Structural characterization and RAW264.7 murine macrophage stimulating activity of a fucogalactoglucan from *Colpomenia peregrina*

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Abstract Water-soluble polysaccharides were isolated from *Colpomenia peregrina* to determine their chemical characteristics and immunomodulatory properties. High extraction yields were obtained for CP₁ (17.6%) and CP₂ (5.2%) polysaccharides. Polysaccharides were mainly consisted of neutral sugars (67.01–73.79%), uronic acids (9.43–14.89%), proteins (3.44–14.89%) and small amounts of sulfates (4.87–4.91%). Polysaccharides were composed of fucose (20.62–24.56%), galactose (25.5–26.94%) and glucose (50.00–52.91%) residues. The average molecular weights of the CP₁ and CP₂ polysaccharides were 1890×10^3 g/mol and 639×10^3 g/mol, respectively. The polysaccharides exerted a relatively low cytotoxicity against HeLa cancer cells (< 40%). The CP₁ and CP₂ polysaccharides were nontoxic and induced RAW264.7 murine macrophage cells to release considerable amounts of nitric oxide (NO). Inflammatory cytokines including IL-1 β , TNF- α , IL-6, IL-10 and IL-12 from were secreted from RAW264.7 cells induced with CP₁ polysaccharides. As the most immunostimulating fraction, CP₁ polysaccharides were homogeneous and formed of 1,3-linked galactose, 1,4-linked glucose and 1,3-linked fucose residues. Overall, these findings suggested that the polysaccharides isolated from *C. peregrina* can be utilized as potential natural

immunostimulant in functional foods or pharmaceutical industries.

Keywords *Colpomenia peregrina* · Polysaccharide · Molecular weight · Structure · Immunostimulating activity

Introduction

Fucoidan is a general name used for sulfated and fucose containing polysaccharides isolated from the cell wall of brown seaweeds. As it appears from the definition given above, these polysaccharides are consisted of different chemical and molecular structures depending on seaweed species (Yuguchi et al. 2016). However, growing conditions of the seaweeds and the method of choice employed to isolate the fucoidans add more complexity and diversity to their structures (Saravana et al. 2016).

Some of the well-established chemical structures of fucoidans which have been found in brown seaweeds include fucan sulfate with a (1→3)- α -Fucp backbone, fucogalactan with a (1→6)- β -Manp and (1→2)- β -Manp backbone, fucoglucuronomannan with a (1→2)- α -Manp, (1→4)- β -GlupA and α -Fucp backbone, and fucoglucuronan with a (1→3)- β -GlupA or (1→4)- β -GlupA and α -Fucp backbone (Duarte et al. 2001; Li et al. 2006; Bilan et al. 2010; Shevchenko et al. 2015).

Sulfates, uronic acids, proteins and monosaccharides as well as molecular weights and types of glycosidic linkages are the structural features of fucoidans varying among different species (Wu et al. 2016; Li et al. 2008). Any variations in the chemical structures of fucoidans have determinant effects on their bioactivities either through enhancing the activity or compromising the function (Li et al. 2008). This was evidently shown by an investigation

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conducted on the bioactivities of fucoidans from nine brown seaweeds including *Laminaria*, *Cladosiphon*, *Fucus* and *Ascophyllum* genera (Cumashi et al. 2007). The study reported various levels of fucose (23.0–58.7%), uronic acids (1.0–23.4%) and sulfates (15.1–36.3%) for the isolated fucoidans and then concluded that the discrepancy in the chemical structure is responsible for their different anti-inflammatory and anticoagulant properties (Cumashi et al. 2007). Although, the chemical complexity of fucoidans has made their structural elucidation rather a tedious and laborious task, it resulted in a wide spectrum of therapeutic activities such as anticancer, immunomodulatory, antithrombotic and anti-inflammatory (Cumashi et al. 2007; Shao et al. 2015). In spite of numerous researches conducted on the structural characterization and biological properties of fucoidans from brown seaweeds, there are many more species remaining to be explored for their bioactive polysaccharides with novel structural specifications and health promoting effects.

