Structural characterization of a polysaccharide from *Cetraria islandica* and assessment of immunostimulatory activity

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**A R T I C L E   I N F O**

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**A B S T R A C T**

In this study, *Cetraria islandica* polysaccharide (CIPs) was extracted by hot water extraction method and its effect on structural modification of immunomodulatory activities was investigated. This polysaccharide mainly consisted of carbohydrates (97.0%), Sulfate (1.2%) with one type of glucose. The average molecular weight (328.7 \times 10^3 \text{ g/mol}) was determined by size exclusion chromatography. The structure of the polysaccharide was found to be composed of (1→3) and (1→4)-\(\beta\)-D-glucopyranosyl units. The immunomodulatory activities of the crude polysaccharide and its derivatives, over-sulfated (OS1,2,3) and hydrolyzed (H1,2,3), were tested against NK-92 cells and RAW264.7 cells. The results obtained clearly demonstrated that over-sulfated (OS1,2,3)-treated NK-92 cells induced cytotoxicity in HeLa cells through the expressions of IFN-γ, NKP44, NKP30, and FasL. On the other hand, the hydrolyzed derivatives (H1,2,3) activated RAW264.7 cells through production of nitric oxide (NO) and mRNA expression of iNOS, IL-1, TNF-α, IL-6, IL-10 and IL-12 through the nuclear factor kappa-B (NF-κB) and mitogen activated protein kinases (MAPK) pathways. Our findings suggest that the presence of sulfate in the polysaccharide played a pivotal role in NK-92 cell however the Mw was a determinant factor in RAW264.7 cell activation, in which both cells were activated through the CR3 and TLR-4 signaling pathways.

1. Introduction

Lichens are slow-growing and composite organisms mostly living among fungi in a symbiotic relationship [1]. Lichens are ubiquitously found with variations in colors, sizes, and forms [1]. Although lichens are not plants, they exhibit plant-like properties, such as flat leaf-like structures with leafless branches and flakes on the leaf surface, like peeling paint [2]. *Cetraria islandica* (Iceland moss) is a type of lichen species which grows throughout the world. It has been used for several centuries as a traditional medicine in various countries against a variety of diseases, including tuberculosis, throat irritation, asthma, gastritis, viruses, bacteria, and tumors [3]. Its medicinal activities are mainly due to the presence of constituents and secondary metabolites, such as polysaccharides, polyketides, protolichesterinic acid, and fumarprotocetraric acid [4]. Among the various metabolites of *C. islandica*, polysaccharides are the major constituent, comprising up to 20%, and show strong immunomodulating effects through induction of phagocytosis and complement activation [5]. Furthermore, a previous study reported that the polysaccharides had effects on human dendritic cells to secrete various cytokine products and inhibit inflammatory disease [6].

The polysaccharides obtained from *C. islandica* are known to be lichenan and isolichenan [5,6]. Lichenan is a cold-water insoluble glucan, consisting of \(β-(1→3)\) and \(β-(1→4)\) linkages. In contrast, the main chain of isolichenan is a cold-water soluble \(α-(1→3)\) and \(α-(1→4)\) linkage [7]. In addition to the two glucans, galactomannans from *C. islandica* have been reported [8]. The biological activity of lichen...
polysaccharides was closely interrelated to their structure. Sulfate substitution on linear β-D-glucans was shown to enhance the inhibition of HIV replication in vitro, and suppress the cytopathogenic effect of HIV and its antigen expression in Molt-4 cells, without affecting HIV-reverse transcriptase [9].

