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Chemical structure and biological activity of the (1 → 3)-linked β-D-glucan isolated from marine diatom *Conticribra weissflogii*

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ABSTRACT

Several polysaccharides are considered to be "biological response modifiers" (BRM) — these refer to biomolecules that augment immune responses and can be derived from a variety of sources. Microalgae produce a diverse range of polysaccharides and could be an excellent source of BRM. Here, we describe the chemical structure and biological activity of water-soluble polysaccharide isolated from the marine diatom *Conticribra weissflogii*. Using chemical and NMR spectroscopic methods, the polysaccharide was identified as a (1 → 3)-linked β-D-glucan with a low proportion of C-6 substitution by single β-glucose units. The biological activity of this low molecular weight β-glucan (11.7 kDa) was investigated with respect to glioblastoma cell lines (U87 MG and U251) and macrophages (RAW 264.7). We observed that this β-D-glucan did not exhibit cytotoxic activity against glioblastoma cells, but did enhance the phagocytic activity of macrophages, suggesting that it possesses immunomodulatory properties.

1. Introduction

The potential of microalgae has been the subject of great interest from several areas of research and industry, such as human and animal nutrition. Microalgal biomass represents an additional source of carbohydrates [1], single-cell protein production [2], important fatty acids, natural pigments [3], vitamins, and other substances that can enrich the nutritional value of food while possessing health-promoting effects, such as improved immune responses [4]. Polysaccharides are among the compounds that can be derived from microalgae and are of interest to the food, chemical, and pharmaceutical industries [1,5,6].

β-D-(1 → 3) and β-D-(1 → 3, 1 → 6)-glucans are known to have the ability to enhance the immune system and are well-established as immunostimulants [7]. These polysaccharides are considered biological response modifiers (BRM) due to their interaction with specific cell receptors and proteins, and exhibit antibacterial, antiviral, and antitumor effects [8–11]. The immunological activities of β-glucans obtained from *Euglena gracilis* have been well documented by Barsanti and Gualtieri [12] and Russo et al. [13], and have been shown to mediate Natural Killer (NK) cell activation as well as increase levels of pro-inflammatory mediators.

Gliomas are malignant brain tumors that occur in the central nervous system; they originate in the brain and spread to glial tissue. The global incidence rate of all gliomas is 4.67–5.73 per 100,000 people, regardless of age. Glioblastoma (astrocytoma grade IV) is the most common and most deadly glioma subtype in adults (incidence ranging from 0.59 to

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3.69 per 100,000 people). It has a poor prognosis, with a low mean survival after surgery (approximately 15 months of survival), and a high mortality rate despite current optimal surgical and chemoradiotherapy regimens. Glioblastoma has many genetic alterations that result in deregulated signaling pathways, the evasion of apoptosis, and increased proliferation. These factors necessitate the search for novel therapies for glioblastoma [14–16]. Microalgae represent an economically viable source of polysaccharides with potential biologic effects [17].

β-glucans have been found to have inhibitory effects on different types of tumoral cells, even in the absence of immune cells. Several direct effects have been described in the literature, with apoptosis being the most direct. This can be triggered by the depolarization of the mitochondrial membrane and the activation of the caspase pathway in H1299 cells (human non-small cell lung cancer) [18]; by increasing the expression of p53 and Bax/Bcl-2 in SNU-C4 cells (human colon cancer) [19]; the alteration of the G0 and G1 phases in the cell cycle; and the activation of apoptotic signaling in B16-F10 cells (murine melanoma) [10]. Another potential effect is the alteration of membrane properties by acting as cell adhesion proteins. Zhao et al. [20] reported that the proliferation of B16-BL16 melanoma cells was inhibited due to the interference of β-glucans as cell adhesion proteins. In this study, we isolated and characterized a β-D-glucan extracted from the diatom *Conticribra weissflogii* and evaluated its *in vitro* biological activity with respect to two different biological cell types: glioblastoma and macrophages.

2. Material and methods

2.1. Microalgal strain and culture maintenance

The *C. weissflogii* microalga strain was obtained from the Elizabeth Aidar Microalgal Culture Collection (Fluminense Federal University, Brazil). The microalgae inoculum was maintained in sterilized f/2 culture medium [21] at 19 ± 1 °C under continuous light.

2.2. Extraction and purification of C. weissflogii polysaccharide

The extraction of polysaccharide was carried out following the methodology described by Granum and Myklestad [22]. Briefly, dry biomass was extracted with 0.05 M H_2SO_4 at 60 °C for 10 min. The extract was then centrifuged to separate the supernatant, which was dialyzed with distilled water and ultrapure water (cut-off 1 kDa), concentrated, and freeze-dried, producing the *C. weissflogii* acid (CAC) extract. CAC was purified by microfiltration using a 0.1 μm membrane (Millipore).

2.3. Biochemical analyses

The total carbohydrate content was estimated using the phenol-sulfuric acid method [23] and quantified spectrophotometrically using a standard glucose curve. The protein content was measured using a Folin-Ciocalteu reagent with a bovine serum albumin standard [24].

The monosaccharide composition was determined after acid hydrolysis (2 M TFA at 120 °C for 2 h). Hydrolysis products were reduced with $NaBH_4$, for 12 h. The $NaBH_4$ excess in the reaction was converted into boric acid by adding acetic acid to pH 5. Boric acid was eliminated by co-distillation with methanol (4×). After acetylation with acetic anhydride (1 h at 120 °C), the resulting alditol acetate derivatives were analyzed by Gas Chromatography-Mass Spectrometry (GC–MS). GC–MS analyses were performed using a Varian 3800 chromatograph equipped with a fused-silica capillary column (30 m × 0.25 mm) coated with DB-225 ms (Durabond), and a Varian Saturn 2000 R ITD spectrometer. The chromatograph was programmed to run at 50 °C for 1 min, then ramped from 50 to 215 °C at 40 °C.min^{-1}. Helium was used as carrier gas with a flow rate of 1 mL.min^{-1} [25].

2.4. Controlled Smith degradation

CAC (80 mg) was oxidized with 0.05 M $NaIO_4$ (40 mL) for 72 h at 25 °C under dark and agitated conditions. Following this, the solution was treated with ethylene glycol, reduced with $NaBH_4$ for 15 h, neutralized with acetic acid, dialyzed, and freeze-dried [26]. The oxidized and reduced material was subjected to partial acid hydrolysis (TFA, pH 2 at 100 °C for 1 h). After neutralization with NaOH, dialysis (1 kDa cut-off), and freeze-drying, the Smith-degraded fraction (CAC-s; 40 % yield) was obtained.