Colpomenia peregrina is one of the brown seaweeds widely distributed along the coastal lines of Caspian Sea in North and Persian Gulf and Oman Sea in South of Iran. Despite the abundance and easy access, seaweeds have not been used as food materials in Iran due to strong reliance on land vegetables. Hence, it seems exploring new bioactive compounds from available species and introducing seaweed-based functional foods and nutraceuticals could promote seaweed consumption. Therefore, in the present study, water-soluble polysaccharides were isolated from *C. peregrina* to evaluate their potential utilization as functional foods with immunostimulatory properties. To that end, chemical compositions, monosaccharide components, molecular weights and glycosidic linkages were determined for the isolated polysaccharides. In vitro anticancer activity in association with immunomodulatory properties of the polysaccharides were evaluated on both cellular and molecular levels.

Materials and methods

Materials

Colpomenia peregrina samples were randomly collected from the coast of Caspian Sea, Noor, Iran in January 2016. Sample species was identified in the College of Marine Sciences at Tarbiat Modares University through morphological studies. The raw material was washed with tap water and air dried at 60 °C. The dried seaweed was milled using a blender, sieved (< 0.5 mm) and kept in plastic bags at – 20 °C. All other chemicals and reagents were of analytical grade. RPMI-1640 medium and fetal bovine serum (FBS) used in cell culture were purchased from

Lonza (Walkersville, MD, USA). Alcalase was purchased from Sigma-Aldrich (USA). The following antibodies (Abs) were used in this study: anti-phospho-NF- κ B rabbit polyclonal, anti-phospho-JNK rabbit polyclonal, anti-phospho-pERK1/2 rabbit polyclonal, anti-phospho-p38 rabbit polyclonal (Cell Signaling Technology) and anti- β -actin mAb (Sigma).

Isolation and fractionation of the polysaccharides

Seaweed powder (20 g) was treated with ethanol (80% EtOH, 200 mL) under constant stirring overnight at room temperature to remove pigments, lipids and low molecular weight compounds. The supernatants were discarded after centrifugation at 10 °C and 6080 $\times$ g for 10 min. The sediment was rewashed with EtOH (99%) under the same conditions, rinsed with acetone (99%), centrifuged at 10 °C and 6080 $\times$ g for 10 min and dried at room temperature in a fume hood. Polysaccharide extraction was carried out from 20 g of depigmented material with alcalase, Sigma (5% W:W, pH 8.0, 50 °C, 24 h). Enzymes were inactivated by keeping the extraction mixture at 100 °C for 10 min. The supernatants were collected after centrifugation at 10 °C and 6080 $\times$ g for 10 min. The extraction was carried out twice and the supernatants were combined and concentrated by evaporation under reduced pressure at 60 °C. Then, 1% CaCl₂ was added into the supernatants and kept at 4 °C overnight. The precipitated alginates were discarded after centrifugation. The polysaccharide precipitation was conducted in two steps using EtOH (99%) to obtain a final EtOH concentration of 30% (CP₁) and 70% (CP₂). The mixture was kept at 4 °C overnight and the precipitate was obtained after centrifugation at 10 °C and 6080 $\times$ g for 10 min. Polysaccharides were washed and dehydrated with EtOH (99%), acetone (99%), and then dried at room temperature. Polysaccharides were dissolved in water and dialyzed against distilled water using filter membrane (3500 Da MWCO) for 3 days after which solutions freeze dried to obtain polysaccharide powder (Borazjani et al. 2017). The yields of isolated polysaccharides were calculated in relation to the depigmented material obtained after 80% EtOH treatment.