Recently, two lichen polysaccharides, partially O-acetylated pustulan (GE-3) and a lichenan-type glucan (UR-1-1), were extracted from *Gyrophora esculenta* MIYOSHI (GE-3) and *Usnea rubescens* STIRT (UR-1-1) and were tested for cytotoxic activity against sarcoma 180 solid tumors in mice. The combined treatment of GE-3 and UR-1-1 with urea exhibited no remarkable changes in their activity. However, structurally-modified polysaccharides with carbomethoxy and lauroyl derivatives showed an excellent level of cytotoxic activity [10]. These results suggest that the bioactivities of lichen polysaccharides may be improved by means of various chemical modifications. Therefore, in the current study, the structure and immunomodulating activity of lichen polysaccharides extracted from *C. islandica* were investigated, followed by sulfur and partial acid modification of the polysaccharides which resulted in a series of sulfated derivatives (OS$_{1,2,3}$) and different molecular weights (H$_{1,2,3}$). In this study, natural killer cells (NK-92 cells) and RAW264.7 cells were employed to evaluate the immunostimulatory activities.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture media RPMI-1640 (#CP18-2326) and α-MEM (#1967520), and supplement reagents were procured from Gibco Life Technologies (New York, USA). The WST-1 assay kit was obtained from Daeilab Service Co., Korea. Anti-α-CR3 (ab52920), Anti-TLR-4 (ab47093) antibodies were obtained from Abcam (St. Louis, Missouri, USA). All other chemicals used in this experiment were of analytical grade.

2.2. Cell lines and cell culture

This study used three types of cells, RAW264.7 cells, natural killer cells (NK-92 cells), and cancer cells (HeLa cells), obtained from the ATCC (Rockville, MD, USA). RPMI-1640 medium was used to culture the HeLa and RAW264.7 cells and α-MEM medium was used to culture NK-92 cells. All cells were cultured at 37 °C with 5% CO$_2$.

2.3. Extraction of polysaccharides

The isolation of polysaccharide was performed as previously described [11]. Briefly, 30 g of fine powder was dissolved in 300 mL of ethanol (99%) with constant stirring at 60 °C for 2 h, followed by hot water extraction. The crude polysaccharide was collected after ethanol precipitation. Free proteins were removed using the Sevag method [12]. The crude *C. islandica* polysaccharide (CIPS) was used for further analyses.

2.4. Preparation of polysaccharide derivatives

The polysaccharide was over-sulfated, according to the Seoda method, [13] with minor modification. Sulfated groups were introduced into the polysaccharide using a dimethylformamide and sulfur trioxide-trimethylamine-mediated reaction (Table 3). At the end of the reaction, the solution was precipitated with saturated sodium acetate in ethanol. The over-sulfated sample obtained was dialyzed and lyophilized, and classified as OS$_1$, OS$_2$, and OS$_3$. Similarly, structural modification (H$_{1}$, H$_{2}$, and H$_{3}$) was performed by partial hydrolyzation using a low concentration of hydrochloric acid (0.01 M). The polysaccharide was treated for different times (10, 15, and 20 min) under hot water (100 °C) conditions to obtain polysaccharides of different molecular weights (Table 3). Then, the samples were neutralized with 0.01 M sodium hydroxide, dialyzed, and freeze-dried.

2.5. Physico-chemical characterisation

The total sugar, sulfate, and uronic acid content of the polysaccharides was estimated according to a standard method, using glucose, K$_2$SO$_4$, and glucuronic acid as standards [14–16]. Total watersoluble protein was estimated with a DC protein assay kit (Bio-Rad, USA) [17]. Monosaccharide composition analysis was carried out using a gas chromatography-mass spectrometer (GC–MS, 6890N/MSD5973, Agilent Technologies, Santa Clara, CA), as previously described [18]. Average molecular weight (Mw) and gyration (Rg) analyses were conducted on high-performance size exclusion chromatography, combined with an ultraviolet, multi-angle laser light scattering and refractive index detection system (HPSEC-UV-MALLS-RI), as previously described [19]. The Mw and Rg values were determined using ASTRA software version 6.1. FT-IR spectra of the polysaccharides were recorded using a Tensor 27 spectrophotometer (Bruker, Germany). The glycosidic-linkage was determined by the Ciucanu method, with slight modification [20]. NMR spectroscopic analysis was performed using the JEOL ECA-600 spectrometer (JEOL, Akishima, Japan). The CIPs was dissolved in D$_2$O, then loaded onto the spectrometer at 50 °C. $^1$H and $^{13}$C NMR spectra were recorded at a base frequency of 150 MHz for $^{13}$C, and 600 MHz for $^1$H.

