Isolation And Characterization of Biopolymers From *Chenopodium Quinoa* And Their Immunostimulatory Activity

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Research Article

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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 RAW264.7 cells. The chemical composition of CQNP and deproteinated-CQNP (D-CQNP) were spectrometrically analysed that revealed the presence of carbohydrate (22.7 ± 0.8% and 39.5 ± 0.8%), protein (41.4 ± 0.8% and 20.8 ± 0.5%) and uronic acid (8.7 ± 0.8% and 6.7 ± 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 CD40.

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 m [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 (Alvarez-
Jubete, Wijngaard, et al., 2010). 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 are reported 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]. Polysaccharides from various natural sources such as plants [13], algae [14], fungi [15] stimulated the innate and cellular community via interactions with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes [16]. 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 (L. Zhang & Wang, 2014). 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].

In this study, we reported the extraction of non-starch polysaccharides (CQNP) and deproteinated CQNP from *C. quinoa* and their structural characterization and immunomodulatory properties.

**Material And Method**

**2.1 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, United Kingdom. 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.

**2.2 Isolation of non-starch polysaccharide**

A non-starch polysaccharide (CQNP) was isolated from *C. quinoa* using the 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 × 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.

2.3 Deproteination of CQNP

About, 1mg 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 (10000 rpm for 10 min) and the supernatant was dialyzed against distilled water and freeze-dried [21]. The deproteinated CQNP was named as D- CQNP.

2.4 Chemical and Monosaccharide composition analysis

The presence of carbohydrate, protein and uronic acid were examined by phenol-sulfuric acid (Dubois et al., 1956), folin-phenol reagent (Lowry et al., 1951) and m-hydroxydiphenyl reaction [24], 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 NaBD₄ and then acetylated using acetic anhydride. The monosaccharide composition analysis was carried out by a gas chromatography mass- spectrometry analysis (GC-MS, 6890 N/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).

2.5 Molecular weight analysis

The average $M_w$ and $R_g$ values were estimated by following the method of Tabarsa et al (Tabarsa, et al., 2020). Briefly, the sample was solubilized in distilled water (2 mg/mL) and heated for 30 S using a microwave bomb (#4872; 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; Tosoh 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 (#2414; Waters, Milford, MA, USA) system (HPSEC-UV-MALLS-RI). The $M_w$ was calculated using ASTRA 5.3 software (Wyatt Technology Corp.).

2.6 Glycosidic linkage analysis

The glycosidic linkage profile of the highest immunostimulant D-CQNP was investigated using the method of Ciucanu and Kerek [26]. 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 NaBD₄ and acetylated using acetic anhydride at 100°C. The partially methylated alditol acetates were subjected to GC-MS analysis (6890 N/MSD 5973, Agilent Technologies) equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies).
2.7 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 (Green et al., 1982).

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\text{Cell proliferation} \, (\%) = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100
\]

2.8 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′-GGCTGTCAGAGCCTCGTGCTGTTTGG-3′ (reverse)    |
| IL-1  | 5′-ATGGCAACTATCTCTGAACTCAACT-3′ (forward)     |
|       | 5′-CAGGACAGGGTATAGTCTTCTTTCTTT-3′ (reverse)   |
| IL-6  | 5′-TTCTCTCTGCAAGAGACT-3′ (forward)            |
|       | 5′-TGTATCTCTCTGAAGGACT-3′ (reverse)           |
| IL-10 | 5′-TACCTGGTAGAAGTGATGC-3′ (forward)           |
|       | 5′-CATCAGGTATGCTTCTATGC-3′ (reverse)          |
| TNF-β | 5′-ATGAGCAGAAAAGCATGTC-3′ (forward)           |
|       | 5′-TACAGGGTGTCACTCGAATT-3′ (reverse)          |
| β-actin| 5′-TGGAATCCTGTGCCATCCATGAAAC-3′ (forward)     |
|       | 5′-TAAAACGCAGCTCAGTAACAGTCCCG-3′ (reverse)    |

2.9 Western blot analysis

RAW 264.7 cells (1×10⁶ 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 14000 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, United Kingdom) 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).

2.10 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, United Kingdom), and the data were analyzed using the CytExpert 2.3.0.84 software (Beckman Coulter).
2.11 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.