Purification of polysaccharides

The CP₁ polysaccharides (250 mg) were dissolved in distilled water (10 mL) and fractionated on DEAE Sepharose fast flow column (17-0709-01; GE Healthcare Bio-Science AB, Uppsala, Sweden) using water and different concentrations of NaCl (from 0.5 to 2 M) as eluents. Only a single fraction was obtained after ion exchange chromatography. Therefore, CP₁ polysaccharides were eluted on a Sepharose CL-2B column (65099-79-8; Sigma) using water. To

prepare the sample, 250 mg of CP₁ polysaccharides were dissolved in distilled water (10 mL) at 65 °C for 15 min and filtered using a 3.0 µm filter. The elution of CP₁ polysaccharides also resulted in one fraction.

Chemical compositions

Lowry method was employed to measure the amount of protein using a DC protein assay kit (Bio-Rad, CA, USA) (Lowry et al. 1951). The determination of neutral sugar amount of the isolated polysaccharides was carried out using the phenol–sulfuric acid method and D-glucose as a standard (Dubois et al. 1956). After hydrolysis of polysaccharides (3 mg) with 0.5 M HCl and addition of BaCl₂ containing gelatin, the amount of sulfate was evaluated using K₂SO₄ as a standard (Dodgson and Price 1962). The amount of uronic acid was determined by sulfamate/*m*-hydroxydiphenyl method and glucuronic acid was used as a standard (Filisetti-Cozzi and Carpita 1991).

Monosaccharide composition of polysaccharides

The monosaccharide composition of polysaccharides (3 mg) extracted from *C. peregrina* were determined after hydrolysis with 4 M trifluoroacetic acid at 100 °C for 6 h. This was followed by reduction reaction using NaBD₄ (5 mg) at room temperature. Then, acetylation process was carried out by acetic anhydride (0.5 mL) at 100 °C (Ciucanu and Kerek 1984). Finally, a gas chromatography–mass spectrometry (6890N/MSD5973, Agilent Technologies, Santa Clara, CA) equipped with HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies, Santa Clara, CA) was used to analyze the derivatives.

Glycosidic linkage analysis

The methylation analysis of polysaccharides were carried out based on Ciucanu and Kerek method (1984). Briefly, 0.5 mL of DMSO (dimethyl sulfoxide) was added to lyophilized samples (3 mg) under nitrogen, followed by NaOH (20 mg) addition. Methyl iodide (CH₃I, 0.5 mL) was added after 15 min and the reaction proceeded for 45 min. The methylated derivatives were hydrolyzed by 0.5 mL of 4 M TFA at 100 °C for 6 h. The hydrolyzed derivatives were reduced by NaBD₄ (5 mg) and acetylated with 0.5 mL of acetic anhydride at 100 °C. A gas chromatography mass spectrometry (GC–MS) system (6890N/MSD 5973, Agilent Technologies, Santa Clara, CA) equipped with HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies, Santa Clara, CA) was used to identify the partially methylated alditol acetate derivatives. The carrier gas was Helium which was used as at a constant flow rate of

1.2 mL/min. The oven temperature was set as follows: from 160 to 210 °C for 10 min and then to 240 °C for 10 min. The temperature gradient was 5 °C/min and the inlet temperature was kept constant at 250 °C. The mass range was set to measure between 35 and 450 *m/z* (Tabarsa et al. 2017).

Molecular properties

Polysaccharides were solubilized in distilled water (2 mg/mL) and heated for 30 s in a microwave bomb (#4872; Parr Instrument Co., Moline, IL, USA) for complete solubilization (Rahimi et al. 2016). Prior to injection, samples were filtered through a 3 µm cellulose acetate filter unit (ADVANTEC) to remove any large contaminants or dust particles. Twenty microliters of each sample were loaded onto the size exclusion chromatography system operating at a flow rate of 0.4 mL/min with 0.15 M NaNO₃ and 0.02% NaN₃. The high performance size exclusion chromatography (HPSEC) system was composed of a TSK G5000PW column (7.5 × 600 mm; Toso Biosep, Montgomeryville, PA, USA) connected to a multi-angle laser light scattering (HELEOS; Wyatt Technology Corp, Santa Barbara, CA, USA) and a refractive index detector (Waters, 2414) (HPSEC-MALLS-RI). The volume delays among the MALLS and RI detectors were determined using bovine serum albumin (BSA). The weight average molecular weight (*M_w*) and radius of gyration (*R_g*) was calculated by ASTRA 5.3 software (Wyatt Technology Corp.) (Anvari et al. 2016).