2.5. Methylation analysis

CAC was methylated according to the method described in Ciucanu and Kerek [27]. Briefly, the sample (5 mg) was dissolved in DMSO (1 mL), after which NaOH (30 mg) was added. After being stirred for 30 min at 25 °C, iodomethane (0.2 mL) was added, and the reaction was allowed to proceed. The methylation process was repeated twice, and the reaction was interrupted by the addition of distilled water and neutralized with acetic acid. The methylated polysaccharide was dialyzed and freeze-dried. Partially methylated alditol acetates were generated by hydrolysis using 90 % formic acid (0.5 mL) for 6 h at 100 °C using the method for glucans as described by Bao et al. [28], followed by $NaBH_4$ reduction and acetylation. The products were analyzed by GC–MS and identified by their mass spectra and retention times [25,29] using the same column and conditions as described in Section 2.3.

2.6. High-pressure size exclusion chromatography (HPSEC) analysis

The analysis was performed using 1 mg.mL^{-1} of polysaccharide solution in the eluent (0.1 M $NaNO_3$ solution containing NaN_3 0.2 g.L^{-1}) with a Waters high-performance size exclusion chromatography (HPSEC) instrument coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS) detector adapted for on-line use. Four Waters Ultrahydrogel columns (2000, 500, 250, and 120) were connected in series and coupled to the multi-detection equipment. The specific refractive index increment (dn/dc) was determined using five concentrations between 0.2 and 1.0 mg.mL^{-1} (dn/dc value 0.136). HPSEC data were collected and analyzed using the Wyatt Technology ASTRA program. All experiments were carried out at 25 °C.

2.7. Nuclear magnetic resonance (NMR) spectroscopy

Each lyophilized sample was dissolved in deuterium oxide (D_2O) (30 mg.mL^{-1}) and assessed at 70 °C using a Bruker Advance DRX400 NMR spectrometer equipped with a 5 mm multi-nuclear inverse detection probe; a base frequency of 100.63 MHz and 400 MHz was used for ^{13}C and ^{1}H nuclei, respectively. Chemical shifts were expressed in ppm using acetone as an internal standard at 30.2 and 2.225 ppm for ^{13}C and ^{1}H, respectively. For ^{1}H and 2D NMR experiments, samples were deuterium-exchanged by successive freeze-drying steps in D_2O (99.9 %) and then dissolved in D_2O (30 mg.mL^{-1}). Heteronuclear single quantum coherence spectroscopy (HSQC) spectra were obtained using the pulse program supplied with the Bruker spectrometer. Samples dissolved in dimethyl sulfoxide-d_6 (Me_2SO-d_6, 99.9 %, Sigma-Aldrich) had their spectra calibrated to the solvent peak at 39.51 and 2.50 ppm for ^{13}C and ^{1}H, respectively.

2.8. Biological activity assays

2.8.1. Cells and culture conditions

The biological activity assays were performed using the mouse macrophage-like cell line RAW 264.7 transformed by the Abelson Leukemia virus [30], the human glioblastoma cell lines U87 MG and U251,

and the mouse hippocampal neuronal-derived cell line HT22. All cell lines were cultivated in DMEM supplemented with 10 % SFB and were maintained in an incubator at 37 °C and 5 % CO_2.

2.8.2. Cell metabolic activity assay

Cell lines were incubated in a culture medium in either the presence or absence of CAC β-D-glucan (50 and 100 μg.mL^{-1}) over different lengths of time. Metabolic activity was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma–Aldrich Chemical Co., St. Luis, MO, USA) as described by Reilly et al. [31]. Absorbance was analyzed by spectrophotometry (Bio-Tek Synergy H1 hybrid reader model) at 570 and 630 nm.

2.8.3. Morphology alteration and cell proliferation assay

All macrophages (5 × 10^5 cells per well) were plated in xCELLigence plates and maintained in the presence or absence of CAC β-D-glucan (0.5, 1.0, and 2.5 μg. mL^{-1}) for 30 h. An xCELLigence real-time cell impedance system (Aligent Bioscience, San Diego, CA, USA) was used to monitor the changes in the number and morphology of macrophages by varying impedance in the culture media of a 96-well microelectronic plate after treatment with the CAC β-D-glucan. Measurements of macrophage impedance were normalized to the time of treatment (normalized cell index), where $t = 0$ h was defined as the starting point of the experiment.

2.8.4. Phagocytosis assay

Phagocytic activity was assessed using yeasts as phagocytic particles [32,33]. Briefly, a macrophage monolayer (5 × 10^5 cells per well) was adhered to a 24-well glass coverslip and was incubated with the standard medium in the absence (control) or presence of varying concentrations of CAC (0.5, 1.0, and 2.5 μg. mL^{-1}). After 24 h, cells were washed three times with a MEM medium, following addition of 10 yeast cells per macrophage (macrophage to yeast ratio of 1:5), following incubation under the same conditions for 120 min. After each incubation cycle, the samples were washed with PBS medium to remove non-phagocytic yeasts. Coverslips were fixed with Bouin's fixative, stained with Giemsa for 2 h, and dehydrated in acetone. Slides were mounted with Entelan and examined microscopically. Following this, the total number of macrophages, the number of phagocytic macrophages, and the number of phagocytized yeasts were quantified. The phagocytic activity and index were evaluated by determining the number of yeasts phagocytosed by macrophages for every 200 macrophages on each coverslip.

2.8.5. Flow cytometry assays

U87 MG cells (4 × 10^4 cells per well) were seeded into a 24-well plate. After 24 h, the cells were treated with CAC β-D-glucan (100 μg. mL^{-1}) and incubated for 72 h. To evaluate cell death, the cells were collected and stained with Annexin V FITC and PI from the Dead Cell Apoptosis Kit (Thermo Fisher Scientific, Waltham, US), according to the manufacturer's instructions. For cell cycle analysis, cells were fixed in 4 % PFA for 15 min on ice. The cells were then washed with PBS and stained with DAPI (1 μg.mL^{-1} in PBS, 0.01 % TRITON-X-10) on ice for 30 min in the dark. Samples from both techniques were analyzed in a Beckman Coulter CytoFLEX S Flow Cytometer (Beckman Coulter, Woerden, the Netherlands), and the data were reviewed in the FlowJo 10.5 software.

2.9. Statistical analyses

A Shapiro-Wilk normality test was used to verify the distribution of the data. Significant differences were determined using a one-way analysis of variance (ANOVA). Differences between mean values were tested using Tukey's *post hoc* test. Data with $p < 0.05$ were considered statistically significant. All graphs were created using GraphPad – Prism 5 and 8.

Table 1
Yield, chemical analyses, and monosaccharide composition of the polysaccharide fraction obtained from diatom *C. weissflogii*.

