ORIGINAL PAPER

Structural Elucidation and Immunostimulatory Activities of Quinoa Non-starch Polysaccharide Before and After Deproteinization

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Abstract

Non-starch polysaccharides derived from natural resources play a significant role in the field of food science and human health due to their extensive distribution in nature and less toxicity. In this order, the immunostimulatory activity of a non-starch polysaccharide (CQNP) from *Chenopodium quinoa* was examined before and after deproteination in murine macrophage RAW 264.7 cells. The chemical composition of CQNP and deproteinated-CQNP (D-CQNP) were spectrometrically analysed that revealed the presence of carbohydrate ($22.7 \pm 0.8\%$ and $39.5 \pm 0.8\%$), protein ($41.4 \pm 0.5\%$ and $20.8 \pm 0.5\%$) and uronic acid ($8.7 \pm 0.3\%$ and $6.7 \pm 0.2\%$). The monosaccharide composition results exposed that CQNP possesses a high amount of arabinose (34.5 ± 0.3) followed by galactose (26.5 ± 0.2), glucose (21.9 ± 0.3), rhamnose (7.0 ± 0.1), mannose (6.0 ± 0.1) and xylose (4.2 ± 0.2). However, after deproteination, a difference was found in the order of the monosaccharide components, with galactose (41.1 ± 0.5) as a major unit followed by arabinose (34.7 ± 0.5), rhamnose (10.9 ± 0.2), glucose (6.6 ± 0.2), mannose (3.4 ± 0.2) and xylose (3.2 ± 0.2). Further, D-CQNP potentially stimulate the RAW 264.7 cells through the production of nitric oxide (NO), upregulating inducible nitric oxide synthase (iNOS) and various pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-alpha (TNF-α). Moreover, stimulation of RAW 264.7 cells by D-CQNP takes place along the NF-κB and the MAPKs signaling pathways through the expression of cluster of differentiation 40 (CD40). This results demonstrate that RAW 264.7 cells are effectively stimulated after removal of the protein content in *C. quinoa* non-starch polysaccharides, which could be useful for develop a new immunostimulant agent.

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Graphical Abstract
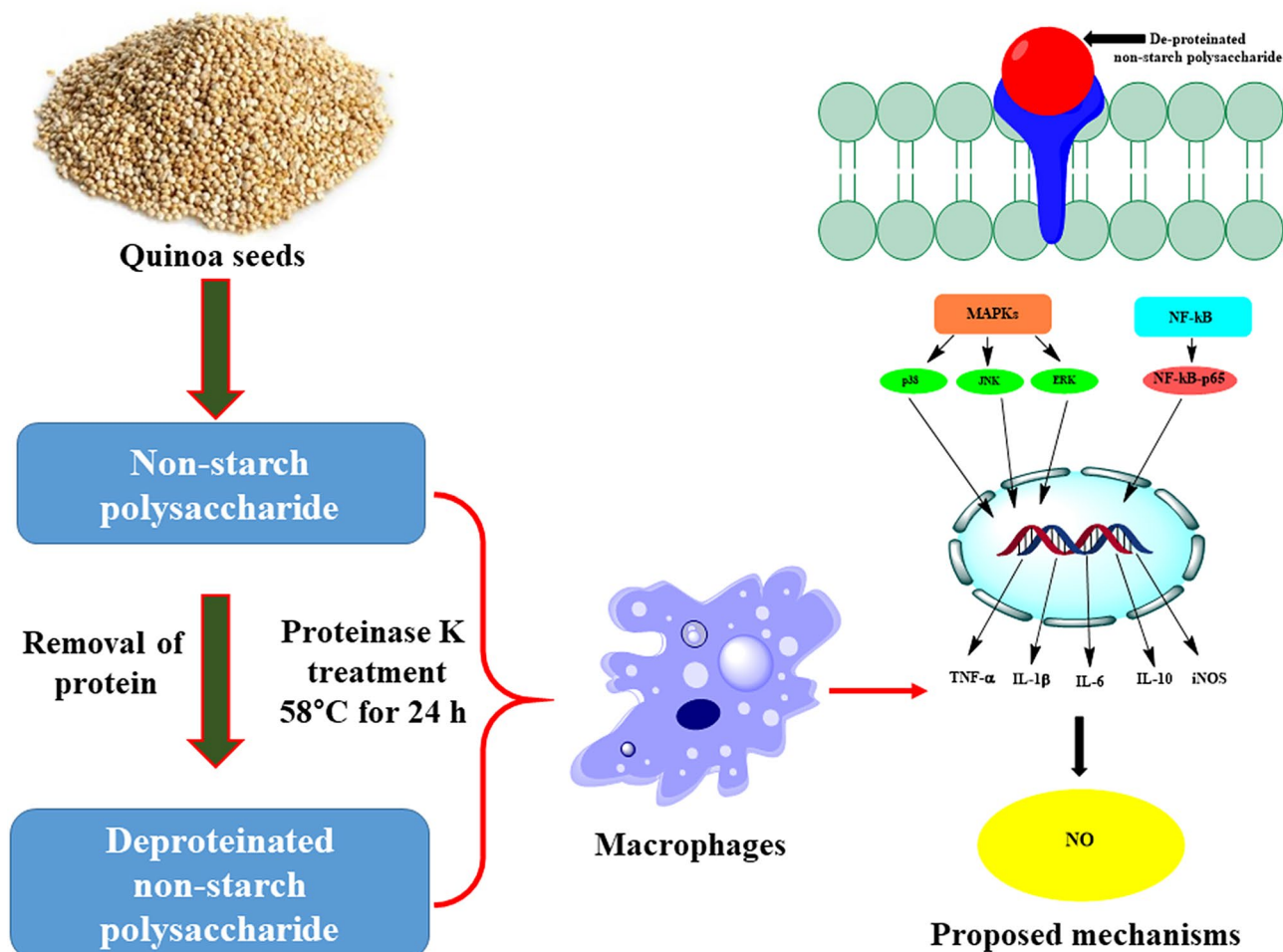
Introduction

Quinoa (*Chenopodium quinoa* Willd.) is one of the ancient Pseudo-cereal crops belonging to the family of Chenopodiaceae. It is a broadleaf non-grasses plant originated from South America and their seeds have been included in cereal-based foods. It is largely produced by small farms and associations in the Andean region of South America. Nevertheless, it is currently cultivated in more than 70 countries, including Kenya, India, United States and many European countries. It considers as a starch-rich component that is high in carbohydrates and also contains starch and small amounts of sugars. Quinoa starch content varies from 51 to 61% and its granule diameter is ˂3 µm [1]. Currently, quinoa is gaining more attention in the nutrition and pharmaceutical industries because it is rich in nutrients, especially the dietary fiber content (1.1% to 16.3%)

is higher than other cereals such as rice (0.4%), wheat (2.7%) and corn (1.7%). Also, there is no gluten compared to other grains [2]. Quinoa is important for patients with celiac disease because it is believed that gluten-free fiber deficiency can be prevented by adding quinoa seeds to the diet [2].