2.6. NK cell activity assay

Cells viability (NK-92) was tested using the WST-1 assay. In this assay, cells were seeded onto a 96-well plate (1 × 10$^5$ cells/mL) and treated with crude polysaccharide and its derivatives at different concentrations (10–100 μg/mL). The optical density (OD value) at 450 nm was recorded using a microplate reader. In the cytotoxicity assay, cells treated with different doses of polysaccharides were co-cultured with Hela cells at a 25:1 ratio of effector to target cell. After a 24 h incubation, the Hela cells were analyzed by the WST-1 assay. The samples were read at 450 nm and the percentage of cytotoxicity was calculated based on the formula:

\[
\text{Cytotoxicity (％)} = 100 \times (1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}})
\]

2.7. RAW264.7 cell activity assay

The cell viability of RAW264.7 cells was determined using the WST-1 assay. Briefly, cells were seeded at a density of 1 × 10$^6$ cells/mL onto 96-well plates and incubated with varying doses (50–500 μg/mL) of polysaccharides for 24 h. The production of nitric oxide (NO) was estimated using the Griess reaction method [21].

2.8. Receptors neutralization

Surface receptor blocking experiments were performed by the method of Huyan [22]. Before treatment with polysaccharides, the cells were pre-treated with 20 μg/mL anti-TLR4, anti-TLR2, or anti-CR3 antibodies. Cytotoxicity and NO activities were determined by the methods described above.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Briefly, NK and RAW264.7 cells were treated with polysaccharides and maintained at 37 °C for 18 h. Positive (LPS for RAW264.7 cells) and negative controls (RPMI for RAW264.7 cells, MEM-α for NK-92 cells) were similarly maintained. RNA was harvested using the TRIzol method (Invitrogen Carlsbad, CA, USA). cDNA was synthesized using an oligo-
The glycosidic pattern and ratio of the polysaccharide were also inferred from 1D and 2D NMR spectra, including 1H, 13C and DQF-COSY, TOCSY, HMQC, and HMBC to support the GC-MS results. Gorshkova et al. (1997) also reported that the polysaccharide extracted from C. islandica was a cold water-insoluble lichenan β-glucan linearly linked by (1→3) and (1→4) glucopyranosides at a linkage ratio of 3:7 [28]. However, an isolichenan type of α-glucan with (1→3) and (1→4)-glycosidic linkages from C. islandica was also reported with linkage ratios varying from 3:2 to 1:1 [8]. It was, therefore, suggested that the polysaccharides from C. islandica were mixtures of lichenan and isolichenan in various amounts, and their isolation could be largely achieved by modification of the extraction temperature, or by soaking the sample in water at room temperature.

### 3.3. Preparation of over-sulfated and hydrolyzed derivatives

Table 3 shows the preparation conditions for the over-sulfated polysaccharides from C. islandica. The sulfate content was increased from 1.2% to 5.2% in the first one hour of reaction and it continually increased to 8.3% and 11.2% after 3 h and 6 h respectively, resulting in the production of OS1, OS2; and OS3. Basically, reaction conditions, such as temperature, time, and the ratio of sulfur-trioxide-trimethylamine (STMA) are important factors affecting the degree of sulfate substitution on the polysaccharides, in which an optimal temperature of 80 °C was desirable to obtain better-sulfated derivatives [30]. Moreover, reaction time was considered another important factor to increase the sulfate content [31,32]. Increased sulfated levels appeared in the FT-IR spectra (Fig. 1D). Compared to the CIPs, two characteristic peaks were observed in the region of 820 cm⁻¹ and 1240 cm⁻¹, corresponding to asymmetrical C-O-S vibration associated with a C-O-SO₃⁻ group and an asymmetrical S=O stretching vibration, respectively. These findings confirmed that the sulfate groups were successfully bonded to CIPs by the over-sulfation process. However, the reaction led to a significant reduction in the Mw of the polysaccharides from 328.7 × 10³ g/mol to 215.1, 167.2, and 130.5 × 10³ g/mol after 1, 3, and 6 h treatments, respectively (data not shown). The molecular degradation appeared to be inevitable due to the high treatment temperature [33]. The Mw of polysaccharides has been directly related to their biological activities [34]. Therefore, the effect of the Mw of the polysaccharides from C. islandica on the bioactivity was also investigated after their partial acid hydrolysis. The acid hydrolysis treatment yielded H₂,₃,₄ derivatives by heating in hot water (100 °C) for 10, 15, and 20 min. Decreases in the hydrolyzed derivatives were observed on the RI chromatograms (Fig. 1E), in which the CIPs appeared as a single peak between 28 and 50 min of elution time. After hydrolysis for 10 min (H₁), the peak slightly moved to 31–50 min. When the reaction was allowed to proceed for 15 and 20 min (H₂ and H₃), the resulting peaks shifted to 32 and 33 min, respectively. The shifted peaks indicated Mw degradation of the CIPs. As shown in Table 3, the Mw was significantly decreased from 328.7 × 10³ g/mol (CIPs) to 130.5, 82.1, and 77.1 g/mol (H₁, H₂, and H₃), respectively. These results suggested...
successful yield of polysaccharide derivatives with different Mw. Overall, the sulfated and hydrolyzed derivatives of the polysaccharides enabled evaluation of the effects of sulfate substitution and Mw of the polysaccharides from *C. islandica* on the biological activities.

