNF-α by a mechanism involving transcriptional regulations.

3.8. The protein expression of NF-κB

Previous study suggested that polysaccharide induced NO and cytokines production from the RAW264.7 cells through the activation of transcription factor NF-κB (Lee et al., 2004). NF-κB usually stays quietly as an inactive, non-covalent binding form with p50-p65-IκB trimer in the cytoplasm. When signals for the activation of NF-κB are received, serine residues in IκB are phosphorylated and dissociated from NF-κB, which are then transferred to the nucleus as an activated transcription factor (Ley, 2004). After the degradation of IκB, the binding sites of p50–p65 dimer are then exposed to combine with the κB motif. Then the NF-κB p65 subunit exhibits a potent activity in regulating many important biological and pathological processes and modulating the transcription of a large number of genes involving iNOS, IL-1β and TNF-a. Thus, NF-κB p65 in nuclear was examined using Western-blotting.

The outcome shown in Fig. 5D suggested that NF-κB was tremendously activated in OFPS11-treated RAW264.7 cells when comparing with normal control group, and the extent of the activation were in an evidently concentration-dependent manner, which indicated that NF-κB activation was required for OFPS11-induced macrophage activation. Thus, the results demonstrated that NF-κB p65 in nuclear was significantly increased after stimulation with OFPS11, which suggests that OFPS11 can stimulate the release of NF-κB from IκB and transfer from cytoplasm to cytoplast. In summary, the results suggested that OFPS11 could potentially be used as an immune-modulatory agent, and it induced antitumor responses by activated RAW264.7 macrophages, at least in part, by up-regulating the expressions of NO and TNF-α through NF-κB signaling pathway.

4. Conclusion

OFPS11 is a water-soluble polysaccharide mainly composed of galactose and rhamnose in a molar ratio of 2.23:1 with molecular mass of 1700 kDa. The results revealed that OFPS11 exhibited an indirect cytotoxic effect on HepG-2 cancer cells by activating RAW264.7 cells. Meanwhile, OFPS11 could promote the phagocytosis, production of NO and cytokines of RAW264.7 cells when comparing with normal control groups. In addition, the expressions of iNOS in both protein and mRNA level, TNF-α in mRNA level and NF-κB in protein level were enhanced in OFPS11-treated groups. Thus, this study provides evidence of plant-derived polysaccharide OFPS11 with non-toxic nature, which could be used as a potential antitumor immunomodulatory agent used in health food and pharmaceutical therapy. Since activities of polysaccharides depend on their chemical structure, the future work will be conducted to determine the concrete structure of OFPS11 and its targets in macrophages.

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