Result And Discussion

3.1 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. 1. The amount of extracted CQNP was 1.7% of the starting raw material. Primarily, the CQNP constituted by carbohydrate (22.7 ± 0.8%), protein (41.4 ± 0.5%) and uronic acid (8.7 ± 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 [27]. 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 ± 0.8% carbohydrate, 20.8 ± 0.5% protein, and 6.7 ± 0.2% uronic acid.

The monosaccharide composition analysis is one of the recognized tools to determine the quality of polysaccharide [28]. In this study, the monosaccharide composition of CQNP and D-CQNP was analyzed using GC-MS analysis. As shown in Table. 1 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).
Table 2
Total yield, chemical composition, and monosaccharide content and molecular weight analysis of CQNP and D-CQNP obtained from *Chenopodium quinoa*.

| Chemical Composition (%) | Sample       | CQNP          | D-CQNP       |
|--------------------------|--------------|---------------|--------------|
| 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 (%)**

| Monosaccharide | CQNP       | D-CQNP       |
|----------------|------------|--------------|
| 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   |

**Molecular weight (Mw) and Radius of gyration (Rg)**

| Samples | Mw (kDa) | Rg (nm) |
|---------|----------|---------|
|         | Peak I   | Peak II | Peak III | Peak I | Peak II | Peak III |
| 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 |

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

### 3.2 Molecular weight analysis

The molecular weight of polysaccharide is an important factor to be associated with various biological activities [29]. The average molecular weight of CQNP and D-CQNP were examined using a multi angle laser light scattering technique (MALLS) through a high-performance size exclusion column (HPSEC). As shown in Figure 2 and Table 1, 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 HBSEC 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 and 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 [30]. Further, $R_g$ values were calculated from the spectrum, which showed approximate sizes of the molecules ranging from 60.3–74.6 nm for CQNP and 57.5–62.3 for D-CQNP.

### 3.3 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 (Kohchi et al., 2004). 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 D-CQNP for 24 h and assess the cell proliferation activity using WST-1 assay. The cell proliferation results were compared with LPS treatment (1µg/mL). As shown in Figure. 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 [33]. 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 (Figure. 3b).

### 3.4 Expression of iNOS and cytokines

iNOS is one of the major enzyme involved in the NO production in stimulated macrophages [34]. 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 [35–37]. 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.

### 3.5 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 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 (Liu et al., 2017). Similarly, the pathways of MAPKs also play a major role in regulating cellular functions and regulating pro-inflammatory responses (Kim & Choi, 2010). 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.

3.6 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 (Figure. 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 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.

3.7 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 (Figure. 7 and Table. 2). 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, (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 glucopyranosyl.
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<sub>f</sub>(1→3) | 13.4          |
| 2    | 6.91                 | 1,5-di-O-acetyl-2,3,4-tri-O-methyl-Rha | Rha-(1→2) | 9.5           |
| 3    | 7.83                 | 1,3,4-tri-O-acetyl-2,5-di-O-methyl-Ara | →3)-Ara<sub>f</sub>(1→2) | 7.4          |
| 4    | 8.37                 | 1,4,5-tri-O-acetyl-2,3-di-O-methyl-Ara | →5)-Ara<sub>f</sub>(1→3) | 7.7          |
| 5    | 9.10                 | 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-Glc | Glcp-(1→2) | 4.4           |
| 6    | 9.46                 | 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-Gal | Galp-(1→2) | 9.7           |
| 7    | 9.96                 | 1,2,4,5-tetra-O-acetyl-3-O-methyl-Ara | →2,5)-Ara<sub>f</sub>(1→3) | 1.8          |
| 8    | 10.62                | 1,2,4-tri-O-acetyl-3,5-di-O-methyl-Ara | →2)-Ara<sub>f</sub>(1→3) | 3.1           |
| 9    | 10.75                | 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-Gal | →4)-Galp-(1→2) | 1.3          |
| 10   | 10.88                | 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-Glc | →4)-Glc<sub>p</sub>(1→2) | 4.4          |
| 11   | 11.03                | 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-Gal | →3)-Galp-(1→2) | 9.5          |
| 12   | 11.74                | 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-Gal | →6)-Galp-(1→2) | 10.0         |
| 13   | 13.45                | 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-Gal | →3,4)-Galp-(1→2) | 17.8         |

**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**
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Conflicts of Interest Statement

The authors declare that there is no conflict of interest.