Sample[a]	Yield[b] (%)	Carbohydrate[c] (%)	Protein[c] (%)	Monosaccharides (mol %)[d]			
				Rha	Ara	Xyl	Glc
CAC	29.9	89.9	2.1	2.1	0.3	1.6	95.0

[a] CAC sample is defined in the text.
[b] Percentage based on dried microalgae biomass.
[c] Carbohydrate and protein contents were determined by the Dubois et al. [23] and Lowry et al. [24] methods, respectively.
[d] Monosaccharides quantified in mol% in the acetate alditol form. Rha: rhamnose, Ara: arabinose, Xyl: xylose, Glc: glucose.

3. Results and discussion

3.1. Extraction and characterization of the water-soluble polysaccharide from C. weissflogii

Water-soluble polysaccharide (CAC) was isolated from the marine diatom *C. weissflogii* following the conditions previously reported [22]. The yield, analyses, and monosaccharide composition of CAC are presented in Table 1. The polysaccharide fraction contained glucose as major neutral sugar (95.0 mol%), indicating the presence of a glucan. HPSEC-MALLS-RID analysis of CAC showed a symmetric peak centered at 55 min, suggesting a homogeneous profile (Fig. 1) with a molecular weight of 11.7 kDa.

3.2. NMR spectroscopic analyses

The ^{13}C NMR spectrum of CAC exhibited six main signals (Fig. 2a). The 102.5 ppm signal in the anomeric region was attributed to C-1 in the β-glucosyl units. The resonance at 84.8 ppm was attributed to C-3 in the (1 → 3)-linked β-D-glucosyl units, while the signals at 73.2, 68.4, and 75.7 ppm were assigned to C-2, C-4, and C-5, respectively. The 61.0 ppm signal corresponds to the non-linked C-6 in the (1 → 3)-linked β-D-glucosyl units as confirmed by the inverted signal in the ^{13}C-DEPT NMR spectrum (Fig. 2b). This NMR spectrum is representative of chrysolaminaran similar to those previously extracted from other diatoms [7,34]. Low-intensity signals observed at 95.9 and 92.2 ppm respectively correspond to the C-1 of β- and α-anomers of the 3-linked glucosyl reducing terminal unit [9]. These results together with HPSEC-RID data, indicate that a (1 → 3)-linked β-D-glucan with a relatively low molecular weight was extracted from the marine diatom *C. weissflogii*.

The main ^{13}C/^{1}H correlations were observed in the HSQC spectrum of CAC (Fig. 3). The C1/H1 anomeric correlation at 102.5/4.78 ppm confirmed the β-anomericity of the glucosyl units. The correlation at 84.8/3.78 ppm was attributed to the substituted C3 and its hydrogen

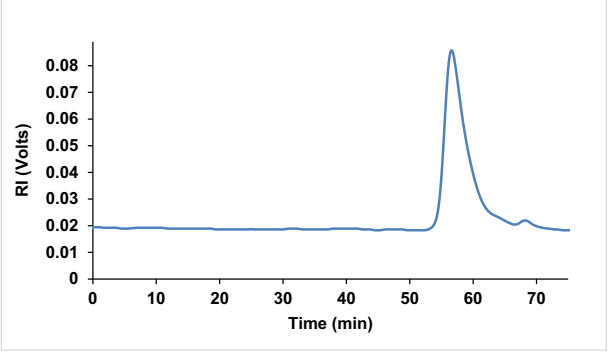

Fig. 1. HPSEC elution profile of CAC, obtained with refractive index (RI) detector.

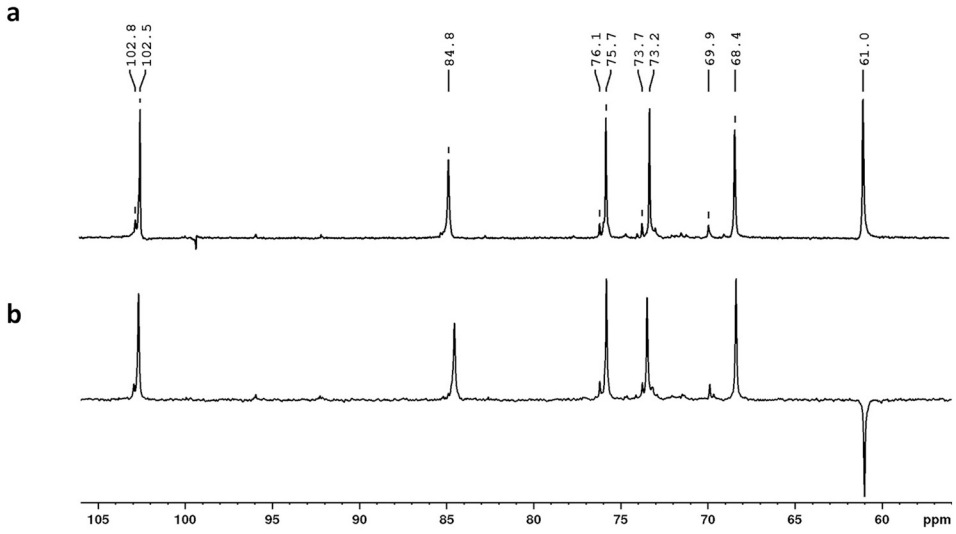

Fig. 2. ^{13}C NMR spectrum (a) and ^{13}C DEPT NMR spectrum (b) of the polysaccharide CAC (solvent: D_2O, at 70 °C).

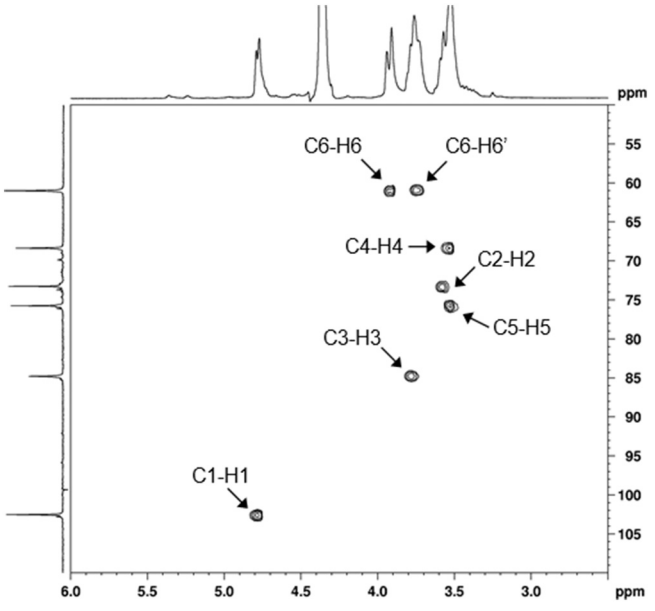

Fig. 3. HSQC spectrum of polysaccharide CAC (solvent: D_2O, at 70 °C).