Previous studies on quinoa proteins have shown that it contains a balanced essential amino acid composition and high levels of essential amino acids. Its value is higher than that of common grains [3]. The lipid content of quinoa is two to three times higher than that of general grains and contains high levels of unsaturated fatty acids, which contribute an important role in nutrition [2]. In addition, it contains some important micronutrients such as minerals and vitamins, as well as significant amounts of bioactive components such as polyphenols, flavonoids that exhibit various biological activities [4, 5]. It has been

reported that quinoa can help reduce the risk of complications such as diabetes, heart disease, obesity, anemia and dyslipidemia [6].

Non-starch polysaccharides are the important components of dietary fiber that are formed by the binding of many monosaccharide units by glycosidic bonds, which play an important role in regulating cellular growth and development. Several studies report that non-starch polysaccharides of cereals have shown countless biological activities including immunogenic [7], anti-oxidants [8], anti-atherosclerogenic [9], anti-cancer [10] and anti-diabetics [11]. Of these, immunostimulatory activities are directly connected to health benefits. Promoting innate immunity in a controllable way will enhance the host defence activities [12]. Recently, Barbosa and de Carvalho Junior stated that the immunomodulatory potential of polysaccharides is used to assess the ability of these biopolymers to enhance the body immunity towards viral infections, especially SARS-CoV-2 [13]. Polysaccharides from various natural sources such as plants [14], algae [15], fungi [16–18] stimulates the innate and cellular community via interactions with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes [19]. When stimulated, the macrophage can destroy the pathogens directly by phagocytosis and indirectly through the secretions of NO and various cytokines TNF-α, IL-1β, and IL-6 [20]. Usually, non-starch polysaccharide stimulate the macrophages by binding to pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), Dectin-1, and complement receptor type 3 (CR3), and trigger the signal transduction pathways including phosphoinositide-3-kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPKs), as well as transcription factors such as nuclear factor (NF)-κB and activator protein (AP) [7]. Numerous researches have been done to evaluate the immunomodulatory activity of non-starch polysaccharides derived from various natural resources [7, 21–24]. However, there are only a few investigations into the effect of structural changes in polysaccharide on macrophage activity.

Hence, we aimed to study the structural characteristics of non-starch polysaccharides (CQNP) and deproteinated CQNP (D-CQNP) from *C. quinoa*, and explored the possible immunostimulatory activity on the murine RAW 264.7 macrophage cell line. We inspected the immunogenic effect of CQNP and D-CQNP by examining the production of NO, cytokines, inducible nitric oxide synthase (iNOS), and TNF-α. We also determined the signaling pathway associated with the immunostimulatory activity of CQNP and D-CQNP by examining the NF-κB and MAPK via a cluster of differentiation 40 (CD40) and CD11b expression.

Materials and Methods

Chemical and Reagents

Cell culture media and its supplements were purchased from Lonza, Inc. (Walkersville, MD, USA). The major enzymes such as α-amylase and proteinase K were attained from Sigma-Aldrich, St. Louis, MO, USA. The primary antibodies such as anti-phospho-NF-κB p65, anti-phospho-c-Jun N-terminal kinase (JNK), anti-phospho-extracellular signal-regulated kinase (ERK1/2), and anti-phospho p38, and secondary antibody (horseradish peroxidase-conjugated anti-rabbit antibody) were obtained from Abcam, Cambridge, UK. The antibodies for flow cytometry analysis such as anti-CD40-APC (1C10) and anti-CD11b (M1/70) were procured from ThermoFisher Scientific, Waltham, MA, USA.

Isolation of Non-starch Polysaccharide

In this study, the *C. quinoa* non-starch polysaccharide (CQNP) was isolated by hot water extraction and α-amylase treatment. The *C. quinoa* seeds were collected from the local market of XiNing City, QingHai Province, China. The seeds thoroughly cleaned with distilled water, dried at 45 °C, and were milled into a fine powder. For CQNP extraction, 20 g of powdered sample was extracted with 200 mL of distilled water at 65 °C. The collected extract was centrifuged at $4000 \times g$ for 15 min. Afterwards, the extract was mixed with an equal volume of phosphate-buffered saline (PBS, pH 6.0) and incubated with 4 mg of α-amylase (Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 55 °C. The reaction was arrested by raising the temperature to 100 °C for 10–15 min. Finally, the reaction mixture was filtered through a 110 nm size of the membrane, dialyzed against distilled water and freeze-dried.

Deproteination of CQNP

About, 1 mg of CQNP was dissolved in 0.1 M sodium phosphate buffer and then incubated with 10–20% of proteinase K (w/v) in a water bath at 58 °C for 24 h. The reaction was stopped by keep the sample at 100 °C for 10 min. Afterwards, the reaction mixture was centrifuged (10,000 rpm for 10 min) and the supernatant was dialyzed against distilled water and freeze-dried [25]. The deproteinated CQNP was named as D-CQNP.

Chemical and Monosaccharide Composition Analysis

The presence of carbohydrate, protein and uronic acid were examined by phenol–sulfuric acid [26], Folin-phenol reagent [27] and m-hydroxydiphenyl reaction [28], respectively. For the monosaccharide composition analysis, three milligrams of the sample was hydrolyzed with 0.5 mL of 4 M trifluoroacetic acid (TFA) at 100 °C for 6 h. The hydrolyzed product was reduced by $NaBD_4$ and then acetylated using acetic anhydride. The monosaccharide composition analysis was carried out by a gas chromatography–mass spectrometry analysis (GC–MS, 6890N/MSD 5973, Agilent Technologies, Santa Clara, CA, USA) equipped with the HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies).

Molecular Weight Analysis

The average M_w and R_g values were estimated by following the method of Tabarsa et al. [29]. Briefly, the sample was solubilized in distilled water (2 mg/mL) and heated for 30 S using a microwave bomb (Parr Instrument Co., Moline, IL, USA). Then, the samples were filtered through a cellulose acetate membrane (3.0 µm pore size; Whatman International) and injected into a TSK G5000 PW column (7.5 × 600 mm; Toso Biosep, Montgomeryville, PA, USA) coupled with a multi-angle laser light scattering (MALLS) detection (HELEOS; Wyatt Technology Corp., Santa Barbara, CA, USA) and refractive index (RI) detection (Waters, Milford, MA, USA) system (HPSEC–UV–MALLS–RI). The M_w was calculated using ASTRA 5.3 software (Wyatt Technology Corp.).