The specific volume of gyration (*SV_g*) is another important property of the polysaccharide molecules that can be estimated using *M_w* and *R_g* values by the following equation (You and Lim 2000):

$$SV_g = 4/3\pi(R_g \times 10^8)^3 / (M_w/N) = 2.522R_g^3/M_w$$

where the units for *SV_g*, *M_w* and *R_g* are cm³/g, g/mol and nm respectively, and *N* is Avogadro's number (6.02 × 10²³/mol).

In vitro anticancer activity assay

The anticancer activity of the polysaccharides was evaluated using a human cervical cancer cell line (HeLa, ATCC). The HeLa cells were seeded in a 96 well microplate and incubated for 4 h at 37 °C in the presence of 5% CO₂. The polysaccharide solutions (100 µL) were added into the wells at concentrations of 100, 200 and 400 µg/mL. 5-fluorouracil was used as positive control (10 µg/mL). After incubation for 72 h, the anticancer activity of the polysaccharides was determined using the WST-1

colorimetric assay kit (Roche Diagnostics, Madison, WI, USA).

RAW264.7 macrophage proliferation and nitric oxide production assays

Aliquots (1×10^4 cells/well, 100 μ L volume) of a RAW264.7 macrophage cells grown in an RPMI-1640 medium containing 10% fetal bovine serum (FBS) were plated in a 96-well microplate. One hundred microliter of samples with different concentration (10, 25 and 50 μ g/mL) was incubated with cells for 24 h at 37 °C in humidified atmosphere containing 5% CO₂. Then, the WST-1 solution (20 μ L) was added to each well and incubated for 4 h at 37 °C. The optical density of the mixture was determined at 450 nm using a microplate reader (EL-800; BioTek Instruments, Winooski, VT, USA) (Tabarsa et al. 2015). The level of macrophage proliferation was calculated using the following equation:

Macrophage proliferation (%) = $A_t/A_c \times 100$, in which A_t the absorbance of the test group and A_c is the absorbance of the control group (cells without samples addition).

To indicate immunoenhancing activity of polysaccharides extracted with different methods, the nitric oxide (NO) secreted by RAW264.7 cells into culture supernatants was measured by Griess reagent (Green et al. 1982). In brief, RAW264.7 cells (1×10^5 cells/well) in a 96-well plate were treated with varying concentration of either polysaccharide samples (10, 25 and 50 μ g/mL) or LPS (1.0 μ g/mL) at 37 °C for 18 h. The level of NO production was quantified using the Griess reagent. A range of 1–200 μ M NaNO₂ in culture medium was used as reference to quantify the NO amount produced by macrophages (Tabarsa et al. 2015).

Cytokine gene expression

Different concentrations of either polysaccharide or LPS were added to RAW264.7 macrophage cells with density of 1×10^5 cells/well and incubated at 37 °C for 18 h. The total RNA was extracted from RAW264.7 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and according to the manufacturer's protocol. The isolated RNA concentration was determined using a spectrophotometer and construction of cDNA was carried out with an oligo-(dT)₂₀ primer and Superscript III RT (Invitrogen, Carlsbad, CA, USA). The GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) and specific primers were employed for PCR amplification. The primers used were as follows: iNOS, 5'-CCCTTCCGAAGTTTCTGGCAG-CAGC-3' (forward) and 5'-GGCTGTCA-GAGCCTCGTGGCTTTGG-3' (reverse); IL-1 β , 5'-ATGGCAACTATTCTGAAGTCAACT-3' (forward) and