(C3/H3), which is consistent with the presence of (1 → 3)-glycosidic linkages. The carbon at 61.0 ppm (DEPT inverted) was correlated with hydrogens at 3.91 and 3.75 ppm; these correlations were attributed to the unsubstituted C6/H6,H6'. All the other (1 → 3)-linked glucan correlations observed in the CAC spectrum were assigned and presented in Table 2.

NMR analyses of β-glucans from different sources are frequently carried out using deuterated DMSO (Me_2SO_4-d_6) as a solvent. To compare the ^{13}C NMR chemical shifts of the (1 → 3)-linked β-D-glucan extracted from *C. weissflogii* with other β-glucans described in the literature, the CAC fraction was also subjected to NMR analysis using Me_2SO_4-d_6 as a solvent (Table 3). These results show the similarities in the ^{13}C NMR chemical shifts of β-glucans isolated from different sources.

Table 2
NMR chemical shift assignments of polysaccharide CAC isolated from diatom *C. weissflogii*.

Sample	$^{13}C/^{1}H$ chemical shifts (ppm)[a]					
	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6,H6'
CAC	102.5/ 4.78	73.2/ 3.57	84.8/ 3.78	68.4/ 3.52	75.5/ 3.54	61.0/ 3.91,3.75

[a] Solvent D_2O, temperature: 70 °C.

Table 3
^{13}C NMR assignments of polysaccharide CAC (in Me_2SO-d_6) compared with (1 → 3)-linked β-glucans from other sources.

(1 → 3)-linked β-glucan source	^{13}C chemical shifts (ppm)					
	C1	C2	C3	C4	C5	C6
C. weissflogii (marine diatom)[a]	103.1	73.0	86.3	68.6	76.5	61.0
Ganoderma resinaceum (fungus)[b]	102.9	72.8	86.2	68.5	76.4	60.9
Pleurotus tuberregium (fungus)[c]	102.6	72.8	85.9	68.1	75.6	60.3
Chaetoceros müilleri (marine diatom)[d]	102.7	73.4	84.4	68.3	75.8	60.9

[a] Present study.
[b] Amaral et al. [35].
[c] Chenghua et al. [36].
[d] Størseth et al. [7].

3.3. Methylation analysis of CAC

Results from the methylation analysis were consistent with the NMR experiments. The molecule primarily consists of (1 → 3)-linked glucopyranosyl units, which account for 92.3 % of the polymer (Table 4). However, methylation also shows a small degree of branching (4.9 %) in C-6. There is also a small amount of (1 → 4)-linked glucopyranosyl units, which represent 1.8 %.

The results of the ^{13}C NMR spectroscopy and methylation analyses suggest that the minor signals at 102.8 (C-1), 76.1 (C-5), and 73.7 (C-2) ppm could be attributed to the β-(1 → 6)-linked units. The presence of β-(1 → 4)-linked units was not detected by NMR, although they were identified by methylation analysis. These units could be part of the structure or be originated from a minor contamination during the extraction process, as previously reported by Vogler et al. [37] for microalga *Nannochloropsis gaditana*.

3.4. Analysis of the Smith-degraded product

To determine the fine structure of the polysaccharide, a CAC-s fraction was produced by subjecting CAC to a controlled Smith degradation. The ^{13}C NMR spectrum of the CAC-s fraction revealed an abundance of

Table 4
Methylation analysis of polysaccharide fraction CAC from diatom *C. weissflogii*.

Derivative[a]	Rt[b]	Mass fragments (*m/z*)	Deduced linkage	CAC (mol %)[c]
2,3,4,6-Me_4-Glc	1	101; 113,129; 145	Glc*p*-(1→	1.0
2,4,6-Me_3-Glc	1.269	101; 117; 129; 145	→3)-Glc*p*-(1→	92.3
2,3,6-Me_3-Glc	1.393	87; 103; 115; 128	→4)-Glc*p*-(1→	1.8
2,4-Me_2-Glc	1.945	87; 117; 129; 143	→3,6)-Glc*p*-(1→	4.9

[a] 2,3,4,6-Me_4-Glc analyzed as 1,5-di-*O*-acetyl-2,3,4,6-*O*-methyl glucitol, *etc*.
[b] Retention time of derivatives in relation to 2,3,4,6-tetra-*O*-methyl-Glc.
[c] mol % of monosaccharide quantified in the form of partially methylated alditol acetate, analyzed by GC–MS.

signals that corresponded to (1 → 3)-linked β-glucopyranosyl units (Fig. 4) that were identical to those observed in the native polysaccharide CAC (Fig. 2). However, the low-intensity signals in the ^{13}C spectrum differed from those present in the native polysaccharide (CAC). One of these resonances occurred at 63.0 ppm (Fig. 4b) and was attributed to the C-1 of the reducing terminal unit that was reduced with $NaBH_4$ during the controlled Smith process. Furthermore, the signals at 104.1 and 61.5 ppm were attributed to the C-1 and C-6 of the non-reducing terminal (NRT) units, suggesting that the polysaccharide had partially depolymerized [28]. The presence of (1 → 4)-linked units in the backbone of the native polysaccharide, not confirmed in the present study, could explain the partial depolymerization of the β-glucan. The proposed structure of the controlled Smith-degradation product (CAC-s) is presented in Fig. 5.

The NMR spectra and the results of the methylation analyses indicate that the water-soluble polysaccharide extracted from the microalga *C. weissflogii* is chrysolaminaran, consisting of a (1 → 3)-linked β-D-glucan backbone (97.2 %, 3-linked plus 3,6-linked) with low proportion of substitution in C-6 by single glucose units (Fig. 6).

Polysaccharides in diatoms are often divided into three groups: cell wall polysaccharides, extracellular mucilages, and reserve polysaccharides [22]. The cell wall of diatoms is composed of silica and organic wrappers containing heteropolysaccharides and can vary widely in terms of the proportion of galactose, glucose, mannose (and their corresponding uronic acids), xylose, fucose, rhamnose, and other residues, which vary depending on species [22,38]. Extracellular mucilage has a variable composition; however, glucose is the most abundant monomer as it is excreted during the cultivation exponential phase as

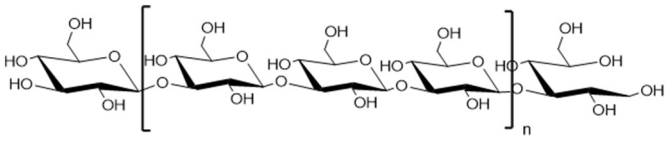

Fig. 5. Proposed structure for the Smith-degraded product (CAC-s).

observed for three diatoms species [39]. Reserve polysaccharides consist of a soluble polysaccharide derivative of glucose formed by β-(1 → 3) glycosidic linkages [40]. β-(1 → 3)-glucan competes with cellulose as the most abundant carbohydrate on earth and is the main food reserve for diatoms [41,42]. β-glucans in diatoms are known as chrysolaminaran (previously known as leucosin) [22]; this differs from laminaran, which is found in brown algae, due to the absence of mannitol [41]. The extraction of diatoms usually involves the disruption of cells by dilute acid treatment, as was the case in this paper. Acid extraction can isolate crystalline laminaran in diatoms, of which 99.5 % is composed of D-glucose [41,42]. However, the extraction of (1 → 3)-linked β-D-glucans from some species of diatoms, such as *Cylindrotheca fusiformis*, *Craspedostauros australis*, and *Thalassiosira pseudonana*, using a hot aqueous medium could indicate the presence of these molecules in the intracellular vacuoles of those organisms [43,44].