Glycosidic Linkage Analysis

The glycosidic linkage profile of the highest immunostimulant D-CQNP was investigated using the method of Ciucanu and Kerek [30]. Nearly, 3 mg of D-CQNP was fully dissolved in 0.5 mL dimethyl sulfoxide under a nitrogen stream. Add, 20 mg of NaOH to this solution. The methylation process was carried out by mixing of methyl iodide for 45 min and then hydrolyzed with 4 M TFA at 100 °C for 6 h. The hydrolysates were reduced in distilled water with $NaBD_4$ and acetylated using acetic anhydride at 100 °C. The partially methylated alditol acetates were subjected to GC–MS analysis (6890N/MSD 5973, Agilent Technologies) equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies).

Cell Proliferation and NO Production

In this study, the immunostimulatory activities of CQNP and D-CQNP were tested on RAW 264.7 macrophage cells (ATCC; Manassas, VA, USA and ATCC, Rockville, MD, USA). RAW 264.7 cells were grown in RPMI-1640 medium in the presence of 10% fetal bovine serum (FBS) under 5% CO_2 atmosphere condition. The cell proliferation activity was tested using WST-1 assay. RAW 264.7 cells were cultured in a 96-well microplate (1×10^6 cells/mL) with different concentration of CQNP or D-CQNP (12, 25 and 50 µg/mL) for 24 h at 37 °C. Add 100 µL of 10% WST-1 solution into the wells and extend the incubation for 1 h. The absorbance was read at 450 nm and the proliferation activity (%) was calculated using the following equation. Similarly, the cells were treated with CQNP or D-CQNP and the NO production was measured using Griess reaction [31].

$$Cell\ proliferation\,(\%) = Absorbance\ of\ sample\,/$$
$$Absorbance\ of\ control \times 100.$$

Real-Time PCR Analysis

RAW 264.7 cells (1×10^6 cells/mL) were incubated with polysaccharide or LPs (1 µg/mL) at the concentration of 50 µg/mL in a 96-well microplate under 5% CO_2 atmosphere for 18 h at 37 °C. The total RNA content was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was constructed using oligo-(dT) 20 primer and Superscript III RT (Invitrogen) by following the steps provided in the kit. PCR amplification was performed by a Real-Time PCR system using Fast Start DNA Master TB Green II kit (Takara Bio, Inc., Shiga, Japan) using the specific primers (Table 1).

Table 1 Sequences of the primers used in real-time PCR analysis

Gene	Sequences of the primers
iNOS	5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (forward)
	5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′ (reverse)
IL-1β	5′-ATGGCAACTATTCCTGAACTCAACT-3′ (forward)
	5′-CAGGACAGGTATAGATTCTTTCCTTT-3′ (reverse)
IL-6	5′-TTCC TCTCTGCAAGAGACT-3′ (forward)
	5′-TGTATCTCTCTGAAGGACT-3′ (reverse)
IL-10	5′-TACCTGGTAGAAGTGATGCC-3′ (forward)
	5′-CATCATGTATGCTTCTATGC-3′ (reverse)
TNF-α	5′-ATGAGCACAGAAAGCATGATC-3′ (forward)
	5′-TACAGGCTTGTCACTCGAATT-3′ (reverse)
β-Actin	5′-TGGAATCCTGTGGCATCCATGAAAC-3′ (forward)
	5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (reverse)

Western Blot Analysis

RAW 264.7 cells (1×10^6 cells/mL) were treated with CQNP or D-CQNP (50 µg/mL) for 18 h at 37 °C. Then, the cells were lysed in RIPA buffer for 30 min and centrifuged at $14{,}000 \times g$ (4 °C) for 15 min. The protein content was estimated by Micro BCA™ Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's guidelines. Simultaneously, the cell lysates containing nearly 30 µg of protein was loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein band was transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with particular primary antibodies such as anti-phospho-NF-κB p65, anti-phospho-c-Jun N-terminal kinase (JNK), anti-phospho-extracellular signal-regulated kinase (ERK1/2), and anti-phospho p38 (Abcam, Cambridge, UK) followed by secondary horseradish peroxidase conjugated anti-rabbit antibody. The proteins were identified by Pierce ECL Plus Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA, USA) and the expression was visualized by Bio-Rad image analysis system (Bio-Rad Laboratories).

Flow Cytometry Analysis

The polysaccharides treated RAW 264.7 cells (50 µg/mL) were washed with flow cytometry staining buffer and then incubated with specific antibodies such as anti-CD40-APC (1C10) and anti-CD11b (M1/70) for 30 min at 4 °C under the dark condition. The flow cytometry analysis was carried out by CytoFLEX flow cytometer (Beckman Coulter, High Wycombe, UK), and the data were analyzed using the CytExpert 2.3.0.84 software (Beckman Coulter).

Statistical Analysis

All experiments were performed in triplicate (n = 3), and the value was expressed as the average and standard deviation (SD). The statistical analysis was carried out using SPSS software (Version 16; SPSS, Inc., Chicago, IL, USA). The differences between groups were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple-range test, and a p-value of < 0.05 was considered statistically significant.

Table 2 Total yield, chemical composition, and monosaccharide content and molecular weight analysis of CQNP and D-CQNP obtained from *Chenopodium quinoa*

	Sample	
	CQNP	D-CQNP
Chemical composition (%)		
Yield (%)	1.7	20.4
Carbohydrate	22.7 ± 0.8	39.5 ± 0.8
Protein	41.4 ± 0.5	20.8 ± 0.5
Uronic acid	8.7 ± 0.3	6.7 ± 0.2
Monosaccharide content (%)		
Rhamnose	7.0 ± 0.1	10.9 ± 0.2
Arabinose	34.5 ± 0.3	34.7 ± 0.5
Xylose	4.2 ± 0.2	3.2 ± 0.2
Mannose	6.0 ± 0.1	3.4 ± 0.2
Glucose	21.9 ± 0.3	6.6 ± 0.2
Galactose	26.5 ± 0.2	41.1 ± 0.5

Samples	M_w (kDa)			R_g (nm)		
	Peak I	Peak II	Peak III	Peak I	Peak II	Peak III
Molecular weight (M_w) and radius of gyration (R_g)						
CQNP	148.5 ± 36.6	24.9 ± 1.9	n.d	60.3 ± 3.9	74.6 ± 2	n.d
D-CQNP	774.3 ± 13.5	90.9 ± 7.7	77.2 ± 6.8	57.5 ± 0.3	62.3 ± 0.4	62.3 ± 0.1

CQNP non-starch polysaccharide of *Chenopodium quinoa*, *D-CQNP* deproteinated non-starch polysaccharide of *Chenopodium quinoa*, *n.d.* not detected