### 3.4. NK-92 cell activation

NK-92 cells are important contributors to innate immunity in human, which eliminates pathogen-infected or cancer cells [35]. In this study, the effect of CIPs and its derivatives on NK-92 cell activity was tested against HeLa cells by investigating cell cytotoxicity and expression of cytokine mRNAs, cytoplasmic granules, and activating receptor. The treatment of NK-92 cells with CIPs and its derivatives showed a considerable increase in cell viability (120%) without toxic effect up to 100 μg/mL (Fig. 2A).

In cytotoxicity studies, the negative control group (the medium, MEM-α) showed 20.7% of NK cell cytotoxicity against HeLa cell, suggesting that NK-92 cell, itself, possessed some direct cytotoxic effect against HeLa cell. NK-92 cells treated with 100 μg/mL of CIPs showed an increased level of cytotoxicity (33%), which exhibited that the CIPs were able to stimulate NK-92 cells. When treated with the over-sulfated (OS1,2,3) derivatives (100 μg/mL), the higher level (55.0%) of NK cell cytotoxicity was recorded. Similarly, in our previous study, the over-sulfated derivatives of *Polygonatum sibiricum* polysaccharides showed the increased level of NK cell cytotoxic activity (42.5% to 51.6%) than the hydrolyzed derivatives [36]. This result suggested that the over-sulfation might be beneficial for the improvement of NK-92 cell activation. However, a considerably lower level of NK cell cytotoxicity was observed with H1,2,3 treatment, implying that the Mw of CIPs did not play a critical role in the enhancement of NK-92 cell activation.

#### Table 1
Glycosidic linkage analysis of constituent sugar of polysaccharides from *C. islandica*.

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Methylation</th>
<th>Glycosidic linkage</th>
<th>Peak ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.3</td>
<td>1,3,5-tri-acetyl-2,4,6-tri-methyl-Glu</td>
<td>→3)-Glu (1→</td>
<td>36.3</td>
</tr>
<tr>
<td>12.5</td>
<td>1,4,5-triacetyl-2,3,6-tri-methyl-Glu</td>
<td>→4)-Glu (1→</td>
<td>56.6</td>
</tr>
</tbody>
</table>
Gene expression (IFN-\(\gamma\), granzyme-B, NKp44, NKp30, NKG2D, perforin, and FasL) after treatment with IM and OS1,2,3 are shown in Fig. 2C-D. The mRNA level of IFN-\(\gamma\) was significantly increased by treatment with OS1,2,3, compared to CIPs. Similarly, the expression of NKp44 and NKp30 were also up-regulated by treatment with CIPs and OS1,2,3. Moreover, CIPs and its over-sulfated derivatives strongly induced the apoptosis-inducing ligand, FasL, mRNA expression. However, the expression of NKG2D and the cytoplasmic granule, perforin, were not considerably increased. Granzyme-B expression was relatively high in both the control and other polysaccharide-treated cells. Co-culture of NK-92 with HeLa cells have been reported to cause the secretion of cytoplasmic granules [23]. As shown in Fig. 2C, the expression of cytoplasmic granules was significantly increased by treatment with OS1,2,3, which indicates that OS1,2,3 might possess an excellent capacity to activate NK-92 cells. However, the results also showed that the presence of a sulfate groups in the polysaccharide backbone increased the cell signaling ability of NK-92 cells. This improved activity may be due to the increased electrostatic interaction between the sulfate groups of the polysaccharide and the surface receptors of NK-92 cells as the previous study [37].