In this study, a β-(1 → 3)-glucan with a molecular weight of 11.7 kDa was isolated. According to McConville et al. [5] as well as Paulsen and Myklestad [6], isolated chrysolaminaran is expressed as a β-(1 → 3)-glucan with a molecular weight of 10.1 kDa with a small degree of binding variation in the β-(1 → 2) and (1 → 6) positions. However, the polysaccharide in this strain was found to have a small degree of β-(1 → 4) glycosidic linkages. Vogler et al. [37] characterized a β-(1 → 3), (1 → 6)-glucan from *N. gaditana*, also containing 4 linkage residues (9.1 %). NMR analysis presented in the study as well did not show the presence of (1 → 4) glycosidic linkages although they were detected by methylation analysis and attributed to cellulose contamination. Størseth et al. [7] identified the presence of reserve polysaccharides such as chrysolaminaran in the diatom *Chaetoceros mulleri* and characterized them as a branched β-(1 → 3)-glucan. This suggests that the structure of chrysolaminaran may vary according to species and that the yield varies depending on the stage of microalgae growth. Størseth et al. [7] also identified a (1 → 3)-linked β-glucan present in *Chaetoceros debilis* that contained a large number of (1 → 6)-linked β-glucose units, which had not previously been reported in diatoms. This emphasizes the variety of

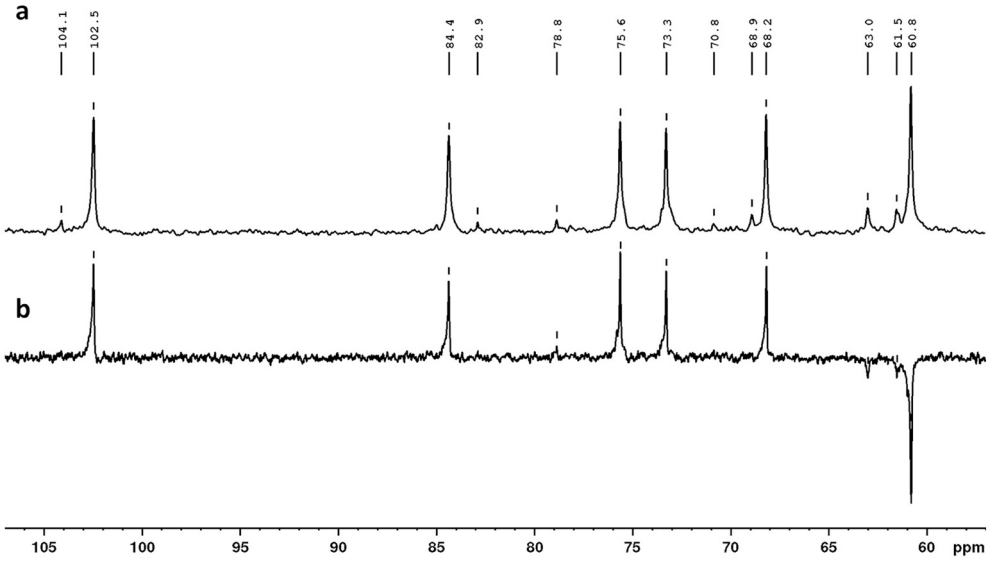

Fig. 4. NMR analyses of the Smith-degraded product (CAC-s). ^{13}C (a) and ^{13}C DEPT (b) NMR spectra (solvent: D_2O, at 70 °C).

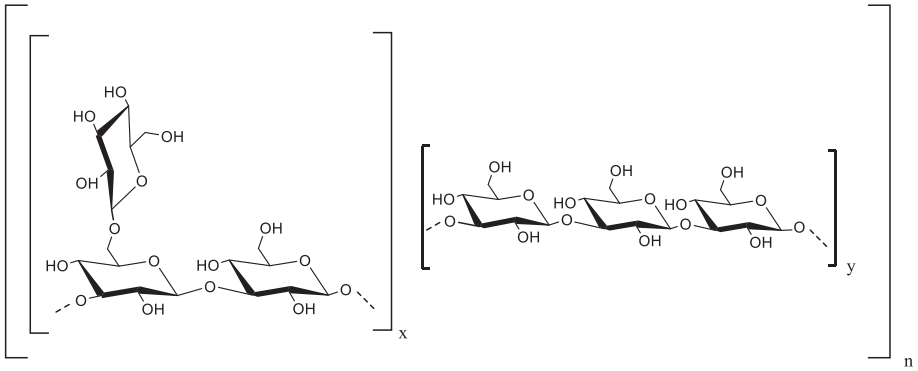

Fig. 6. Proposed structure for the water-soluble polysaccharide extracted from diatom *C. weissflogii*. Molar proportions: x: 0.05; y: 1.

chrysolaminaran structures that can be found within the same microalgae group.

3.5. Biological activity of polysaccharide extracted from C. weissflogii

3.5.1. Effects of CAC β-D-glucan on glioblastoma and neuronal cells

To investigate whether CAC β-D-glucan affects cell growth and survival, we treated human glioblastoma cell lines (U87 MG and U251) and a mouse hippocampal neuronal-derived cell line (HT22) with different concentrations (50 and 100 μg.mL^{-1}) of CAC β-D-glucan for 24, 48, and 72 h. MTT assays and flow cytometric analysis were performed. The results suggested that CAC β-D-glucan had no significant cytotoxic effects on the U87 MG and U251 cells (Fig. 7a, b, c, d). Indeed, after 24 h of CAC β-D-glucan treatment, U87 MG cells in the experimental group exposed to the highest concentration of CAC β-D-glucan (100 μg.mL^{-1}) were found to have slightly higher metabolic activity compared to the control group. However, this metabolic effect was not maintained after longer exposure times (48 and 72 h), indicating that there was no statistical difference in the percentage of metabolically active cells observed in the treated groups compared to the control groups for each of the different CAC β-D-glucan concentrations used. Similarly, there was no significant change in the metabolic activity of U251 MG cells and HT22 non-tumor cells under the same conditions (Figs. 7b and S1a, b, c).