Fig. 1 Monosaccharide composition analysis of non-starch polysaccharides extracted from *Chenopodium quinoa* using a gas chromatography–mass spectrometry analysis. (**A**) CQNP and (**B**) D-CQNP. *CQNP* non-starch polysaccharide of *Chenopodium quinoa*, *D-CQNP* deproteinated non-starch polysaccharide of *Chenopodium quinoa*
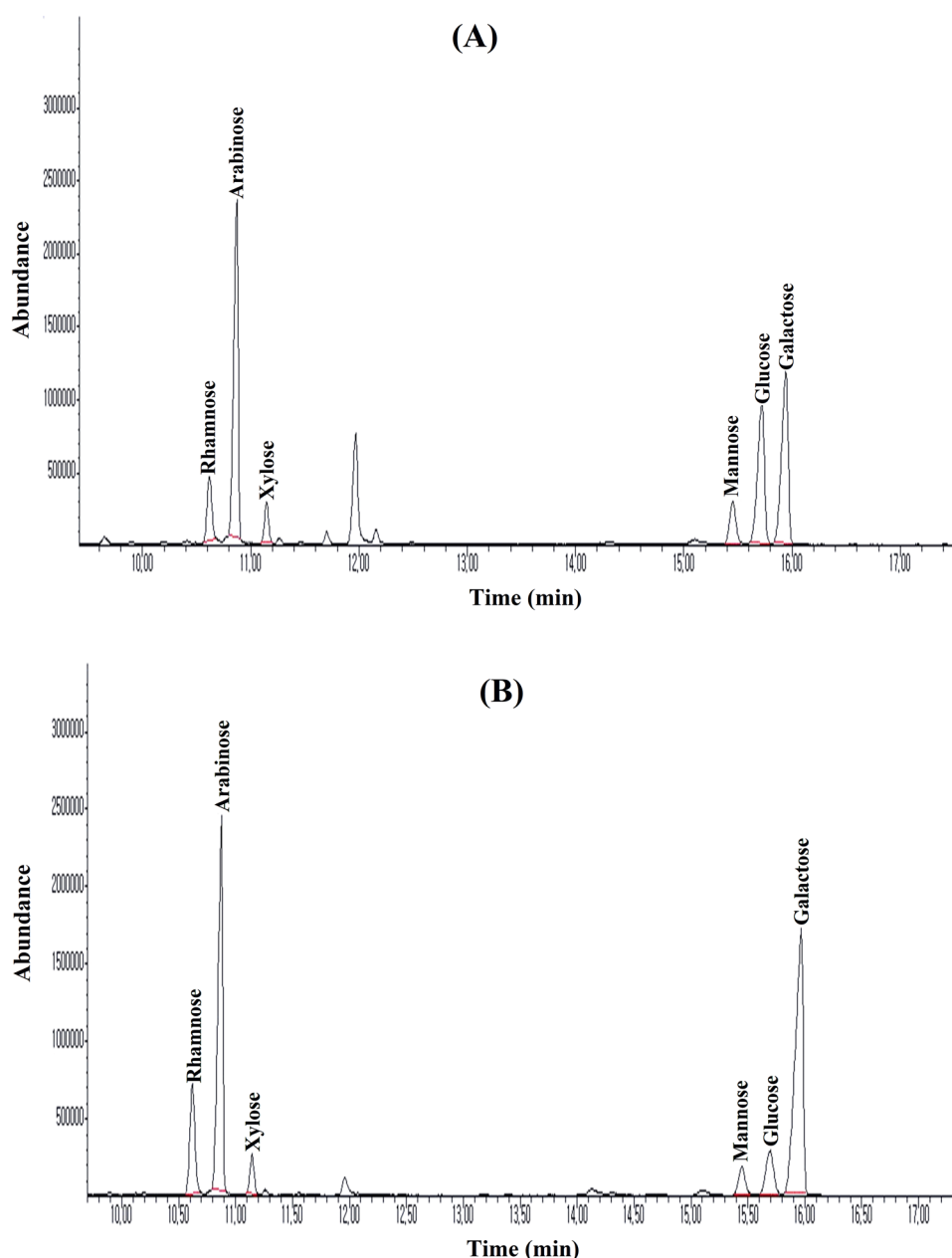
Results and Discussion

Proximate Composition of CQNP and D-CQNP

The non-starch polysaccharide from *C. quinoa* was extracted by hot water extraction and α-amylase treatment. The yield and chemical composition of CQNP was displayed in Table 2. The amount of extracted CQNP was 1.7% of the starting raw material. Primarily, the CQNP constituted by carbohydrate ($22.7 \pm 0.8\%$), protein ($41.4 \pm 0.5\%$) and uronic acid ($8.7 \pm 0.3\%$). Of these result, CQNP has a high protein content because the protein is not hydrolyzed before the extraction of the non-starch

polysaccharides [32]. Further, the existence of free protein in the polysaccharide maybe disturbs the structural and pharmaceutical properties. Hence, the protein content was removed from CQNP by proteinase K that showed notable changes in protein content. Totally, 20.4% of D-CQNP was obtained, which consisted of $39.5 \pm 0.8\%$ carbohydrate, $20.8 \pm 0.5\%$ protein, and $6.7 \pm 0.2\%$ uronic acid.
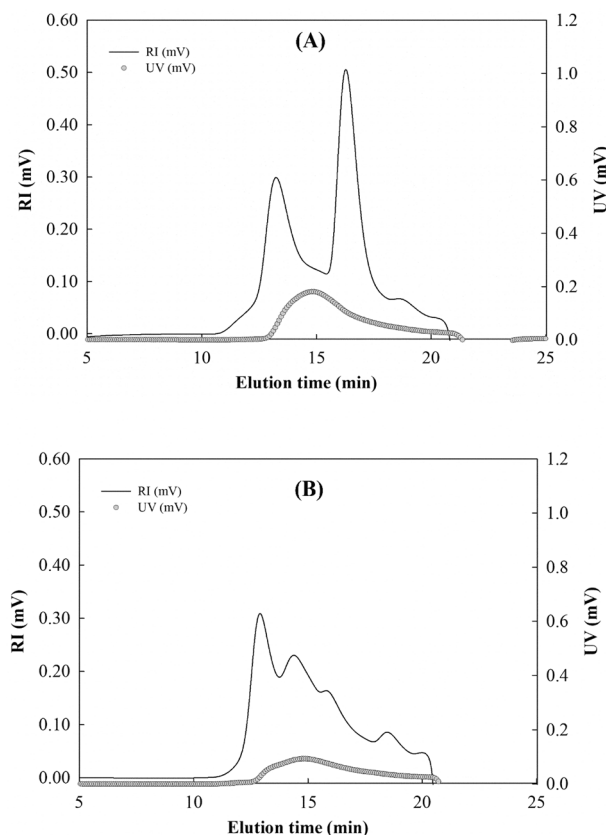
The monosaccharide composition analysis is one of the recognized tools to determine the quality of polysaccharide [33]. In this study, the monosaccharide composition of CQNP and D-CQNP was analyzed using GC–MS analysis. As shown in Table 2 and Fig. 1, the major constituents of CQNP were arabinose (34.5 ± 0.3), galactose

(26.5 ± 0.2), glucose (21.9 ± 0.3), rhamnose (7.0 ± 0.1), mannose (6.0 ± 0.1) and xylose (4.2 ± 0.2). However, these compositions were varied in D-CQNP which showed galactose (41.1 ± 0.5) as a major sugar unit followed by arabinose (34.7 ± 0.5), rhamnose (10.9 ± 0.2), glucose (6.6 ± 0.2), mannose (3.4 ± 0.2) and xylose (3.2 ± 0.2).