Availability of data and material

Not appropriate

Code availability

Not appropriate

Authors' contributions

Rong-An Cao: Conceptualization, Methodology and Writing-Original Draft. Subramanian Palanisamy: Investigation, Validation, Writing-Review & Editing. Nan Ma: Conceptualization, Methodology, Writing-Review & Editing. Natchanok Talapphet: Investigation, Validation and Formal analysis. JiaMiao Zhang: Writing-Review & Editing. ChangYuan Wang: Conceptualization, Methodology, Supervision, Writing-Review & Editing. SangGuan You: Conceptualization, Methodology, Supervision, Writing-Review & Editing.

References

1. Jan R, Saxena DC, Singh S (2015) Physico-chemical and textural property of starch isolated from Chenopodium (Chenopodium album) grains. Cogent Food Agric 1:1095052. https://doi.org/10.1080/23311932.2015.1095052
2. Alvarez-Jubete L, Arendt EK, Gallagher E (2010) Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. Trends Food Sci Technol 21:106–113
3. Drzewiecki J, Delgado-Licon E, Haruenkit R et al (2003) Identification and Differences of Total Proteins and their Soluble Fractions in some Pseudocereals Based on Electrophoretic Patterns. J Agric Food Chem 51:7798–7804. https://doi.org/10.1021/jf030322x
4. Alvarez-Jubete L, Wijngaard H, Arendt EK, Gallagher E (2010) Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. Food Chem 119:770–778. https://doi.org/10.1016/j.foodchem.2009.07.032
5. Hirose Y, Fujita T, Ishii T, Ueno N (2010) Antioxidative properties and flavonoid composition of Chenopodium quinoa seeds cultivated in Japan. Food Chem 119:1300–1306.
6. Noratto GD, Murphy K, Chew BP (2019) Quinoa intake reduces plasma and liver cholesterol, lessens obesity-associated inflammation, and helps to prevent hepatic steatosis in obese db/db mouse. Food Chem 287:107–114. https://doi.org/10.1016/j.foodchem.2019.02.061

7. Li M, Chen LX, Chen SR et al (2017) Non-starch polysaccharide from Chinese yam activated RAW 264.7 macrophages through the Toll-like receptor 4 (TLR4)-NF-κB signaling pathway. J Funct Foods 37:491–500. https://doi.org/10.1016/j.jff.2017.08.025

8. Wu DT, Liu W, Han QH et al (2019) Physicochemical characteristics and antioxidant activities of non-starch polysaccharides from different kiwifruits. Int J Biol Macromol 136:891–900. https://doi.org/10.1016/j.ijbiomac.2019.06.142

9. Subba Rao MVSST, Muralikrishna G (2002) Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (ragi, Eleusine coracana Indaf-15). J Agric Food Chem 50:889–892. https://doi.org/10.1021/jf011210d

10. Kumar V, Sinha AK, Makkar HPS et al (2012) Dietary Roles of Non-Starch Polysaccharides in Human Nutrition: A Review. Crit Rev Food Sci Nutr 52:899–935

11. Rodríguez R, Jiménez A, Fernández-Bolaños J et al (2006) Dietary fibre from vegetable products as source of functional ingredients. Trends Food Sci Technol 17:3–15. https://doi.org/10.1016/j.tifs.2005.10.002

12. Schepetkin IA, Xie G, Kirpotina LN et al (2008) Macrophage immunomodulatory activity of polysaccharides isolated from Opuntia polyacantha. Int Immunopharmacol 8:1455–1466. https://doi.org/10.1016/j.intimp.2008.06.003

13. Tabarsa M, Dabaghian EH, You SG et al (2020) Inducing inflammatory response in RAW264.7 and NK-92 cells by an arabinogalactan isolated from Ferula gummosa via NF-κB and MAPK signaling pathways. Carbohydr Polym 241:116358. https://doi.org/10.1016/j.carbpol.2020.116358