In contrast, Pires Ado et al. [45] showed that β-D-glucans derived from mushrooms *Lactarius rufus* and *Agaricus bisporus* exhibited a cytotoxic effect on HepG2 hepatocarcinoma cells without affecting the primary hepatocyte, albeit in higher concentrations than those tested here (>100 and 200 μg.mL^{-1}, respectively). Interestingly, our data did not show any changes in cell death or alterations to cycle progression in CAC β-D-glucan-treated U87 MG cells even at the highest concentration used (100 μg.mL^{-1} for 72 h) (Fig. 7c, d, e). Choromanska et al. [46], Hussain et al. [47] reported the cytostatic action of a β-D-glucan extracted from the plant species *Avena sativa* on the Me45 (human melanoma), A431 (epidermal carcinoma), Colo-205 (human colon cancer carcinoma), MCF-7 (human breast carcinoma), and T47D (human ductal breast epithelial tumor) cell lines. There was a drop in cell metabolism without inducing cell death or alterations to the cell cycle when exposed to polysaccharides at concentrations of 50 and 100 μg.mL^{-1}.

The antitumor mechanisms proposed for most polysaccharides include direct killing as well as indirect antitumor activity by improving immune functions [4,8,10,17,19,20]. There are several examples of polysaccharides in the literature that are non-cytotoxic or exhibit cytotoxicity in higher concentrations (above 400 μg.mL^{-1}) that still have clear immunostimulatory effects [48–51]. Li et al. 2019 [48] and 2020 [49] reported that the treatment of MCF7 and 4 T1 breast cancer cells with different concentrations of α-(1 → 6)-glucan from the medicinal herb *Astragalus membranaceus* (100 to 1000 μg.mL^{-1}) did not inhibit proliferation or increase apoptosis. On the other hand, this glucan was able to activate murine macrophages, promoting up-regulation of TNF-α and NO production, which may lead to repression of cell proliferation in a time- and concentration-dependent manner, cell cycle arrest and apoptosis in MCF7 cells. In addition, Wufuer et al. [50] showed that an α-(1 → 4) glucan from the plant species *Brassica rapa* L. showed cytotoxic activity more pronouncedly at high concentrations (500–2000 μg.mL^{-1}), leading to apoptosis only on A549 lung adenocarcinoma cells. Interestingly, five different α-glucans from *B. rapa* L. had no cytotoxic effect even at the highest concentration used (2000 μg.mL^{-1}) in both, AGS gastric adenocarcinoma and HepG2 hepatocarcinoma cell lines, showing that cytotoxic effects are dependent of cell-type or cellular context. However, these five α-glucans were able to promote the growth of murine macrophage cells and also stimulate the released of NO and TNF and IL-6 cytokines.

Concerning β-glucans, Rutcheviski et al. [51] showed that β-(1 → 6)-D-glucan extracted from the mushroom *Agaricus bisporus* inhibit cell viability in MCF-7 breast cancer cells already at 100 μg.mL^{-1}, while MDA-MB-231cells (a more aggressive cell line, derived from Triple-negative breast cancer -TNBC) showed more resistance phenotype even at higher concentration (300 μg.mL^{-1}). Interestingly, the authors indicate that MCF-7 cell line express estrogen receptors while MDA-MB-231 do not express such receptor [51,52]. According to this data, Xu et al. [53] described that a β-glucan from the mushroom *Lentinus edodes* reduced MCF-7 cell viability in a concentration-dependent manner but did not affect the TNBC cell line, suggesting that cytotoxic-responsiveness to β-glucans could be related to estrogen-receptor expression in breast cancer model. It is important to note that, in addition to direct tumor cell cytotoxicity, β-(1 → 6)-D-glucan extracted from *A. bisporus* was able to stimulate human THP-1 macrophages, leading to increased levels of pro-inflammatory cytokines (TNF-α, IL-1β and CXCL10) at lower concentrations of the polysaccharide (30 and 100 μg.mL^{-1}).

The literature also shows that β-glucans obtained from different sources often exhibit different biological responses despite having the same basic structures, suggesting that there are essential aspects of the β-glucans structures that affects cellular response. These include the degree of polymerization, branching, molecular weight, and the presence, abundance or absence of specific functional groups at the backbone and side chains (as sulfate, phosphate, carboxymethyl, among others) that will rule the primary and spatial structure of those glucans. These features mainly control the interaction between the polysaccharides and carbohydrate receptors or polysaccharide-proteins complexes and trigger different types of responses [54–56].

3.5.2. Effect of CAC β-D-glucan on the phagocytic activity of macrophages

We then investigated whether CAC β-D-glucan affected murine macrophages in RAW 264.7 cell lines, which are associated with immunomodulatory processes. An analysis of xCELLigence cell-impedance real-time measurements indicates that CAC β-glucan (2.5 μg.mL^{-1}) significantly increases the cell index even after only 14 h