**Table 2**

<table>
<thead>
<tr>
<th>Residue</th>
<th>C-1/H-1</th>
<th>C-2/H-2</th>
<th>C-3/H-3</th>
<th>C-4/H-4</th>
<th>C-5/H-5</th>
<th>C-6/H-6a, H-6b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: (\beta-(1\rightarrow3)) Glu</td>
<td>104.2/4.67</td>
<td>74.1/3.69</td>
<td>88.0/3.75</td>
<td>71.4/3.68</td>
<td>77.6/4.12</td>
<td>62.2/3.90, 3.77</td>
</tr>
<tr>
<td>B: (\beta-(1\rightarrow4)) Glu</td>
<td>103.4/4.61</td>
<td>74.2/3.65</td>
<td>75.1/3.84</td>
<td>80.8/3.69</td>
<td>77.9/4.05</td>
<td>61.0/3.92, 3.85</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temperature (°C)</th>
<th>Reaction time</th>
<th>Yield (%)</th>
<th>Sulfate content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oversulfation of polysaccharides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIPs</td>
<td>–</td>
<td>–</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>OS1</td>
<td>80</td>
<td>1 h</td>
<td>80</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td>OS2</td>
<td>80</td>
<td>3 h</td>
<td>81</td>
<td>8.3 ± 0.8</td>
</tr>
<tr>
<td>OS3</td>
<td>80</td>
<td>6 h</td>
<td>78</td>
<td>11.2 ± 1.1</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>100</td>
<td>10 min</td>
<td>70</td>
<td>130.5 ± 11.1</td>
</tr>
<tr>
<td>H2</td>
<td>100</td>
<td>15 min</td>
<td>61</td>
<td>82.1 ± 14.1</td>
</tr>
<tr>
<td>H3</td>
<td>100</td>
<td>20 min</td>
<td>60</td>
<td>77.1 ± 10.0</td>
</tr>
</tbody>
</table>
determine the possible mechanism involved in the activation of NK-92 cells. Fig. 2E shows that the NK-92 cell cytotoxicity of CIPs and OS1,2,3 were 37.4%, 40.5%, 41.6%, and 44.5%, respectively. At the same time, the untreated (negative control) showed 19.7% cytotoxicity. Among the three pattern recognition molecules (TLR-4 and TLR-2, and CR-3), the anti-CR3 antibody-treated cells showed considerably decreased cytotoxicity. This finding demonstrates that the polysaccharide from *C. islandica* might stimulate NK-92 cell activity through CR3-receptors.

3.5. RAW264.7 cell activation

In this study, immunomodulation of the polysaccharide from *C. islandica* and its derivatives were examined in RAW264.7 cells. Fig. 3A shows the effect of the CIPs and its derivatives on RAW264.7 cell viability at concentrations of 50–500 μg/mL. Cell viability was slightly improved by the presence of polysaccharides, indicating that they were freely toxic to the RAW264.7 cells over the treatment concentration range. The production of NO by the RAW264.7 cells is shown in Fig. 3B. Treatment by CIPs and its sulfated derivatives (OS1,2,3) of RAW264.7 cells demonstrated a very low and constant level of NO production,
clearly indicating that the sulfate-substituted CIPs did not affect RAW264.7 cell activity. In a recent study, it has been suggested that the introduction of sulfated groups into the polysaccharides can improve their biological activities through the increase in the cell-binding capacity [38]. However, the sulfate-substituted polysaccharides in this study appeared not to affect the RAW264.7 cell activation. This may be related with their high Mw. However, a considerably increased amount of NO was observed in cells treated with H1,2,3 compared to crude CIPs. This suggested that the Mw of the polysaccharide was involved in the NO-releasing capacity. Among the H1,2,3, the highest NO production was found following H2 treatment, suggesting that its Mw might be optimal for RAW264.7 cell activation, however, the activation was lower than in the positive control (LPS). According to Lake et al., the low Mw polysaccharide strengthened the binding affinity of the vascular endothelial growth factor 165 (VEGFA165) to its receptor, which suggested that smaller Mw polysaccharides could move freely and reconfigure to facilitate their binding capacity [39]. Therefore, in the RAW264.7 cell activation, the molecular orientation appeared to play an important role in the binding of cell surface receptors with minimal effect of electrostatic interaction by sulfate groups.