Molecular Weight Analysis

The molecular weight of polysaccharide is an important factor to be associated with various biological activities [34]. The average molecular weight of CQNP and D-CQNP were examined using a MALLS technique through a high-performance size exclusion column (HPSEC). As shown in Fig. 2 and Table 2, CQNP exhibited two different peak at the elution time between 11.6 and 14.7 min (peak I) and 15.4 and 17.9 min (peak II). The average molecular weight of CQNP calculated using the MALLS system, which was 148.5 ± 36.6 kDa and 24.9 ± 1.9 kDa, respectively. Next, the D-CQNP emerged from the HPSEC as three distinct peaks between 11 and 13.7 min (peak I), 13.8 and 15.4 min

(peak II), and 15.5 and 17.8 min (peak III) indicating that the molecular weight of 774.3 ± 13.5 kDa, 90.9 ± 7.7 kDa, and 77.2 ± 6.8 kDa, respectively. Similarly, the non-starch polysaccharide has a molecular weight of 15–150 kDa when isolated from green gram using hot water extraction [22]. Further, R_g values were calculated from the spectrum, which showed approximate sizes of the molecules ranging from 60.3 to 74.6 nm for CQNP and 57.5 to 62.3 for D-CQNP.

Cell Proliferation and Nitric Oxide Production

Generally, macrophages or monocytes play a significant role in both immunities, which considered being important immunocytes to protect the host from the pathogen, including cancer [35]. In this study, the immunostimulatory effect of CQNP and D-CQNP were tested on murine RAW 264.7 macrophage cells. Initially, the RAW 264.7 cells were incubated with different concentration of CQNP and

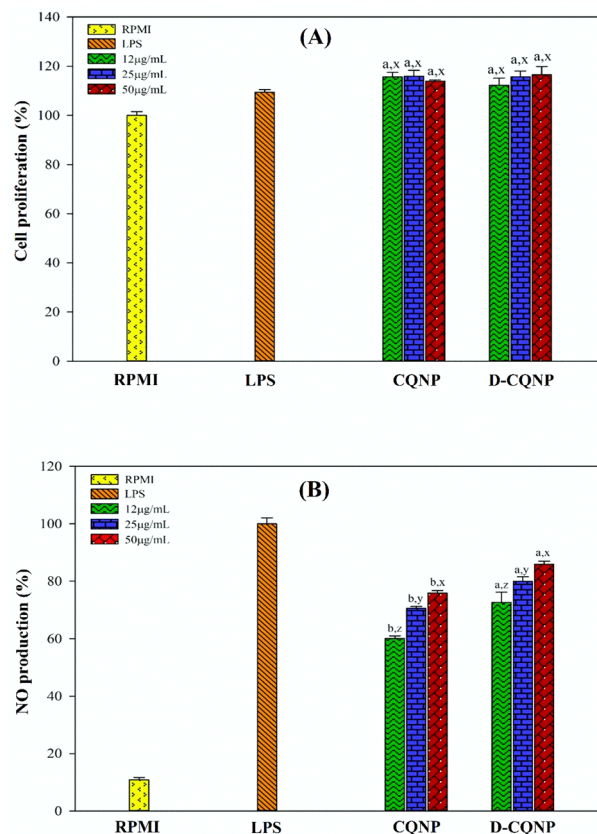Fig. 2 Determination of molecular weight of non-starch polysaccharides extracted from *Chenopodium quinoa* using HPSEC–UV–MALLS–RI spectral analysis. (**A**) CQNP and (**B**) D-CQNP. *CQNP* non-starch polysaccharide of *Chenopodium quinoa*, *D-CQNP* deproteinated non-starch polysaccharide of *Chenopodium quinoa*

Fig. 3 The effects of CQNP and D-CQNP treatments on cell proliferation and NO production in RAW 264.7 cells. (**A**) Cell proliferation and (**B**) NO production. The presence of letters x, y and z indicates a significant difference ($p < 0.05$) between the concentration of polysaccharides and a and b designates a significant difference ($p < 0.05$) between the treatments. *NO* nitric oxide, *RPMI* Roswell Park Memorial Institute medium, *LPS* lipopolysaccharide, *CQNP* non-starch polysaccharide of *Chenopodium quinoa*, *D-CQNP* deproteinated non-starch polysaccharide of *Chenopodium quinoa*

D-CQNP for 24 h and assess the cell proliferation activity using WST-1 assay. The cell proliferation results were compared with LPS treatment (1 µg/mL). As shown in Fig. 3A, RAW 264.7 cells showed an enhanced level of cell proliferation than LPs treatment when incubating with CQNP and D-CQNP. While increasing the concentration the cell proliferation activities were also increased. Further, the extracted non-starch polysaccharides did not show any toxic effects to the RAW 264.7 cells at the tested concentration. Subsequently, the NO production that could occur during treatment with different concentrations of CQNP and D-CQNP was measured in RAW 264.7 cells. NO is one of the primary molecule produced by stimulated macrophages that shows potent cytotoxic effects on pathogen and cancer cells. It is also performed as an intracellular messenger in the regulation of various physiological process [36]. In this study, RAW 264.7 cells treated with D-CQNP exhibited higher NO production than CQNP, demonstrating that the deproteinated non-starch polysaccharide has a strong immunostimulatory function (Fig. 3B).

Expression of iNOS and Cytokines

iNOS is one of the major enzyme involved in the NO production in stimulated macrophages [37]. Therefore, the experiments are being conducted to examine whether the increase in NO production is associated with increased iNOS activity and/or gene expression. Here, the gene expression of iNOS and various cytokines such as IL-1β, IL-6, IL-10 and TNF-α were studied by Real-Time PCR analysis. As shown in Fig. 4, the RAW 264.7 cells treated with 50 µg/mL of D-CQNP revealed significantly higher iNOS expression than those treated with CQNP. These findings are consistent with the result of NO production. Several studies reported that the increase in NO production in RAW 264.7 cells is associated with an increase in iNOS expression [38–40]. Similarly, the mRNA expression of various cytokines including IL-1β, IL-6, IL-10 and TNF-α was significantly increased ($p < 0.05$) when RAW 264.7 cells were treated with D-CQNP (50 µg/mL). These results confirmed that the RAW 264.7 cells are stimulated by D-CQNP by enhance the NO production through the mRNA expression of iNOS and various cytokines.