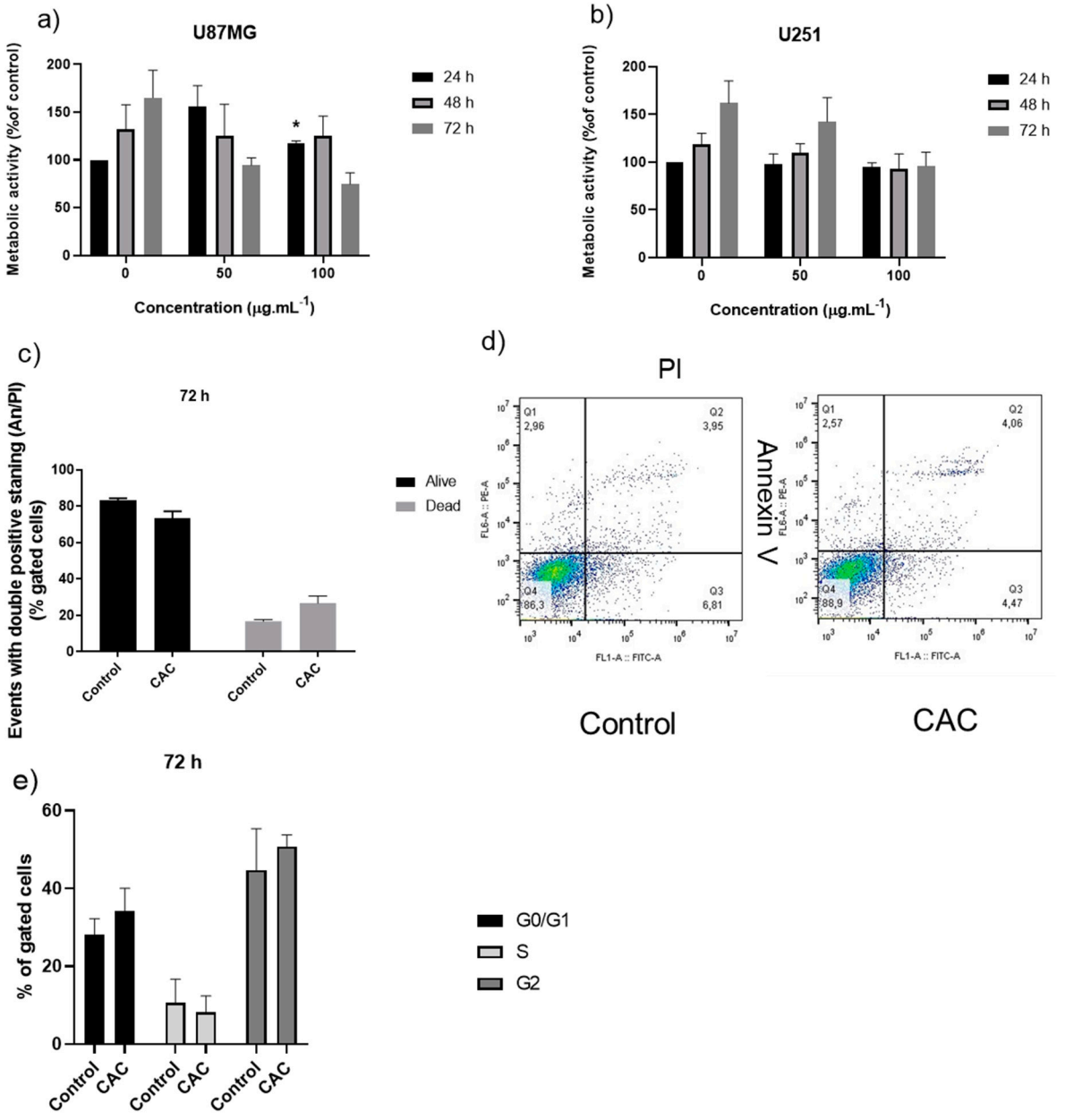

Fig. 7. Metabolic activity determined by MTT test at glioblastoma cells U87 MG (a) and U251 (b) with CAC β-D-glucan at 50 and 100 μg. mL^{-1} concentrations at 24, 48 and 72 h treatment time points. Zero (control) corresponds to 100 % of metabolic activity, normalized to untreated cells at 24 h. These results are expressed as mean ± S.D., three independent experiments with four technical replicates, by ANOVA test. (*) Significant differences observed compared with the control group by *post-hoc* Tukey test ($p < 0.05$). Cell death assay by An-/PI- and An+/PI+ staining with U87 MG cell line (c, d) and cell cycle progression with U87 MG (e), with presence of CAC β-D-glucan compound at 100 μg mL^{-1} concentration for 72 h, expressed as mean ± S.D., three independent experiments each with three technical replicates, by ANOVA test.

following treatment when compared with the controls (Fig. 8a); there were also changes in cell attachment, morphology, and/or growth characteristics as a response to CAC β-D-glucan. These results allow for the study of the biomodulating effect of the polymer as well as the evaluation of the phagocytic activity of macrophages in the RAW 264.7 cell line. Using yeasts as phagocytic particles, a clear increase in the phagocytic capacity of RAW 264.7 cells treated with CAC β-D-glucan was observed in terms of the percentage of phagocytosis activity (Table 5), indicating immunostimulatory activity at all tested concentrations (Fig. 8b, c). It must be pointed out that CAC β-D-glucan already showed phagocytic activity at the lowest concentration that was used (0.5 μg. mL^{-1}). In the literature, a different α-(1 → 4) glucan from *Brassica rapa L* [50] and a branched β-(1 → 3)-glucan from the mushroom *Russula vinosa* [57] were able to induce phagocytic ability of RAW264.7 cells, evaluated by neutral red assay, from concentrations of 62.5 and 25 μg. mL^{-1}, respectively.

Macrophages have an important function in tumoral immunosurveillance. They are present within the tumoral microenvironment and have an important role in tumor immunity. Depending on their activation state, they are capable of phagocytizing cancer cells and presenting tumor-specific antigens to T and B lymphocytes. The activation state of macrophages is modulated by lipopolysaccharides (LPS). LPSs trigger cell signaling events in macrophages, inducing the release of important pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), which stimulates tumoral killing and phagocytosis to produce tumor-specific antigens [58].