5'-CAGGACAGGTATAGATTCTTTCCTTT-3' (reverse); IL-6, 5'-TTCCTCTCTGCAAGAGACT-3' (forward) and 5'-TGTATCTCTCTGAAGGACT-3' (reverse); IL-10, 5'-TACCTGGTAGAAGTGATGCC-3' (forward) and 5'-CATCATGTATGCTTCTATGC-3' (reverse); TNF- α , 5'-ATGAGCACAGAAAGCATGATC-3' (forward) and 5'-TACAGG CTTGTCACTCGAATT-3' (reverse); IL-12, 5'-CCACAAAGGAGGCGAGACTC-3' (forward) and 5'-CTCTACGAGGAACGCACCTT-3' (reverse) and β -actin, 5'-TGGAATCCTGTGGCATCCATGAAAC-3' (forward) and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3' (reverse). To run the reverse transcriptase amplification, a sequential process (30 cycles) of denaturation (94 °C for 30 s), annealing (56 °C for 40 s) and extension (72 °C for 1 min) was carried out which was ended with a final extension step at 72 °C for 10 min. The PCR products were run on a 1% agarose gel electrophoresis and visualized by ethidium bromide staining (Tabarsa et al. 2015).

Statistical analyses

All experiments were conducted in triplicate ($n = 3$) and statistical analysis performed using SPSS software (Version 16; SPSS Inc., Chicago, IL, USA). Significant differences were identified by one-way analysis of variance (ANOVA) and Duncan's multiple-range test. A probability value of $p < 0.05$ was considered to be statistically significant.

Results and discussions

Chemical composition of polysaccharides from *C. peregrina*

The water-soluble polysaccharides extracted from *C. peregrina* were partitioned through stepwise precipitations using 30% and 70% EtOH resulting polysaccharides namely CP₁ and CP₂, respectively. The yields, chemical compositions and sugar constituents of the partitioned polysaccharides are presented in Table 1. As calculated with respect to the dry weight of depigmented seaweed powder, the yield of CP₁ polysaccharides was found to be notably high (17.6%); whereas that of CP₂ polysaccharides was significantly lower (5.2%). So far, a wide range of extraction yields has been reported for the polysaccharides from brown seaweeds ranging from 1.5 to 14.7%. While seaweeds such as *Sargassum muticum* and *Agarum cribrosum* contained higher levels of water soluble polysaccharides, *S. fusiforme* and *S. mclurei* showed lower extraction yields (Cho et al. 2014; Thinh et al. 2013; Flórez-Fernández et al. 2017). The chemical analysis revealed that CP₁ polysaccharides were chiefly consisted of

Table 1 Yield and chemical composition of the polysaccharides from *C. peregrina*

	CP ₁	CP ₂
Chemical compositions (%)		
Yield (%)	17.6	5.2
Neutral sugars	73.79 ± 0.29	67.01 ± 0.31
Protein	3.44 ± 0.76	14.78 ± 1.52
Uronic acid	9.43 ± 0.26	14.89 ± 0.50
Sulfate	4.91 ± 0.12	4.87 ± 0.19
Monosaccharide (%)		
Fucose	24.56 ± 0.09	20.62 ± 0.07
Glucose	50.00 ± 0.15	52.91 ± 0.13
Galactose	25.5 ± 0.11	26.94 ± 0.13
Molecular characteristics		
$M_w \times 10^3$ (g/mol) ^a	1890.95 ± 1.34	639.05 ± 1.62
R_g (nm) ^b	56.60 ± 0.00	57.55 ± 0.63
SV_g (cm ³ /g) ^c	0.24 ± 0.00	0.75 ± 0.02

^aWeight average molecular weight (M_w)^bRadius of gyration (R_g)^cSpecific volume of gyration (SV_g)

neutral sugars (73.79%) with small amounts of uronic acids (9.43%), sulfates (4.91%) and proteins (3.44%). The amount of neutral sugars was slightly lower in CP₂ polysaccharides whereas uronic acids (14.89%) and proteins (14.78%) were measured to be at higher levels. Similar amount of sulfate esters (4.87%) was determined in CP₂ polysaccharides. A similar chemical composition has been reported for fucoidans from *A. cribrosum*, *S. glaucescens* and *S. binderi* (Cho et al. 2014; Huang et al. 2016; Lim et al. 2016).