Western Blot Analysis

In addition, the underlying mechanisms for the activation of RAW 264.7 cells were explored by evaluating the protein expressions of NF-κB and MAPK (p38, ERK, and JNK) signaling pathways using western blot analysis. NF-κB is one of the major transcriptional factors that controls pro-inflammatory gene expression. The treatment of RAW 264.7 cells with CQNP and D-CQNP promote the phosphorylation
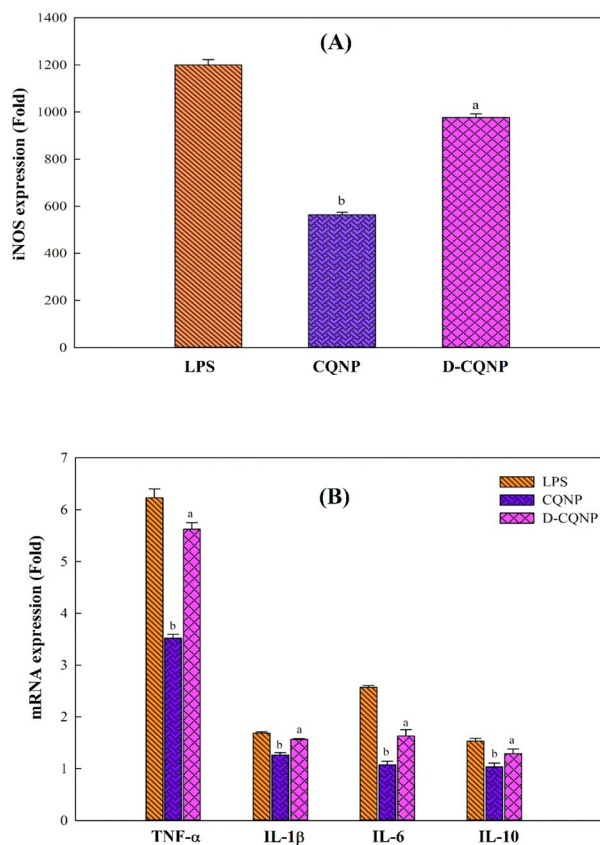

Fig. 4 The expression of inducible nitric oxide synthase and pro-inflammatory cytokines in CQNP and D-CQNP treated RAW 264.7 cells was evaluated through real-time PCR analysis; (**A**) iNOS expression and (**B**) expression of TNF-α, IL-1β, IL-6 and IL-10. The alphabets a and b signify significant differences ($p < 0.05$) between the polysaccharide treatments. LPS-treated cells served as a positive control. *iNOS* inducible nitric oxide synthase, *IL-1β* interleukin 1 beta, *TNF-α* tumor necrosis factor alpha, *CQNP* non-starch polysaccharide of *Chenopodium quinoa*, *D-CQNP* deproteinated non-starch polysaccharide of *Chenopodium quinoa*, *PCR* polymerase chain reaction, *LPS* lipopolysaccharide

of NF-κB (Fig. 5). In a previous study in support of this result, it was reported that the translocated NF-κB induce the pro-inflammatory mediators expression including iNOS and cytokines [7]. Similarly, the pathways of MAPKs also play a major role in regulating cellular functions and regulating pro-inflammatory responses [41]. In particular, p38 MAPKs play an important role in responding to cellular processes [42]. The phosphorylation of MAPKs, p38, ERK, and JNK has remarkably induced in RAW 264.7 cells by the treatment of CQNP and D-CQNP. Of these, the phosphorylation of ERK and JNK was markedly higher than others. Furthermore, D-CQNP showed an expression equivalent to a positive control (LPS). Hence, these results demonstrated that the immunostimulating activity of CQNP and D-CQNP was related to the activation of NF-κB and the MAPKs signaling pathways.

**Fig. 5** The protein expression of p-p38, p-JNK, p-ERK, and p-p65 in CQNP and D-CQNP treated RAW 264.7 cells. In this experiment, α-tubulin served as a control. RPMI represents the cells without treatment of polysaccharides. *p-p38* phosphorylated p38, *p-JNK* phosphorylated c-Jun N-terminal kinase, *p-ERK* phosphorylated extracellular signal-regulated kinase, *p-p65* phosphorylated p65, *RPMI* Roswell Park Memorial Institute medium, *LPS* lipopolysaccharide, *CQNP* non-starch polysaccharide of *Chenopodium quinoa*, *D-CQNP* deproteinated non-starch polysaccharide of *Chenopodium quinoa*
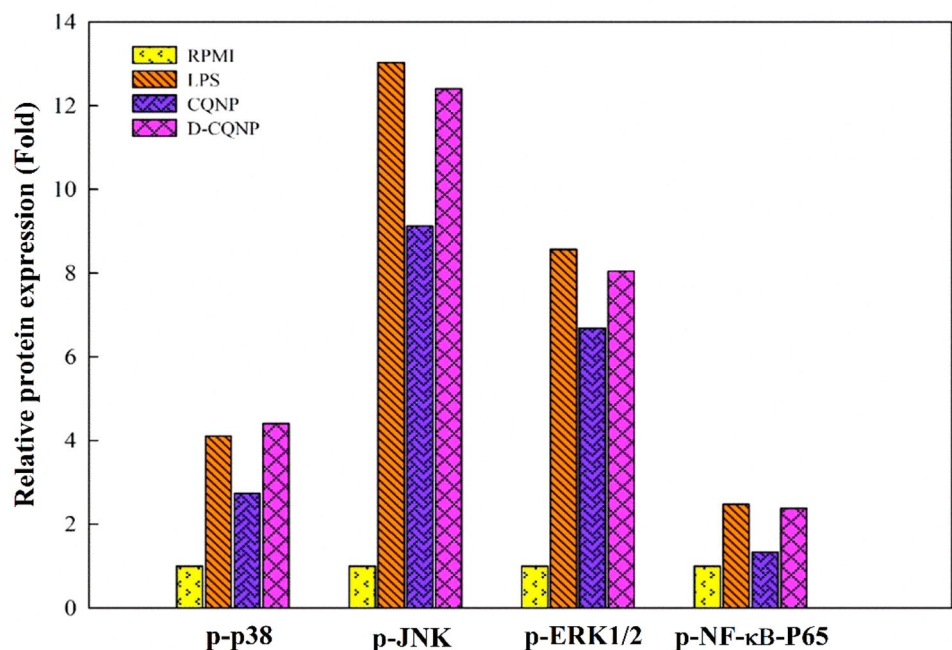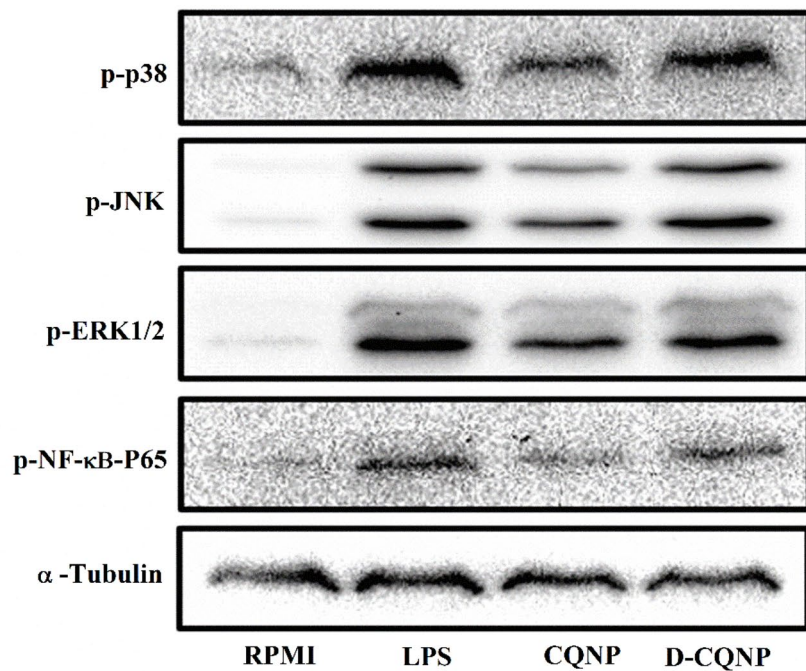
Flow Cytometry Analysis