14. Alboofetileh M, Rezaei M, Tabarsa M et al (2019) Effect of different non-conventional extraction methods on the antibacterial and antiviral activity of fucoidans extracted from Nizamuddinia zanardinii. Int J Biol Macromol 124:131–137. https://doi.org/10.1016/j.ijbiomac.2018.11.201

15. Yelithao K, Surayot U, Lee C et al (2019) Studies on structural properties and immune-enhancing activities of glycomannans from Schizophyllum commune. Carbohydr Polym 218:37–45. https://doi.org/10.1016/j.carbpol.2019.04.057

16. Tzianabos AO (2000) Polysaccharide immunomodulators as therapeutic agents: Structural aspects and biologic function. Clin Microbiol Rev 13:523–533

17. Wu LC, Fan NC, Lin MH et al (2008) Anti-inflammatory effect of spilanathol from Spilanthes acmella on murine macrophage by down-regulating LPS-induced inflammatory mediators. J Agric Food Chem 56:2341–2349. https://doi.org/10.1021/jf073057e

18. Zhang L, Wang CC (2014) Inflammatory response of macrophages in infection. Hepatobiliary Pancreat Dis Int 13:138–152
19. Han SB, Yoon YD, Ahn HJ et al (2003) Toll-like receptor-mediated activation of B cells and macrophages by polysaccharide isolated from cell culture of Acanthopanax senticosus. Int Immunopharmacol 3:1301–1312. https://doi.org/10.1016/S1567-5769(03)00118-8

20. Xie SZ, Hao R, Zha XQ et al (2016) Polysaccharide of Dendrobium huoshanense activates macrophages via toll-like receptor 4-mediated signaling pathways. Carbohydr Polym 146:292–300. https://doi.org/10.1016/j.carbpol.2016.03.059

21. Li C, Talapphet N, Palanisamy S et al (2020) The relationship between structural properties and activation of RAW264.7 and natural killer (NK) cells by sulfated polysaccharides extracted from Astragalus membranaceus roots. Process Biochem 97:140–148. https://doi.org/10.1016/j.procbio.2020.06.021

22. Dubois M, Gilles KA, Hamilton JK et al Colorimetric Method for Determination of Sugars and Related Substances

23. LOWRY OH, ROSEBROUGH NJ FARRAL, RANDALL RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275. https://doi.org/10.1016/s0021-9258(19)52451-6

24. Filisetti-Cozzi TMCC, Carpita NC (1991) Measurement of uronic acids without interference from neutral sugars. Anal Biochem 197:157–162. https://doi.org/10.1016/0003-2697(91)90372-Z

25. Tabarsa M, You SG, Yelithao K et al (2020) Isolation, structural elucidation and immuno-stimulatory properties of polysaccharides from Cuminum cyminum. Carbohydr Polym 230:115636. https://doi.org/10.1016/j.carbpol.2019.115636

26. Ciucanu I, Kerek F (1984) A simple and rapid method for the permethylation of carbohydrates. Carbohydr Res 131:209–217. https://doi.org/10.1016/0008-6215(84)85242-8

27. Miafo APT, Koubala BB, Kansci G, Muralikrishna G (2019) Free sugars and non-starch polysaccharides–phenolic acid complexes from bran, spent grain and sorghum seeds. J Cereal Sci 87:124–131. https://doi.org/10.1016/j.jcs.2019.02.002

28. Yang X, Zhao Y, Wang Q et al (2005) Analysis of the monosaccharide components in Angelica polysaccharides by high performance liquid chromatography. Anal Sci 21:1177–1180. https://doi.org/10.2116/analsci.21.1177

29. Azmi AFMN, Mustafa S, Hashim DM, Manap YA (2012) Prebiotic activity of polysaccharides extracted from Gigantochloa Levis (buluh beting) shoots. Molecules 17:1635–1651. https://doi.org/10.3390/molecules17021635

30. Ketha K, Gudipati M (2018) Immunomodulatory activity of non starch polysaccharides isolated from green gram (Vigna radiata). Food Res Int 113:269–276. https://doi.org/10.1016/j.foodres.2018.07.010

31. Kohchi C, Inagawa H, Hino M et al (2004) Utilization of Macrophages in Anticancer Therapy: The Macrophage Network Theory. Anticancer Res 24