The sugar composition of the polysaccharide chains was determined and the results are presented in Table 1 and Fig. 1. As shown in the GC–MS chromatogram, there were three peaks recorded for the monosaccharide composition of CP₁ polysaccharides where the predominantly occurring sugar was glucose (50.00%). In addition, as the characteristic monosaccharide of brown seaweeds, the fucose content (24.56%) was found to be nearly as high as that of galactose (25.50%) in the polysaccharide chain. Likewise, the CP₂ polysaccharides were mainly formed of glucose (52.91%) with lower levels of fucose (20.62%) and galactose (26.94%).

The fraction with the highest yield, CP₁ polysaccharide, was chosen for further purification. As shown in Fig. 2a, the employment of DEAE Sepharose fast flow column resulted only a single fraction. Similarly, the CP₁ polysaccharides were eluted from Cellulose CL-2B column as one fraction (Fig. 2b). These results implied that CP₁ polysaccharides were formed of molecules with

homogeneous charge intensity and size distribution. Therefore, the rest of experiments were carried out on CP₁ and CP₂ polysaccharides.

Molecular properties of the polysaccharides

The RI (refractive index) chromatograms of CP₁ and CP₂ polysaccharides eluted from the SEC column are presented in Fig. 2c, d. The molecules of CP₁ polysaccharides were eluted from the SEC column as one peak between elution times of 24 and 46 min indicating the presence of one type of polymers with similar molecular weights. In contrast, the polysaccharide polymers of CP₂ were eluted from SEC column as two peaks between elution times of 25 and 51 min which implied the existence of heterogeneous polysaccharides with different molecular weights. The M_w of CP₁ and CP₂ polysaccharides were 1890.95×10^3 and 639.05×10^3 g/mol, respectively (Table 1). Similar molecular sizes were also estimated by the calculated R_g for CP₁ (56.60 nm) and CP₂ (57.55 nm) polysaccharides. The molecular weights of fucoidans reported from brown seaweeds seem to be very diverse and vary greatly from the very high molecular weight polysaccharides of 2420.0×10^3 g/mol from *A. cribrosum* to the very low molecular weight polysaccharides of 27.9×10^3 g/mol from *S. binderi* (Dubois et al. 1956; Lim et al. 2016). These large discrepancies in the molecular weights of the fucoidans on one hand has been attributed to species differences of tested seaweeds and on the other hand were related to the variables involved in the extraction protocols and seaweeds environmental conditions (Saravana et al. 2016; Huang et al. 2016; Fletcher et al. 2017).

Using M_w and R_g , the SV_g values were calculated for CP₁ and CP₂ polysaccharides. Basically, the SV_g value is the theoretical gyration volume per unit of molar mass with an inverse relationship to the degree of molecular compactness. The SV_g value of CP₁ polysaccharide was 0.24 cm³/g; whereas the SV_g value of CP₂ polysaccharide was considerably high (0.75 cm³/g) (Table 1). These findings indicated that the molecules of CP₁ polysaccharides had compact structures and CP₂ polysaccharides had more loosed and expanded conformation.

Bioactivities of partitioned polysaccharides

The effect of extracted polysaccharides on the growth of HeLa cancer cells was studied over the concentration range of 100–400 µg/mL. Both CP₁ and CP₂ polysaccharides exhibited similar inhibitions on the growth rate of HeLa cells (Fig. 3a). The tested polysaccharides exerted relatively lower cytotoxicity against cancer cells which reached nearly 40% at the highest concentrations. An overview of the previous studies shows that there are many