Flow cytometry analysis was used to evaluate the surface biomarkers on RAW 264.7 cells treated with CQNP or D-CQNP (50 μg/mL) for 24 h. In this study, two biomarkers, CD11b and CD40 were used; CD11b is an indicator of activated macrophage that can be detected from the surface of granulocytes, monocytes, macrophages and natural killer cells and subsets of B- and T-cells. However, CD40 is a co-stimulatory molecule that induces the IL-2 and TNF-α

through T- cell activation [43]. When treating RAW 264.7 cells with CQNP or D-CQNP at 50 μg/mL the expression of CD11b and CD40 was 23.82% and 27.90% for CQNP and 80.25% 81.33% for D-CQNP, respectively (Fig. 6). Moreover, the activity of D-CQNP more or less similar to the positive control (LPS). This result was agreed in previous studies, where CD11b and CD40 expression were found to be higher when macrophages were activated by microfibers [44]. Furthermore, the expression of CD40 was higher than CD11b, which demonstrates the stimulation of RAW 264.7
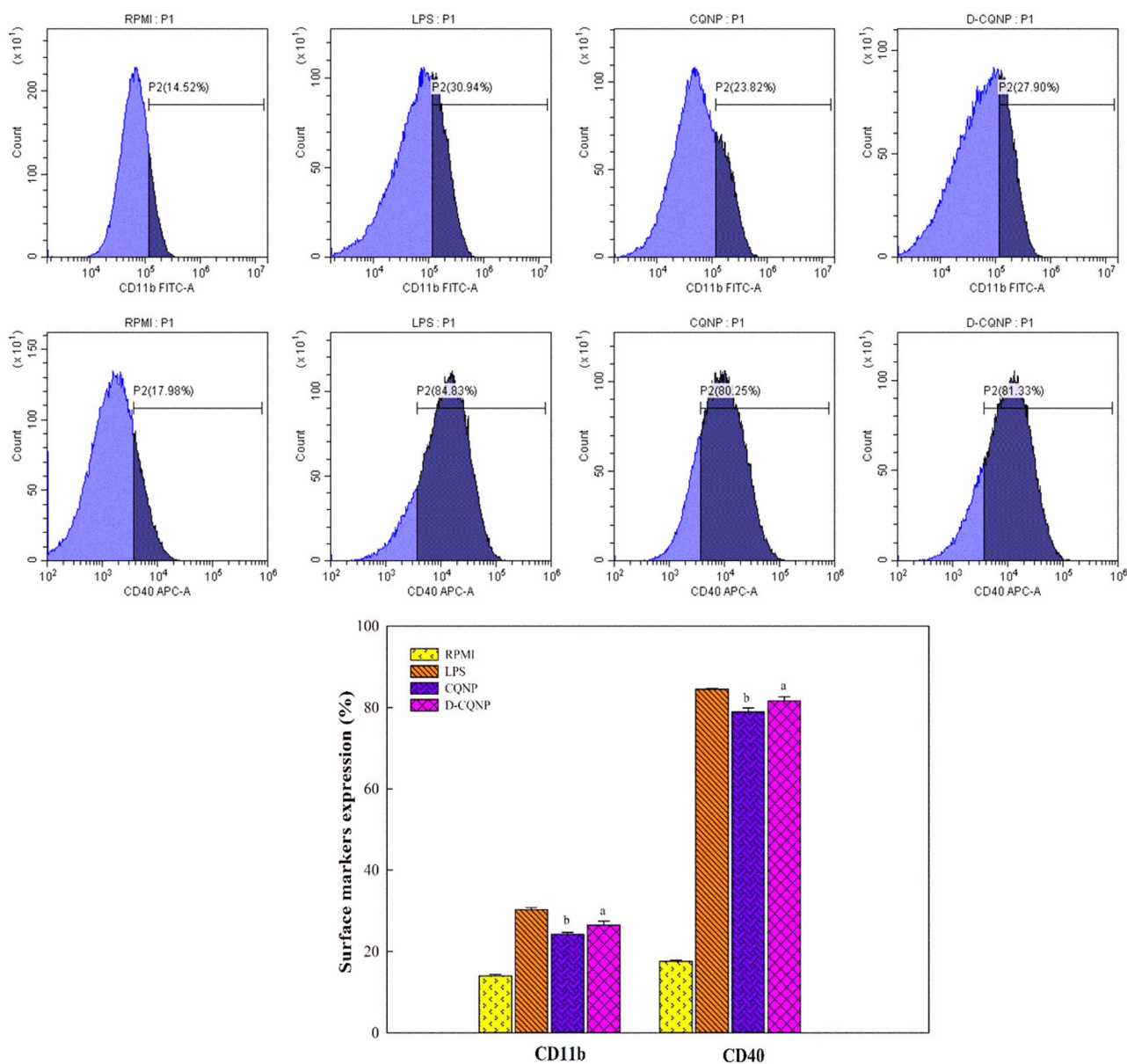
Fig. 6 Evaluation of CD11b and CD40 expression in CQNP and D-CQNP treated RAW 264.7 cells. Cells treated with LPS and the medium alone (RPMI) served as a positive and negative control. The letters a and b indicate significant differences ($p < 0.05$) between the polysaccharide treatments. *CD11b* cluster of differentiation 11b, *CQNP* non-starch polysaccharide of *Chenopodium quinoa*, *D-CQNP* deproteinated non-starch polysaccharide of *Chenopodium quinoa*, *RPMI* Roswell Park Memorial Institute medium, *LPS* lipopolysaccharide

cells by non-starch polysaccharide of C. *quinoa* through the NF-κB and MAPK signaling pathways by the production of pro-inflammatory cytokines and NO production via CD40 expression.