32. Fidler IJ, Kleinerman ES (1993) Therapy of cancer metastasis by systemic activation of macrophages: From the bench to the clinic. In: Research in Immunology. pp 284–287
33. O’Rourke C, Gregson T, Murray L et al (2015) Sugar composition of the pectic polysaccharides of charophytes, the closest algal relatives of land-plants: Presence of 3-O-methyl-d-galactose residues. Ann Bot 116:225–236. https://doi.org/10.1093/aob/mcv089

34. Uehara EU, Shida B, de S, de Brito CA (2015) Role of nitric oxide in immune responses against viruses: beyond microbicidal activity. Inflamm Res 64:845–852

35. Qi J, Kim SM (2018) Effects of the molecular weight and protein and sulfate content of Chlorella ellipsoidea polysaccharides on their immunomodulatory activity. Int J Biol Macromol 107:70–77. https://doi.org/10.1016/j.ijbiomac.2017.08.144

36. Geng L, Zhang Q, Wang J et al (2018) Glucofucogalactan, a heterogeneous low-sulfated polysaccharide from Saccharina japonica and its bioactivity. Int J Biol Macromol 113:90–97. https://doi.org/10.1016/j.ijbiomac.2018.02.008

37. Borazjani NJ, Tabarsa M, You SG, Rezaei M (2018) Purification, molecular properties, structural characterization, and immunomodulatory activities of water soluble polysaccharides from Sargassum angustifolium. Int J Biol Macromol 109:793–802. https://doi.org/10.1016/j.ijbiomac.2017.11.059

38. Li C, Meng M, Chen D et al (2013) The immunostimulatory effect of novel immunostimulator CH2b with a thiazolidin-4-one ring on the functions of LPS-activated RAW 264.7 macrophages in vitro. Int Immunopharmacol 17:698–703. https://doi.org/10.1016/j.intimp.2013.08.011

39. Liu T, Zhang L, Joo D, Sun SC (2017) NF-κB signaling in inflammation. Signal Transduct Target Ther 2:1–9

40. Kim EK, Choi EJ (2010) Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta - Mol Basis Dis 1802:396–405

41. Pearson G, Robinson F, Beers Gibson T et al (2001) Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions*. Endocr Rev 22:153–183. https://doi.org/10.1210/edrv.22.2.0428

42. Zhang M, Chiang YH, Toruño TY et al (2018) The MAP4 Kinase SIK1 Ensures Robust Extracellular ROS Burst and Antibacterial Immunity in Plants. Cell Host Microbe 24:379–391.e5. https://doi.org/10.1016/j.chom.2018.08.007

43. Pyo K-H, Kim M-K, Shin K-S et al (2013) Involvement of Trypsin-Digested Silk Peptides in the Induction of RAW264.7 Macrophage Activation. Nat Prod Commun 8:1934578X1300801.e5. https://doi.org/10.1177/1934578X1300801226

44. Berghaus LJ, Moore JN, Hurley DJ et al (2010) Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. Comp Immunol Microbiol Infect Dis 33:443–454. https://doi.org/10.1016/j.cimid.2009.07.001

Figures
Figure 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.
Figure 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.
Figure 3

The effects of CQNP and D-CQNP treatments on cell proliferation and NO production in RAW 264.7 cells. 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, b, and c designates a significant difference (p < 0.05) between the treatments. NO, nitric oxide; RPMI, Roswell Park Memorial Institute.
Institute medium; LPS, lipopolysaccharide; CQNP, non-starch polysaccharide of Chenopodium quinoa; D-CQNP, deproteinated non-starch polysaccharide of Chenopodium quinoa.

Figure 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.

**Figure 5**

![Image of protein expression](image_url)

Relative protein expression (fold)

- p-p38
- p-JNK
- p-ERK 1/2
- p-NF-κB-p65

|       | RPMI | LPS  | CQNP | D-CQNP |
|-------|------|------|------|--------|
| p-p38 |      |      |      |        |
| p-JNK |      |      |      |        |
| p-ERK |      |      |      |        |
| p-NF-κB-p65 |      |      |      |        |
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.

**Figure 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.

Figure 7

GC-MS chromatogram represents the glycodidic linkage of D-CQNP. GCMS; Gas chromatography mass-spectrometry, D-CQNP, deproteinated non-starch polysaccharide of Chenopodium quinoa.

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