Fig. 1 GC chromatograms of monosaccharides of CP₁ (a) and CP₂ (b) polysaccharides from *C. peregrina* and standard (c)

reports on the effectiveness of fucoidans on the growth inhibition of various cancers through different pathways (Wu et al. 2016). However, it has to be mentioned that the magnitude of fucoidans direct anticancer activities vary significantly depending on polysaccharide structure and cancer type (Wu et al. 2016; Cho et al. 2014). The polysaccharides with little anticancer activities were found to be potent agents in treating cancers through the stimulation of immune system (Tabarsa et al. 2015). The immune system is involved in defense against tumors during which macrophage cells play a pivotal role because of their ability to selectively destroy a broad range of tumor types after specific activation (Weiming et al. 2002). The effect of CP₁ and CP₂ polysaccharides on the proliferation of RAW264.7 macrophage cells was examined over the concentration range of 10–50 µg/mL. As shown in Fig. 3b, CP₁ polysaccharides not only were safe and nontoxic to RAW264.7 cells at all concentrations, but also stimulated the macrophage cells to proliferate up to 118% ($p < 0.05$). Although it was less effective than CP₁ polysaccharides, CP₂ polysaccharides showed significant proliferating effect when incubated with macrophage cells.

The immunostimulatory effect of extracted polysaccharides was evaluated by measuring the amount of NO released from RAW264.7 cells after incubation with CP₁ and CP₂ polysaccharides. The level of NO secreted from macrophage cells after treating with CP₁ and CP₂ polysaccharides at concentrations of 10, 25 and 50 µg/mL

is presented in Fig. 3c. The CP₁ polysaccharides induced a considerable NO release from the cells in a dose dependent manner up to 49.6 µmol ($p < 0.05$). This level of stimulation was comparable with that of lipopolysaccharide (LPS) used at 1 µg/mL. The CP₂ polysaccharides showed weaker effect on macrophage cells releasing only 39 µmol at the highest concentration ($p < 0.05$). In basic, macrophage NO is a free radical gasotransmitter that its regulation is under the transcriptional control of cytokines, LPS and other mediators. Activated macrophages generate large concentrations of NO after which tumor cells can be killed through mechanisms such as inhibition of DNA synthesis and direct damage of DNA (MacMicking et al. 1997).

To further confirm the results obtained above, the RAW264.7 macrophage cells stimulated with polysaccharides and LPS were collected and the whole mRNA contents of the cells were extracted afterwards. Using specific primers, the possibility of mRNA expression of inducible nitric oxide synthase (iNOS) which catalyzes the production of NO from L-arginine, was investigated on the molecular level (Förstermann and Sessa 2011). As shown in Fig. 4a, strong and distinctive bands were observed for the PCR products indicating the expression of iNOS mRNA and thus synthesis of NO after polysaccharide stimulations. The level of iNOS mRNA expression induced by polysaccharides and LPS was consistent with that of NO production, as calculated by graphic analysis of the PCR products on the agarose gel (Fig. 4b). Cytokines are

Fig. 2 The DEAE Sepharose FF (a) and Sepharose CL-2B (b) elution profiles of CP₁ polysaccharides. The HPLC chromatograms of CP₁ (c) and CP₂ (d) polysaccharides determined by a TSK G5000 PW column at a flow rate of 0.4 mL/min

signaling proteins that are released from macrophages and their primary function is to regulate inflammation (Arango Duque and Descoteaux 2014). In the present study, the mRNA expression of cytokines including IL-1 β , TNF- α , IL-6, IL-10 and IL-12 was also studied in the macrophage cells (Fig. 4a, c–g). In accordance with NO production, CP₁ polysaccharides were able to significantly increase the mRNA expressions of these cytokines dose dependently. It is well known that the inflammatory response is triggered by pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF α . Consequently, these cytokines affect other cell types leading to the secretion of other inflammatory mediators and creation of multiple inflammatory cascades (Hernandez-Rodriguez et al. 2003). The overexpression of proinflammatory cytokines leads to sever inflammation symptoms and inflammatory diseases to prohibit which anti-inflammatory cytokines are produced to down regulate their expressions (Sultani et al. 2012). In current study, the mRNA expression of IL-10 was studied and the results showed the potential anti-inflammatory effect of CP₁ and CP₂ polysaccharides (Fig. 4f).