Glycosidic Linkage Analysis

Overall, the immunostimulatory results demonstrate that DQ-CQNP stimulates the RAW 264.7 cells at a higher level than CQNP; hence, the main structural details of DQ-CQNP was further explored by glycosidic linkage analysis. Totally, thirteen derivatives were identified: 1→Araf, 1→Rha, 1→3 Araf, 1→5 Araf, 1→Glc, 1→Gal, 1→2, 5 Araf, 1→2 Araf, 1→4 Gal, 1→4 Glc, 1→3 Gal, 1→6 Gal, 1→3, 4 Gal (Fig. 7; Table 3). Predominantly, galactopyranosyl as a main sugar component in the D-CQNP structure which was connected by (1→3, 4), (1→6), (1→3), (1→4) linkages with the peak area of 17.8%, 10.0%, 9.5% and 1.3%, respectively. Next, the arabinopyranosyl existed in the second residence with the connection of (1→5), (1→3), (1→2), (1→2, 5) linkages. In addition,

Fig. 7 GC–MS chromatogram represents the glycosidic linkage of D-CQNP. *GC–MS* gas chromatography–mass spectrometry, *D-CQNP* deproteinated non-starch polysaccharide of *Chenopodium quinoa*
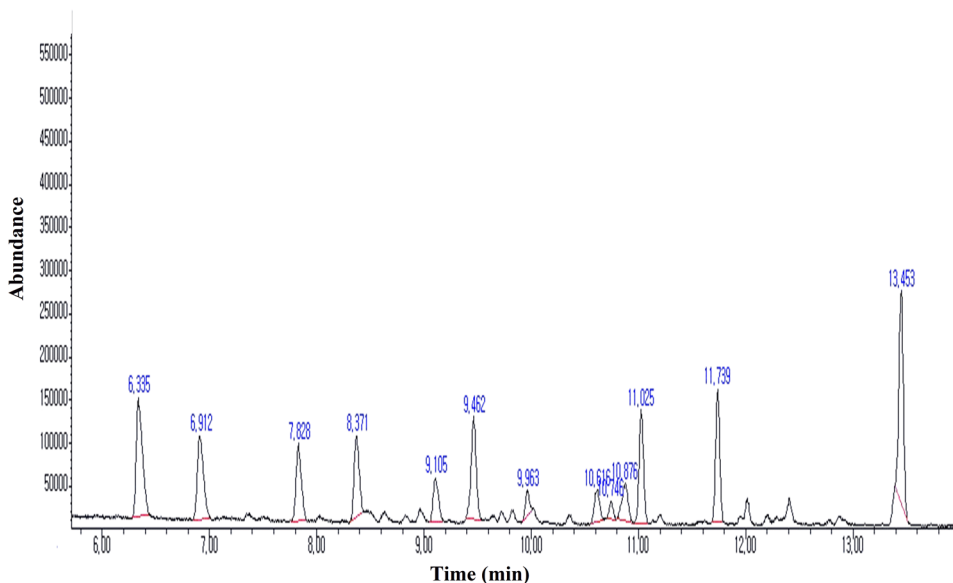
Table 3 Glycosidic linkage analysis of D-CQNP extracted from *Chenopodium quinoa*

S. no	Retention time (min)	Methylation product	Linkage type	Peak area (%)
1	6.34	1,4-Di-O-acetyl-2,3,5-tri-O-methyl-Ara	Ara*f*-(1→	13.4
2	6.91	1,5-Di-O-acetyl-2,3,4-tri-O-methyl-Rha	Rha-(1→	9.5
3	7.83	1,3,4-Tri-O-acetyl-2,5-di-O-methyl-Ara	→3)-Ara*f*-(1→	7.4
4	8.37	1,4,5-Tri-O-acetyl-2,3-di-O-methyl-Ara	→5)-Ara*f*-(1→	7.7
5	9.10	1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-Glc	Glc*p*-(1→	4.4
6	9.46	1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-Gal	Gal*p*-(1→	9.7
7	9.96	1,2,4,5-Tetra-O-acetyl-3-O-methyl-Ara	→2,5)-Ara*f*-(1→	1.8
8	10.62	1,2,4-Tri-O-acetyl-3,5-di-O-methyl-Ara	→2)-Ara*f*-(1→	3.1
9	10.75	1,4,5-Tri-O-acetyl-2,3,6-tri-O-methyl-Gal	→4)-Gal*p*-(1→	1.3
10	10.88	1,4,5-Tri-O-acetyl-2,3,6-tri-O-methyl-Glc	→4)-Glc*p*-(1→	4.4
11	11.03	1,3,5-Tri-O-acetyl-2,4,6-tri-O-methyl-Gal	→3)-Gal*p*-(1→	9.5
12	11.74	1,5,6-Tri-O-acetyl-2,3,4-tri-O-methyl-Gal	→6)-Gal*p*-(1→	10.0
13	13.45	1,3,4,5-Tetra-O-acetyl-2,6-di-O-methyl-Gal	→3,4)-Gal*p*-(1→	17.8

(1→4) linked glucopyranosyl residues were found to be present in the D-CQNP structure. It is noteworthy that D-CQNP contains some types of terminal residues such as arabinopyranosyl, rhamnopyranosyl, galactopyranosyl and glucopyransosyl.

Conclusion

In this study, non-starch polysaccharide CQNP was extracted from *C. quinoa* by hot water extraction and α-amylase treatment. Simultaneously, the protein content of CQNP was removed by enzymatically using proteinase K. Both samples enhanced the RAW 264.7 cells proliferation without toxicity. Among these, the higher RAW 264.7 cells stimulation was found in D-CQNP than CQNP. D-CQNP stimulate the RAW 264.7 cells through NF-κB and MAPK signaling pathways by the production of NO and pro-inflammatory cytokines via CD40 expression. The structure of D-CQNP mainly consisted of (1→3, 4), (1→6), (1→3), (1→4) linked galactopyranosyl with arabinopyranosyl, and glucopyranosyl residues. These findings are helpful for understanding the structural properties of non-starch polysaccharides from *C. quinoa* and their effect on macrophage activation.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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