Further, this study determined NO production-associated gene expression, i.e., inducible nitric oxide synthase (iNOS). Fig. 3C and D show the appearance of DNA bands. The strongest band was observed from treatments with H1,2,3. This result suggested that the NO production might be augmented by iNOS expression resulting from treatment with CIPs and its derivatives (H1,2,3). Similarly, the mRNA expression of other related cytokines, including IL-1β, TNF-α, IL-6, IL-10, and IL-12, in RAW264.7 cells suggested that hydrolysis of the C. islandica polysaccharide was an effective technique to improve RAW264.7 cell activation.

An additional experiment was carried out to investigate the mechanism of CIPs and its derivatives (H1,2,3) to induce inflammatory mediators. As shown in Fig. 3E, expression of p65 was observed upon treatment with H1,2,3, indicating that the derivatives (H1,2,3) induced the phosphorylation of the p65 subunit from the cytosol to the nucleus. At that time, the phosphorylation of p65 led to activation of the NF-κB pathway and stimulation of the RAW264.7 cells. This indicated that not only the transcription of NF-κB, but also the phosphorylation of MAPK protein members, such as ERK, JNK, and p38, participate in macrophage activation.

It is shown in Fig. 3E, treatment with the derivatives induced the phosphorylation of ERK, JNK, and p38. Overall, these results demonstrated that the derivatives (H1,2,3) of the polysaccharides possess the ability to stimulate RAW264.7 cells by activation of the NF-κB and MAPK pathways. The RAW264.7 cell signaling activity was dependent on the Mw of the CIPs. This activity may be due to the interaction between the polysaccharides and its surface pathway receptors. In our study, antibodies (anti-TLR-4 and anti-TLR-2, and anti-CR3) to the pattern recognition molecules on the cell surface were used to determine the possible activation pathway of the RAW264.7 cells. Fig. 3F shows that when surface receptors were blocked with anti-TLR-4, the NO-releasing capacity of the RAW264.7 cells was dramatically decreased. Nevertheless, such activity did not occur with anti-TLR-2 or anti-CR3 treatment. These results demonstrated that CIPs and its derivatives might activate the RAW264.7 cells through a TLR-4-mediated signaling pathway. Toll-like receptor members (TLR-4) participate in recognition activities, activation of dendritic cells and macrophages, and boosting of the adaptive immune system [40].

4. Conclusion

In this study, the extracted C. islandica polysaccharide exhibited molecular features of (1→3)- and (1→4)-β-D-glucans with a Mw 328.7 × 10^6 g/mol. The immunostimulatory properties of the polysaccharide and its derivatives were tested using RAW264.7 and NK cells. A variety of sulfated derivatives improved NK cell activation through the CR3-mediated signaling pathway. At the same time, in RAW264.7 cells, the NF-κB and MAPK pathways were stimulated through TLR-4 receptors. The presence of sulfate groups in the polysaccharide backbone was influential for the cell signaling ability of NK-92 cells, probably due to the increased electrostatic interaction between the sulfate groups of the polysaccharide and the surface receptors of NK-92 cells, however, in the RAW264.7 cell activation, the molecular orientation appeared to play an important role in the binding of their cell surface receptors with minimal effect of electrostatic interaction by sulfate groups. Hence, this study concluded that chemical and structural property modifications of the polysaccharides of C. islandica could be used as functional foods or for pharmacological applications.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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