Glycosidic linkage analysis of CP₁ polysaccharides

The CP₁ polysaccharides were chosen as the most potent macrophage stimulating fraction for further structural analysis. The types and ratios of glycosidic linkages of CP₁ polysaccharides were found from the partially methylated alditol acetate products after GC–MS injection. As presented in Table 2, the most abundant derivatives were 2,3,6-tri-*O*-methyl-Glu and 2,4,6-tri-*O*-methyl-Gal which indicated the presence of 1,4-linked glucose and 1,3-linked galactose residues in the backbone of CP₁ polysaccharides. Lower amounts of 1,3-linked glucose residues were also included in the polysaccharide chain. The existence of 2,6-di-*O*-methyl-Glu derivative showed the inclusion of branch points as 1,3,4-linked glucose in the CP₁ polysaccharide. The inclusion of fucose monosaccharides in the polysaccharide structure was in form of 1,3-linked fucose residues. Besides, some levels of fucose and galactose residues were found to be presented as non-reducing terminals. The ratios of glycosidic linkages of (1 $\rightarrow$)-Fuc, (1 $\rightarrow$ 3)-Fuc, (1 $\rightarrow$)-Gal,

Fig. 3 Anticancer activity against HeLa cells (a), RAW264.7 cells proliferation activity (b) and nitric oxide production (c) of the RAW264.7 macrophage cells after treatment with CP₁ and CP₂ polysaccharides. Superscripts a, b, c, d show significant differences ($p < 0.05$) among different treatments, and A, B, C, D show significant differences ($p < 0.05$) among different concentrations

Fig. 4 Effects of CP₁ and CP₂ polysaccharides on the mRNA expression of cytokines in RAW264.7 cells. Superscripts a–d show significant differences ($p < 0.05$) among different treatments, and A–

D show significant differences ($p < 0.05$) among different concentrations. β -actin was used as a control

Fig. 4 continued

Table 2 Glycosidic linkage analysis of the constituent sugars of the fraction F₁ from *C. peregrina*

Glycosidic linkage	Methylation	Peak ratio (%)
Fuc-(1→	1,5-di- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methyl-Fuc	7.62
→3)-Fuc-(1→	1,3,5-tri- <i>O</i> -acetyl-2,4-di- <i>O</i> -methyl-Fuc	10.25
Gal-(1→	1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl-Gal	6.23
→3)-Gal-(1→	1,3,5-tri- <i>O</i> -acetyl-2,4,6-tri- <i>O</i> -methyl-Gal	20.30
→4)-Glu-(1→	1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl-Glu	29.31
→3)-Glu-(1→	1,3,5-tri- <i>O</i> -acetyl-2,4,6-tri- <i>O</i> -methyl-Glu	9.12
→3,4)-Glu-(1→	1,3,4,5-tetra- <i>O</i> -acetyl-2,6-di- <i>O</i> -methyl-Glu	17.17

(1→3)-Gal, (1→4)-Glu, (1→3)-Glu and (1→3,4)-Glu were calculated to 0.26:0.35:0.21:0.69:1.0:0.31:0.58.

In conclusion, alcalase extraction of polysaccharides from *C. peregrina* resulted in high extraction yield. Step-wise ethanol precipitations produced two fractions with different chemical compositions and molecular weights. The CP₁ polysaccharides contained higher acidic sugars and notably lower molecular weight. The CP₁ polysaccharides were the most potent immunostimulating polysaccharides with the highest potential to release cytokines from macrophage cells via NF-κB and MAPKs signaling pathways. CP₁ polysaccharide was consisted of 1,3-linked galactose, 1,4-linked glucose and 1,3-linked fucose residues. The novel findings of the present study suggested the potential application of fucogalactoglucan isolated from *C. peregrina* as immunostimulatory agent to improve innate immunity function. However, further researches need to be conducted on structure–activity relationships of these polysaccharides before any applications.

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