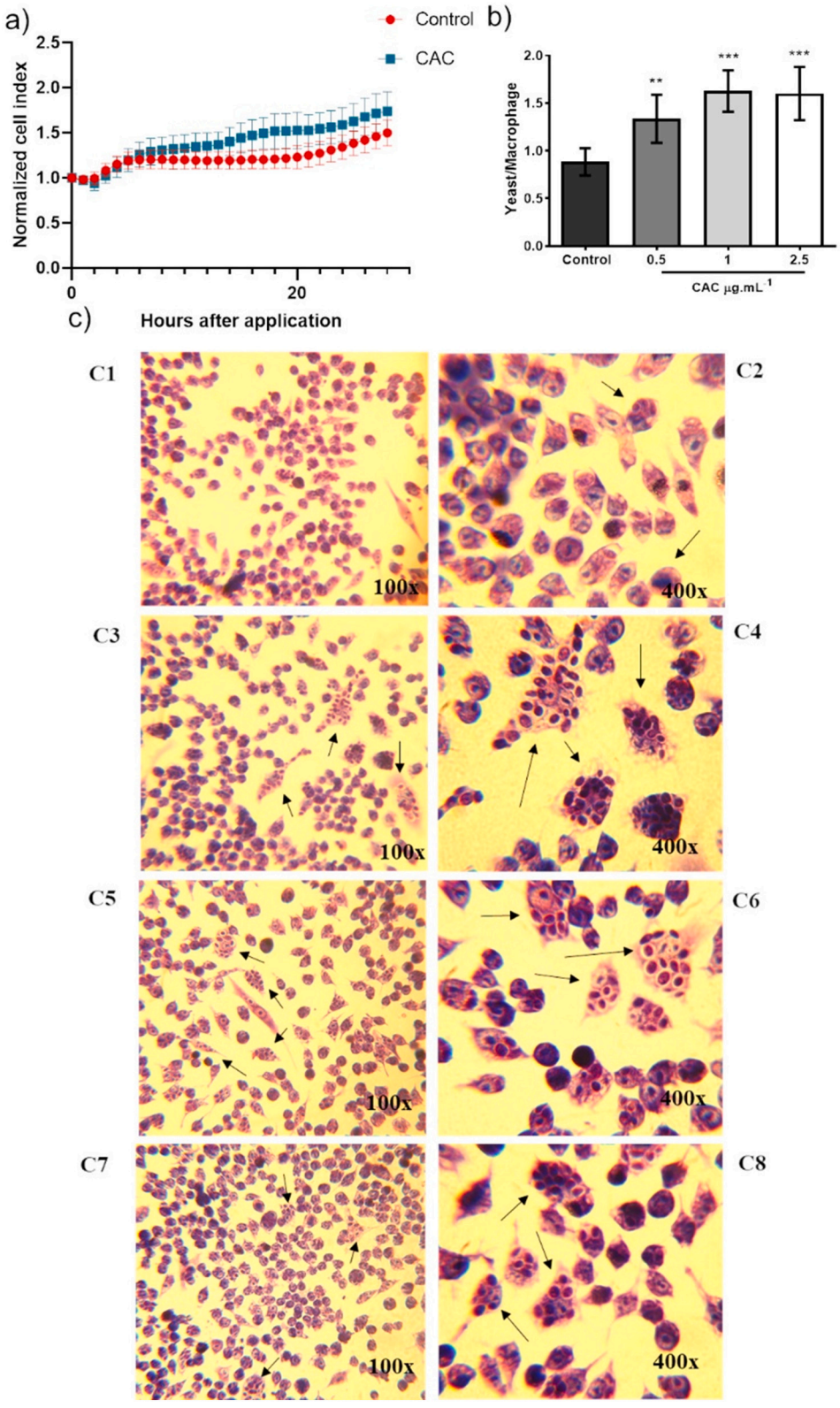

Fig. 8. Macrophage RAW 264.7 cells challenged with CAC β-D-glucan at 2.5; 5.0; 10 and 25 μg. mL^{-1} concentrations. (a) Cell impedance real-time measurements upon CAC β-D-glucan treatment (2.5 μg. mL^{-1}, 30 h) (blue curve) and non-treated control (red). (b) Phagocytic index (yeast/macrophage) upon CAC β-D-glucan treatment (0, 0.5, 1.0 and 2.5 μg. mL^{-1}) for 24 h. (c) Representative pictures of phagocytic activity. Macrophages were cultivated in absence (control) (C1, C2) or presence of CAC β-glucan 0.5 (C3, C4), 1 (C5, C6) and 2.5 (C7, C8) μg. mL^{-1} for 24 h. After treatment, the medium was removed and yeasts (1:5) were added following incubation for 1 h. Arrows point yeasts being phagocytized by macrophages. Original magnification: ×400 (C1, C3, C5, C7) and ×1000 (C2, C4, C6, C8). All results are expressed as mean ± s.d., three independent experiments in triplicate to each concentration. (**) Significant differences between treatment groups and the control group by Tukey *post-hoc* test ($p < 0.01$) (***) Significant differences between treatment groups and the control group by the Tukey test ($p < 0.001$).

It is important to note that CAC β-D-glucan is a partially branched (1 → 3)-linked β-D-glucan with low molecular weight. Many studies have reported the immunostimulating capacity of β-glucans and have related this immunostimulatory activity to their chemical structure [48–51]. Kulicke et al. [58] compared the influence of the molecular structure of four different β-glucans on immune activity and found that β-glucans with low molecular weight are more efficient at activating the immune system by increasing the amount of TNF-α and superoxide radicals released by human monocytes. Furthermore, higher immunomodulatory activities were observed in (1 → 3)-linked β-D-glucans with a lower degree of branching compared to those with higher degrees of branching [28,59]. β-glucans have received increased attention in recent decades

Table 5
Phagocytic activity of RAW 264.7 cells upon yeast with CAC β-D-glucan.

Concentration (μg. ml^{-1})	Phagocytic activity (Mean ± SD)	% of enhancement compared with control
Control (0)	0.88 ± 0.1	–
0.5	1.34 ± 0.3*	54.3
1.0	1.61 ± 0.3***	84.7
2.5	1.63 ± 0.2***	88.8

Three independent experiments in triplicate for each concentration. Significant differences from control group by Dunnett *post-hoc* test (*) ($p < 0.05$) and (**) ($p < 0.01$). Phagocytic activity was calculated as described in the methods section.

as they can be recognized by macrophage-specific receptors and, consequently, can activate this type of cell. The β-D-glucans are phagocytized after activation [60,61]. Consequently, determining the structural characteristics of β-glucans is an essential prerequisite for understanding how glucans are recognized by the immune system.

Immunomodulators primarily operate based on the stimulation of specific and non-specific functions of the native and adaptative immune system [58,59]. β-D-glucans are engulfed by macrophages and carried to the marrow and the endothelium reticular system. There, small fragments from the polymer are released and bind to different receptors such as Dectin-1, toll-like receptor (TLR) 2, 3, 4, and 6, complement receptor 3 (CR3), carbohydrate-binding modules, and the cluster of differentiation 5 (CD5). They also can bind to proteins and glycoproteins [62–66]. The activation of these receptors triggers molecular pathways such as phosphoinositide 3-kinase (PI3K), macrophage inflammatory protein 2 (MIP-2), tyrosine-protein kinase (PTK), releasing diverse cytokines [61,65,66]. In addition, another (1 → 3),(1 → 6)-linked β-D-glucan from the seaweed *Durvillaea antarctica* increased the activation of RAW 264.7 cells by binding to TLR4 and mediating the release of nitric oxide (NO), reactive oxygen species (ROS), MCP-1, TNF-α, and interleukin-1 beta (IL-1β) [67].

4. Conclusion

A (1 → 3)-linked β-D-glucan with a small proportion of C-6 substitution by single β-glucose units was isolated from the marine diatom *C. weissflogii.* This water-soluble polysaccharide, which had a molecular weight of 11.7 kDa, did not had cytotoxic effect on glioblastoma cell lines at any of the tested concentrations. However, this microalgal β-D-glucan was able to enhance the phagocytic activity of the macrophage RAW 264.7 at low concentrations, suggesting that it exhibited important immunostimulatory activity and was thus of biotechnological interest. Further studies are necessary to evaluate its *in vivo* immunostimulating potential.

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CRediT authorship contribution statement

Conceptualization, J.R., G.R.N. and M.D.N; Writing – Original Draft Preparation, J.R., M.E.D. and M.D.N; Writing – Review & Editing, T.R.M., G.A.C., M.T.L., M.E.D., G.R.N. S.M.B.W., M.D.N., and A.M.D.; Supervision, M.D.N., M.E.D., G.R.N. and A.M.D.; Project Administration, M.D.N., M.E.D., G.R.N. and A.M.D.; Funding Acquisition, M.D.N., M.E.D., G.R.N. and A.M.D.; All the authors have read, approved and made substantial contributions to the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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